

**EFFECT OF *CHLORELLA VULGARIS* IN IMPROVING THE
VITAMIN B₁₂ STATUS AMONG THE VEGETARIAN
OBESE AND OVERWEIGHT YOUNG ADULT WOMEN**

**DEEPIKA. M
(16PFN005)**

A THESIS SUBMITTED TO THE
AVINASHILINGAM INSTITUTE FOR HOME SCIENCE AND HIGHER EDUCATION
FOR WOMEN, COIMBATORE- 641043.

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE IN FOOD SCIENCE AND NUTRITION

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Certified as a Bonafide Research Work



Signature of the Supervisor



Signature of the Head of the Department

ACKNOWLEDGEMENT

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First and foremost the investigator expresses her deep sense of gratitude to **God Almighty** for showering His blessings on her who graciously blessed her with good health, strength and wisdom, to complete the study. She thanks Him for blessing her with such great and wonderful parents to guide her.

The investigator expresses her heartfelt thanks and deep sense of gratitude to **PadmashriDr.P.R. KrishnaKumar**, Chancellor, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore for providing the infrastructural facilities for the smooth conduct of the study.

The investigator expresses her reverential gratitude to **Thiru. T.S.K. Meenakshisundaram**, M.A., M.Phil., Ph.D., Managing Trustee, Sri Avinashilingam Education Trust Institutions, Coimbatore, for providing the opportunity to conduct the study.

The investigator owes her special thanks and gratitude to **Dr. (Tmt). PremavathyVijayan**, M.Sc., M.Ed., Dip.Spl.Edn., M.Phil., Ph.D (Avinashilingam), Vice Chancellor, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore for the amenities provided for the successful completion of the study.

The investigator records her sincere gratitude to **Dr. (Tmt).S. Kowsalya**, M.Sc., M.Phil., Ph.D., Registrar, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore for providing all help in the smooth conduct of the study.

The investigator owes her heartfelt thanks and deep sense of gratitude to **Dr. (Mrs). N.Vasugi**, M.Sc., M.Phil., Ph.D., Dean, Faculty of Home Science, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for her kind support and encouragement for the conduct of the study.

The investigator expresses her special thanks and sincere gratitude to **Dr. (Tmt) Thirumani Devi**, M.Sc., M.Phil., Ph.D., Professor and Head, Department of Food Science and Nutrition, Avinashilingam Institute for Home Science and Higher Education

for Women, Coimbatore, for the keen interest, valuable help, concern and encouragement which has helped in the successful completion of the study.

The investigator expresses her special thanks and sincere gratitude to **Dr. (Tmt) M. Amirthaveni, M.Sc. Dip. Ed., M.Phil., Ph.D.**, Former Professor and Head, Department of Food Science and Nutrition, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for the immense support in the conduct of the study.

The researcher is deeply indebted and it gives her an immense pleasure and pride to offer profound gratitude to her guide **Dr. (Tmt). R. Balasasirekha, M.Sc., PGDCA., M.Phil., Ph.D.**, Assistant Professor, Department of Food Science and Nutrition, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for her inspiring, ceaseless and dynamic guidance, supportive wisdom, continued motivation and enduring support from the initiation to the completion of the study.

The investigator expresses her heartfelt thanks to **Tmt. S. Radha Devi, M.Sc. (Kerala), M.Phil, (Madras)**, Associate Professor in Statistics, Department of Food Science and Nutrition, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for her valuable suggestions rendered in Statistics throughout the study.

The investigator owes her sincere thanks to all the **Staff Members** of the Department of Food Science and Nutrition, Avinashilingam Institute for Home Science and higher Education for Women, Coimbatore, for being supportive and understanding.

No words are sufficient to express her deep sense of gratitude to her beloved and respected Parents, and Family members for their affection, care, blessing and co-operation in all walks of her life.

The researcher is grateful to each and every soul who had helped her in one or the other way in making this study a great success.

CONTENTS

CONTENTS

Chapter NO.	TITLE	Page No.
	LIST OF TABLES	
	LIST OF FIGURES	
	LIST OF PLATES	
	LIST OF APPENDIX	
I	INTRODUCTION	1-7
II	REVIEW OF LITERATURE A. Importance of <i>Chlorella Vulgaris</i> B. Nutritional and therapeutic importance of <i>Chlorella Vulgaris</i> C. Obesity and overweight – definition and prevalence D. Health consequences of obesity and overweight E. Importance of vitamin B ₁₂ in nutrition and its association with diet	8-23
III	METHODOLOGY A. Selection and procurement of sample B. Analysis of proximate principles, phytochemicals, antimicrobial activity and toxic substances in <i>Chlorella vulgaris</i> C. . Conduct of supplementation study i. Selection of area and participants ii. Formulation of questionnaire and eliciting the background information iii. Imparting nutrition education to the selected participants iv. Anthropometric assessment of participants v. Conduct of supplementation study D. Statistical analysis and interpretation of data	24-48

IV	<p>RESULTS AND DISCUSSION</p> <p>A. Proximate composition, phytochemicals, antimicrobial activity and toxic substances in <i>Chlorella vulgaris</i></p> <p>B. Background information of the young adult women</p> <p>C. Dietary intake of young adult women</p> <p>D. Impact of nutrition intervention on nutritional knowledge of young adult women</p> <p>E. Anthropometric measurements of the young adult women</p> <p>F. Effect of supplementation of <i>Chlorella vulgaris</i> on the vitamin B₁₂ status of the young adult women</p>	49-70
V	SUMMARY AND CONCLUSION	71-79
	BIBLIOGRAPHY	80
	APPENDICES	94

LIST OF TABLES

Table No.	TITLE	Page No.
I	<i>Proximate composition of Chlorella vulgaris</i>	50
II	<i>Phytochemicals of Chlorella vulgaris</i>	51
III	<i>Antimicrobial activity of Chlorella vulgaris</i>	53
IV	<i>Toxic substance in Chlorella vulgaris</i>	54
V	<i>Socio economic status of the young adult women</i>	55
VI	<i>Physical activity pattern of the young adult women</i>	56
VII	<i>Meal pattern and beverage consumption pattern</i>	57
VIII	<i>Meal skipping pattern of the young adult women</i>	58
IX	<i>Habit of eating outside foods</i>	59
X	<i>Dietary intake of young adult women</i>	60
XI	<i>Nutrition knowledge before and after nutrition education</i>	64
XII	<i>Anthropometric measurements of the participants before and after the supplementation study</i>	66
XIII	<i>Derived body composition variables</i>	67
XIV	<i>Classification of participants according to BMI</i>	68
XV	<i>Effect of supplementation of Chlorella vulgaris on the vitamin B₁₂ status of the young adult women</i>	69

LIST OF FIGURES

Figure No.	TITLE	Page No.
I	Research design	47
II	Conduct of supplementation study	48
III	Dietary intake of the participants	62
IV	Dietary intake of the participants	63
V	Effect of supplementation of <i>Chlorella vulgaris</i> on the vitamin B ₁₂ and Heamoglobinstatus of the young adult women	70

LIST OF PLATES

Plate No.	TITLE	Page No.
1	Sun dried Chlorella powder and analysis of proximate principles	28
2	Analysis of proximate principles	29
3	Phytochemical analysis and antimicrobial activity of chlorella vulgaris	34
4	Nutrition education and anthropometric measurements	43
5	Anthropometric measurements and collection of blood sample	44
6	Supplementation to the experimental and placebo group	45

LIST OF APPENDICES

Appendix No.	TITLE	Page No.
I	Questionnaire to elicit details on socioeconomic background and nutrition knowledge	94
II	Ethical clearance	97

INTRODUCTION

REVIEW OF LITERATURE

METHODOLOGY

RESULTS AND DISCUSSION

SUMMARY AND CONCLUSION

BIBLIOGRAPHY

APPENDICES

I. INTRODUCTION

Nutrition and health are two sides of the same coin and therefore are inseparable. Health and nutritional status of the population are recognized as the prime indicator of development at national and international level (Patil, 2011). An estimated 31 per cent of Indian population is aged between 10-24 years (Office of the Registrar General and Census Commissioner of India, 2011). Young/prime adulthood can be considered as the healthiest time of life (Zastrow *et al.*, 2009) and young adults are generally in good health, subjected neither to disease nor the problems of senescence. Strength and physical performance reach their peak from 18–39 years of age (Arpenning *et al.*, 2004 and Knechtle *et al.*, 2012). Flexibility may decrease with age throughout adulthood (Emilio *et al.*, 2014).

Overall obesity rates increase through adulthood, rising from 23 per cent in those aged 20-24; to 35 per cent among those in their late 20s, 30s and early 40s; and to more than 40 per cent at older ages (Harris, 2010). In a longitudinal study, obesity rates more than tripled from 11 per cent in adolescence in 1995 to 37 per cent by young adulthood in 2008 (Harris *et al.*, 2006). In terms of obesity-related conditions, more than one in four young adults aged 24-32 years were hypertensive, 69 per cent were prehypertensive, 7 per cent were diabetic and 27 per cent were prediabetic with impaired glucose tolerance or hyperglycemia (Gooding *et al.*, 2014; Nguyen *et al.*, 2011). Hypertension and diabetes are known risk factors for stroke and the data reveal troubling increases in hospitalization for and prevalence of stroke among young adults over the past decade (George *et al.*, 2011; National Center for Health Statistics, 2011).

Estimates indicate that 12 per cent of all age-eligible men and 35 per cent of all age-eligible women were unable to meet U.S. Army standards for weight-to-height ratio and per cent body fat in 2007-2008 (Cawley and Maclean, 2012). Furthermore, the Department of Defense reported that between 2006 and 2011, 62,000 individuals who arrived for military training failed their entrance physical because of their weight (Cutler and Miller, 2013).

Research documenting the emergence of the obesity epidemic has found that rising body mass indexes and obesity prevalence first occurred in the 1990s particularly among adolescents and children (Lee *et al.*, 2010). So current cohorts of young adults are entering adulthood with much higher obesity rates relative to previous cohorts of young adults and are the first generation to experience dramatically rising obesity rates in childhood and adolescence and they carry this health burden into adulthood. Importantly, the rate of increase in obesity across the age transition has been found to vary by educational experiences and expectations (Clarke *et al.*, 2013). Vitamin B₁₂ is deficient among obese and overweight individuals. Among the participants 16 % had B₁₂ deficiency and 65 % of them were overweight (Samak *et al.*, 2008).

Some countries have strong cultural or religious traditions that promote vegetarianism, such as in India, while in other countries secular ethical concerns dominate, including animal rights and environmental protection, along with health concerns. In many countries, food labeling laws make it easier for vegetarians to identify foods compatible with their diets (UK Government Food Standards Agency, 2008). A study carried out in 2010 estimated that there are 1,450 million vegetarians of necessity and another 75 million of choice, and constitute 21.8% of the world's population (Leahy *et al.*, 2010). In 2007, UN FAO statistics indicated that Indians had the lowest rate of meat consumption in the world (*Scribd.com.*, 2018). India has more vegetarians than the rest of the world put together (Edelstein , 2013). Other survey cited by FAO (FAO, 2003) estimate that 40 per cent of the Indian population as being vegetarians.

Vitamin B₁₂ is absolutely critical for good mental and physical health. It is essential for the DNA (cell's genetic material) and works with other B vitamins to form all types of blood cells. It helps nerve fibres form and function in our brain, spinal cord and peripheral nerves (<https://www.healthaid.co.uk/healthaid-blog/vitamin-b12-deficiency-healthaid>). Vegans may have a greater challenge in meeting the nutritional adequacy for vitamin B₁₂, protein and calcium compared to lacto-ovo-vegetarians and meat-eaters (Le and Sabate, 2014). Thus, the potential adverse effects of vegan

diets deserve consideration. In the EPIC-Oxford study (Appleby *et al.*, 2007) vegans had 30% higher fracture rates than meat-eaters. However, when adjusted for calcium intake, vegans no longer had higher fracture rate. Higher rates of vitamin B₁₂ deficiency is found among vegans compared with other vegetarians (Dunham and Kollar 2006). Compared with lacto ovo vegetarians and omnivores, vegans typically have lower plasma vitamin B₁₂ concentrations, higher prevalence of vitamin B₁₂ deficiency, and higher concentrations of plasma homocysteine (Majchrzak and Manne, 2006).

In vitamin B₁₂ deficiency, high serum folate is associated with increased homocysteine and methylmalonic acid concentrations (Quinlivan, 2008). Elevated homocysteine in blood indicates a lower serum vitamin B₁₂ levels in most cases (Fakhrzadeh *et al.*, 2006; Meertens *et al.*, 2007). Elevated homocysteine has been considered as a risk factor for CVD (McNulty *et al.*, 2008) and osteoporotic bone fractures (McLean *et al.*, 2008). Micronutrient deficiencies have been observed in obese individuals in many parts of the world, and it is obvious that these may influence several physiological body functions, impair the immune system and increase the risk of comorbidities (García *et al.*, 2009).

Absolute vitamin B₁₂ deficiency was found in 6% patients in India , borderline vitamin B₁₂ deficiency in 9%, absolute folic acid deficiency in 26% and combined vitamin B₁₂ and folic acid deficiency in 10% patients. Values below 200 pg/ml is said to be absolute vitamin B₁₂ deficiency and the value between 200-250 pg/ml is said to be border line vitamin B₁₂ deficeincy and the values between 250-900 pg/ml is considered to be the normal vitamin B₁₂ status (Mahajan *and* Aundhakar, 2015). Vitamin B₁₂ deficiency was observed in 19.4% men and 23.7% women suggesting that risk of developing B₁₂ deficiency is affected by gender (Arora *et al.*, 2011). The magnitude of the prevalence of B₁₂ deficiency estimated in our population strengthens the argument that B₁₂ deficiency is more prevalent in Indians.

A diet based on quantity rather than quality has ushered a new culture onto the world stage; the human being who manages to be both overfed and

undernourished, two characteristics seldom found in the same body in the long natural history of our species (Pollan, 2008). Elevated homocysteine levels have also been identified as an independent risk factor for cardiovascular disease and are thought to promote thrombogenesis, impair endothelial vasomotor function, promote lipid peroxidation, and induce vascular smooth muscle proliferation (Refsum *et al.*, 2006 and Lichtenstein *et al.*, 2006 and Schulz, 2007). In the presence of insufficient vitamin B₁₂, homocysteine levels can rise due to inadequate function of methionine synthase (Clarke, 2008). Vitamin B₁₂ plays an important role in reducing the total homocysteine and thereby reducing the CVD risk (Toole *et al.*, 2004).

Vitamin B₁₂ is naturally found in animal products, including fish, meat, poultry, eggs, milk, and milk products. Vitamin B₁₂ is generally not present in plant foods, but fortified breakfast cereals are readily available source of vitamin B₁₂ with high bioavailability for vegetarians (USDA, 2011). Nutritional needs should be met primarily from foods. Foods in nutrient-dense forms contain essential vitamins and minerals and also dietary fiber and other naturally occurring substances that may have positive health effects (USDA, 2015). Recently researchers hit the jackpot when delving underwater: algae has emerged as the sole, promising candidate for a botanical source of vitamin B₁₂. Out of all the potential herbal B₁₂ sources, *Chlorella* is the most promising at present. However, there are also contradictory studies on this algae. The first American study, carried out in 1968, couldn't prove B₁₂ whatsoever, but current studies show a pleasingly high B₁₂ content, as well as low levels of vitamin B₁₂ analogs at the same time (Katsura *et al.*, 2002, Chen and Jiang, 2008).

An alternative to meat source for vitamin B₁₂ is algae. *Chlorella vulgaris* contain 10-50 microgram of vitamin B₁₂ per 100 g of dry weight (Kohlstadt, 2012). *Chlorella* intake resulted in noticeable reductions in body fat percentage, serum total cholesterol, and fasting blood glucose levels. Genes involved in fat metabolism and insulin signaling pathways, which suggests that these pathways could be physiologically affected by *Chlorella* intake (Mizoguchi *et al.*, 2008). *Chlorella vulgaris* is a unicellular green microalgae that has been widely used for centuries as a food source with complete nutrients, such as carbohydrate, protein, vitamins and

minerals, and is marketed commercially as health supplement or incorporated in food along with cereals (Halperin *et al.*, 2003). *Chlorella vulgaris* has been shown to have anti-atherogenic, anti-cholesterolemic, anti-inflammatory, and antitumor effects (Hasegawa *et al.*, 2000). One of *Chlorella's* most significant health benefits is that it wraps itself around even stubborn toxins residing in our bodies, such as lead, cadmium, mercury and uranium, and keeps them from being reabsorbed. Regular consumption of *Chlorella* can even help keep heavy metals from accumulating in our bodies' soft tissues and organs (Shim *et al.*, 2009 and Queiroz *et al.*, 2003). *Chlorella* enhances the Natural killer cell activity and produces interferon- γ and interleukin-12 as well as interleukin-1 β , the Th-1 cell-induced cytokines in healthy people (Kwak *et al.*, 2012).

In addition, purified peptides from *Chlorella vulgaris* have demonstrated significant protective effects against cellular damage (Sheih *et al.*, 2009). The most important substance in *Chlorella* is β -1,3-glucan, which is an active immunostimulator, a free radical scavenger and a reducer of blood lipids. *Chlorella* can also be used as a food additive owing to the taste and flavour adjusting actions (Spolaore *et al.*, 2006). A diet enriched with the algae, *Chlorella*, reduced oxidative stress and significantly prevented the decline of cognitive ability in an age dependent dementia mouse model. Prolonged consumption of *Chlorella* has the potential to prevent the progression of cognitive impairment (Nakashima *et al.*, 2009). Intake of *Chlorella* containing multicomponent supplement can decrease arterial stiffness (Otsuki *et al.*, 2013) and increases aerobic endurance capacity in young individuals (Umemoto and Otsuki, 2014). Vitamin B₁₂ in *Chlorella* is bioavailable and such dietary supplementation is a natural way for vegetarians and vegans to get the vitamin B₁₂ they need (Merchant *et al.*, 2015).

The present study is conducted to improve the vitamin B₁₂ status among the young adult vegetarian women who are either obese or overweight as vitamin B₁₂ is lower / deficient among this group. The statistics of vegetarianism in India is higher compared to any other countries in the world. At the same time obesity is an

emerging public health problem. As the vitamin B₁₂ is found only in animal source, vegetarians are deficient in this vitamin.

Obesity and overweight leads to further B₁₂ deficiency. Young adults are highly prone to obesity and vitamin B₁₂ is very much important for woman of child bearing age. Today we can see lot of people with cardiovascular diseases. The reason for cardiovascular disease is not only the high cholesterol but also due to stress, which in turn is due to a high circulating homocysteine in blood. One of the main reason for high circulating homocysteine in blood is low vitamin B₁₂. Most of the vegetarians are consuming vitamin B₁₂ supplements when they are diagnosed with vitamin B₁₂ deficiency and also people are consuming fortified foods. Always a natural food consumption is found to be useful and healthy as it does not cause any side effects. The idea was to generate a new food source of vitamin B₁₂ for vegetarians who are highly prone to vitamin B₁₂ deficiency as vitamin B₁₂ is present only in animal source. Dairy products are the only source of vitamin B₁₂ for vegetarians. The vegans are highly susceptible to vitamin B₁₂ deficiency than the lacto-vegetarians. *Chlorella vulgaris* is a vegetarian food but is not commonly consumed in India. Though the benefits of *Chlorella vulgaris* is found out by various scientist it is not used as a supplement for vitamin B₁₂ deficiency by the population.

Since obesity, vitamin B₁₂ deficiency and cardiovascular diseases are in emergence particularly among vegetarians there is an urgency in controlling these conditions. Keeping the above mentioned facts and the benefits of *Chlorella vulgaris*, the purpose of the present study is to test the effect of *Chlorella vulgaris* which is an under utilized algae, would be of great help in order to improve the vitamin B₁₂ status among the obese/overweight young adult vegetarian women.

Hence the null hypothesis of the study is “There is no effect on supplementing *Chlorella vulgaris* in improving the vitamin B₁₂ status among obese and overweight young adult women”.

With this in view, the study was conducted with the following objectives: To

- analyse the proximate principles, phytochemicals and antimicrobial activity of *Chlorella vulgaris*
- provide nutrition education to the participants on the importance of vitamin B₁₂ and *Chlorella vulgaris*
- supplement *Chlorella vulgaris* as a source of vitamin B₁₂ to vegetarian obese and overweight young adult women
- evaluate the effect of supplementation of *Chlorella vulgaris* in improving the vitamin B₁₂ status among the vegetarian obese and overweight young adult women.

II. REVIEW OF LITERATURE

The review of literature pertaining to the study entitled “ **Effect of *Chlorella vulgaris* in improving the vitamin B₁₂ status among Vegetarian obese and overweight young adult women**” is reviewed under the following headings:

- A. Importance of *Chlorella Vulgaris*
- B. Nutritional and therapeutic importance of *Chlorella Vulgaris*
- C. Obesity and overweight – definition and prevalence
- D. Health consequences of obesity and overweight
- E. Importance of vitamin B₁₂ in nutrition and its association with diet

A. Importance of *Chlorella vulgaris*

***i. Chlorella vulgaris* and its emergence**

Chlorella vulgaris is a eukaryotic, unicellular green algae. *Chlorella vulgaris* is estimated to have been on earth for more than 2.5 billion years. It's existence resulting in many of the useful functions that are used today and will be used in the future also (Liebke., 2014). Sacasa and Claudia, 2013 found that the important feature of *Chlorella vulgaris* and its ability to rapidly grow and they were grown in photobioreactors. Tang and Sutter, 2011) in a study examined the cell wall of *Chlorella* and it is found that cell wall is rigid and it cannot be digested easily. For this reason, the *Chlorella* which is harvested should be processed in order to break the cell wall prior using it as a dietary supplement for its nutrient content and a special feature in *Chlorella* is called Chlorella Growth Factor or CGF that can strengthen immunity and prevent or destroy cancer lesions. In an another study conducted by Kent., *et al* (2015) it was reported that the Chlorella Growth Factor and its extracts are capable of promoting growth rate, increasing insulin sensitivity, strengthening

immune system function, preventing stress-induced ulcers, pregnancy-associated anemia and hypertension.

Liebke *et al.*, (2014) found that *Chlorella vulgaris* like most of other green algae, is a freshwater micro-algae and all *Chlorella* species combined together attribute to the largest source of chlorophyll. During its growth period on earth, it has developed many important functions that is valued with regard to human health. *Chlorella vulgaris* can survive under certain stressors such as viruses, bacteria, fungi, and many types of pollutants. The reason for these attributes stems from its ability to rapidly repair the DNA of stressors.

Chlorella vulgaris is sold as a food supplement or additive and it is noted that this is one of the few microalgae which is used as a food supplement (Fradique *et al.*, 2010). Fernandes *et al.*, (2012) in a study mentioned that *Chlorella* can also be used as colourant and food emulsion. Liang *et al.*, (2004) reported that *Chlorella* products come in various forms such as capsules, tablets, extracts and powder. Kitada *et al.*, (2009) in a study on the pharmacological importance of *Chlorella vulgaris*, elucidated that Japan is the world leader in consuming *Chlorella* and is used for medical treatment. *et al.*, 2009 and Justo *et al.*, 2001 reported that it is because of the *Chlorella's* immune-modulating and anti-cancer properties; *Chlorella* powder when administered to rats, mice and rabbits in the form of powder, it showed protection properties against haematopoiesis (Queiroz *et al.*, 2013).

ii.. Antioxidant effect of *Chlorella vulgaris*

Fernandez *et al.*, (2012) found that the chlorophyll pigment found in *Chlorella* have multiple therapeutic properties, such as antioxidant activities and chlorophyll is the highest among the pigments found in *Chlorella* which has a protective effect against retina degeneration. Cha *et al.*, (2008) reported the regulatory effects of *Chlorella* against blood cholesterol, prevention from chronic diseases like cardiovascular and colon cancer and its effect on fortifying the immune system. Shim *et al.*, (2008) found the protective effect of *Chlorella vulgaris* against heavy metals and other harmful compounds like lead, cadmium, and naphthalen by reducing

significantly the oxidative stress induced by these harmful compounds, and increasing the antioxidant activity in the organisms of tested animals

Raghavendran in 2013 proposed that the compounds in *Chlorella vulgaris* like antioxidants and a glycoprotein have an antagonistic effect on the tumor producing factors. Apart from the importance of macroalgae in healthcare and medicine, *Chlorella*, a green microalga, has gained more attention in the pharmaceutical industry because of its ability to enhance the nutritional content of conventional food preparations and positively affect the health of humans and animals. Dantas *et al.*, (2015) studied the solvents with higher efficiency of extraction of compounds with antioxidant activity of the species *Chlorella vulgaris*. The highest phenolic content, hydrogen peroxide radical scavenging activity was also obtained by Manivannan *et al.*, (2012). Antioxidant potential of microalgae in relation to their phenolic and carotenoid content was carried out by Goiris *et al.*, 2012 and found that industrially cultivated samples of *Tetraselmis suecica*, *Botryococcus braunii*, *Neochloris oleoabundans*, *Isochrysis sp.*, *Chlorella vulgaris* and *Phaeodactylum tricorutum* possessed the highest antioxidant capacities and thus could be a potential new source of natural antioxidants. Bhuvana *et al.*, (2017) found out that the methanolic extract of *Chlorella vulgaris* showed potent antioxidant activity.

Miranda *et al.*, (2001) found out the total phenolic compounds in *Chlorella vulgaris* and it was 24.95 mg in 1 g of dry algae matter from methanolic extract and five phenolic acids. The phenolic compounds salicylic, trans cinnamic, synapic, chlorogenic, chamic and caffeic acids found in the methanolic *Chlorella* extract may be responsible for its higher antioxidant activity. Kim *et al.*, (2008) examined various biologically active compounds of *Chlorella vulgaris* including growth factors, anti-inflammatory and wound healing substances, antioxidants, and emollient compounds. According to Wang *et al.*, (2010) the isolated indigenous *Chlorella vulgaris* strain extract obtained from supercritical carbon dioxide extraction exhibits significant antioxidant activities and presents dual inhibitions to lung cancer cell growth and migration ability, which is the index of cancer metastasis. Experimental studies

carried out under *Chlorella* have demonstrated its antitumor effect, cancer chemo prevention properties, anti-inflammatory activity, antioxidant activity, and anti microbial activity (Makridis *et al.*, 2006).

iii. Cholesterol lowering effects of *Chlorella vulgaris*

Hee *et al* conducted an animal study in 2014 and demonstrated that *Chlorella* supplementation reduced the serum cholesterol levels under high-fat or high-cholesterol diets . Small, open-label trials yielded promising results with a focus on the improvement of serum cholesterol. A study of Kourimská *et al* (2014) on the “effect of feeding with algae on fatty acid profile of goat’s milk” with 10 g of *Chlorella* supplementation per day changed the fatty acid profile of Goat’s milk. Tsiplakou *et al.*, (2016) conducted a study on goat based on *Chlorella* diet and results showed that the *Chlorella* diet compared with the control, increased significantly the populations of *Methanosphaera stadtmanae*, *Methanobrevibacter ruminantium* and *Methanogens bacteria* and protozoa in the rumen of goats. There was a significant reduction in the cellulase activity and in the abundance of *Ruminococcus albus*, and a significant increase in the protease activity and in the abundance of *Clostridium sticklandii* in the rumen liquid of goats fed with the chlorella diet, compared with the control, were found.

iv. Hepatoprotective, antimicrobial and antidiabetic effect of *Chlorella vulgaris*

Jae-Young *et al.*, (2008) and Panahi *et al.*, (2012) reported the hepatoprotective effect of *Chlorella vulgaris*. It was reported that *C. vulgaris* has a protective effect against cadmium-induced liver damage by reducing cadmium accumulation and stimulating the expression of metallothioneins II in liver. Study of panahi *et al.*, proven that the *Chlorella vulgaris* extract might be a promising hepatoprotective supplement for patients with non-alcoholic fatty liver disease.

Syed *et al.*, (2015) studied the antimicrobial activity of *Chlorella vulgaris* and proved the inhibitory activity of *Chlorella vulgaris* against some of the common pathogen like *E.coli*, *Klebsilla sp.*, *bacillus sp.* and *Pseudomonas sp.* which are pathegeonic to humans and considered to cause water borne and vector diseases

and is causing drastic impacts with huge impacts. The antibacterial activity of *Chlorella vulgaris* was higher for the aqueous and DMSO extracts, including the acetone extract which has also demonstrated the growth inhibition of some bacteria like *E. faecalis*, *S. enteritidis* and *B. subtilis* (Danielli *et al.*, 2015).

Jeong *et al.*, (2009), proved that the intake of *Chlorella* significantly reduced the insulin/glucagon ratio, the concentrations of fasting plasma glucagon and hepatic triglycerides. Also, the concentrations of fasting blood glucose and plasma insulin, the level of HOMA-IR were slightly decreased by the intake of *Chlorella*. Sun *et al* (2010), found out the compounds of the *Chlorella vulgaris* like carotenoids, linoleic acid, arachidonic acid, eicosapentaenoic acid have an inhibitory effect on the advance glycation end products. Mameghani, (2014) studied the effect of *Chlorella vulgaris* on serum glucose and the results were statistically significant. In a study conducted by Itakura and co workers in 2015 proved that the ingestion of *Chlorella* suppresses resistin and related genes' expression in peripheral blood cells of borderline diabetics.

v. Anticarcinogenic effect of *Chlorella vulgaris*

Jayshree *et al.*, (2016) stated that both *Chlorella vulgaris* and *Chlorella reinhardtii* have a significant level of flavonoids and phenols, which turn out to be potential scavenger of free radicals at the concentration of 1000 µg/ml. The crude extracts tend to inhibit the growth of breast cancer cell lines and human pathogens. Overall comparison between two proposed microalgae reveal that *Chlorella vulgaris* are rich in flavonoid content and are able to exhibit better free radical scavenging and anticancer activity than *Chlorella reinhardtii*. Mohd *et al.*, (2009) explored the chemopreventive role of *Chlorella vulgaris* by inhibiting cellular proliferation (decreasing Bcl-2 expression) and inducing apoptosis (increasing caspase 8 expression) in liver cancer cells. The results of the study justified the role of *Chlorella Vulgaris* in the treatment of thousands of liver cancer patients. Sheih *et al.*, (2010) proved that the peptide fraction had much better antioxidant activity toward peroxy radicals and LDL than those of Trolox.

Balaji *et al.*, (2017) investigated algal biomass in methanol extracts of *Chlorella vulgaris* was screened. The antioxidant property of the methanolic extracts of these green microalgae was evaluated by measuring the free radical scavenging activity by DPPH assay method. The DPPH radical scavenging activity was found to be higher in *Chlorella vulgaris* (RSA 53.96%) at a concentration 20µg compared to standard ascorbic acid (RSA 59.42%) and it inhibited the growth of tumor cells (MCF7).

B. Nutritional and therapeutic importance of *Chlorella vulgaris*

***i. Chlorella vulgaris* as a source of protein**

Chlorella from several large producers have “GRAS” designations [Generally Recognized As Safe (FDA 2016)]. Large-scale production of both “spirulina” and *Chlorella* occurs throughout the world, and these well-domesticated crops are added to many foods to increase their protein and other nutritional contents e.g., salad dressings, beverages, baked goods, and/or sold as protein supplements (Safi *et al.*, 2014). Aarstad *et al.*, (2012), reported that green algae *Chlorella* constitute up to 70 % dry weight protein; these micro algae have an amino acid profile that compares well with egg, notably containing all of the essential amino acids (EAA) that humans cannot synthesize and must obtain from foods. Enyidi, (2017) found that the microalgae *Chlorella vulgaris* has good-quality protein with amino acids rich in methionine, lysine and alanine. In the same study it was proved that *Chlorella vulgaris* is a good protein source for African catfish and can also substitute fishmeal in the catfish diets.

Klamczynska and Mooney in 2017 suggested that *Chlorella* grown vi photosynthesis in open pond systems is commonly used today as supplements and whole algae protein delivers over 60% protein. Klamczynska and Mooney mentioned that with a global population growing and demanding more protein, heterotrophic microalgae is a scalable and sustainable source of protein. Michel, 2015 reports that *Chlorella* has all essential amino acids required for the nutrition of heterotrophic organisms. Priyadharshini and Rath, (2012) reported the protein quality

in *Chlorella vulgaris* and its highest bioavailability. Becker (2007) and Christi (2007) studied the protein content of *Chlorella vulgaris* and found that 51-58 g of protein. *Chlorella*, contains about 50-60% of proteins, whose quality is comparable to the proteins of yeast, soy flour and milk powder (Blazencic, 2007).

ii. Polysaccharides in *Chlorella vulgaris*

Compositional analysis of *Chlorella* and similar microalgae began more than 60 years ago, and an impressive number of biological processes are now reported to be influenced by ingestion of whole algae or polysaccharide extracts as food or supplements (Pulz and Gross, 2004; Plaza *et al.*, 2009; Chacon-Lee and Gonzalez-Marino, 2010; Lordan *et al.*, 2011; Vo *et al.*, 2011). Species of *Chlorella* being recognized as particularly rich in polysaccharides (Chacon-Lee and Gonzalez-Marino, 2010). This putative bioactivity includes anticancer properties, cytokine modulation, anti-inflammatory effects, macrophage activation, and inhibition of protein tyrosine phosphatase (Kralovec *et al.*, 2005; Sheng *et al.*, 2007; Hsu *et al.*, 2010).

iii. *Chlorella vulgaris* and active vitamin B₁₂

Katsura *et al.*, (2002) reported that *Chlorella vulgaris* contain vitamin B₁₂. LCMS,SIR,MRM and RNA aptamer along with other conventional analytical techniques such as HPLC, microbiological assay and chemiluminescence were used to confirm the presence of methylcobalamin, a form of vitamin B₁₂ in *Chlorella vulgaris* (Kumutha *et al.*, 2015). Michel (2015) proposed that detoxication of metals and pesticides performed by *Chlorella* is associated with porphyrin rings in chlorophyll or glutathione-induced pathway production by vitamin B₁₂. Watenabe *et al* in 2002 found that substantial amounts of vitamin B₁₂ in chlorella using the *Lactobacillus delbrueckii* subsp. *lactis* ATCC7830 microbiological assay method. The Corrinoid compounds were separated and characterized from these algae to test the chemical properties and also the bioavailability of the algal vitamin B₁₂ was checked. These tests found the true vitamin B₁₂ is available in *Chlorella*.

v. Therapeutic values of *Chlorella vulgaris*

Bae *et al.*, (2013) investigated the effects of *Chlorella vulgaris* aqueous extracts on *in vitro* immuno-allergic responses using rat peritoneal mast cells, and *in vivo* through evaluation of plasma markers. Data showed that the aqueous extract is capable of suppressing histamine release via modulation of T helper 1 (Th1) activity, thus attenuating allergenic responses in animals. In addition, *C. vulgaris* dry extracts modulate oxidative damage in chronically stressed individuals. A study providing 3600 mg/day (six weeks) dry *Chlorella vulgaris* extract to non-comorbidity bearing smokers showed significant decrease in lipid peroxidation and optimized antioxidant status of participants (Merchant and Andre, 2001).

Grames *et al.*, (2013) found that supplementing *Chlorella vulgaris* in the diet of Atlantic salmon (*Salmo salar* L.), attenuated *in vivo* gut inflammatory symptoms. Suggesting applicability in the treatment of human gastrointestinal diseases, such as Crohn's disease and hypersensitivity to prolamins (e.g. wheat gliadin, commonly known as gluten). In a randomized, double-blind clinical trial by Kwak *et al.*, (2012) showed that supplementation with *Chlorella vulgaris* can optimize innate immune response, stimulating the activity of natural killer cells and raising the concentration of interleukins associated to defense against pathogens. *Chlorella vulgaris* may be incorporated to food products, such as pasta, thus enhancing both nutritional and sensorial quality without affecting processing (Fradique *et al.*, 2010). *Chlorella* may be added to cookies (Bang *et al.*, 2013), yellow layer cake (Kim and Chung, 2010), imitation processed cheese (Shalaby and Yasin, 2013), and others, without modifying sensorial/nutritional properties.

C. Obesity – definition and prevalence

i. Definition of obesity

Ofei (2005) defined obesity as a condition of abnormal or excessive fat accumulation in adipose tissue, to the extent that health is impaired. The amount of excess fat in absolute terms, and its distribution in the body - either around the waist and trunk that is android obesity or peripherally around the body that is gynoid obesity have important health implications. As per current World Health Organisation (WHO) criteria, a BMI $<18.5\text{kg/m}^2$ is considered underweight, $18.5\text{--}24.9\text{ kg/m}^2$

as ideal weight and 25–29.9kg/m² as overweight or pre-obese. The obese category is sub-divided into obese class I (30–34.9kg/m²), obese class II (35–39.9kg/m²) and obese class III (\geq 40kg/m²). Kumaroff (2016) in their study “historical review of extra body weight definitions” collected many historical definitions of obesity”. Definition given by Hippocrates for health as a balance of four humors are fluids blood, black bile, yellow bile, and phlegm, and any deficiencies or extras were considered causes of diseases. Obesity was defined as the surplus of humors. Hippocrates was the first and realized that obesity leads to infertility and early mortality. Overweight was defined as weight that exceed the threshold from the reference value, where reference value was derived from the distribution of population.

ii. Prevalence of obesity at global level

Apovian, (2016) examined the worldwide prevalence rate for being overweight or obese between 1980 and 2013 increased 27.5% for adults and 47.1% for children, for a total of 2.1 billion individuals considered overweight or obese. These increase were seen in both developed and developing countries. However, the prevalence of overweight and obesity is higher in developed countries than in developing countries at all ages (data from 2013). In a review study on adult obesity found that obesity prevalence rates have increased worldwide in the last three decades from 1980 to 2008, reaching a prevalence of 10–14% among the world’s adult population in 2008 (Karageorgi *et al.*, 2013). Afshin *et al.*, (2017) found that the global prevalence of obesity in 2015 was 107.7 million among children and 603.7 million among adults. The overall prevalence of obesity was 5.0% among children and 12.0% among adults.

Misra and Khurana, (2008) opined that waist circumference data from 63 countries has shown highest prevalence of abdominal obesity among South Asians when compared with North Europeans and other Asian ethnic populations. Data from countries in sub-Saharan Africa, South America, and the Middle East also showed similar high prevalence of abdominal obesity with 67 per cent among women and 18 per cent among men in urban Cameroon, 43 per cent in Venezuela, and 24.6 per

cent in Oman. In an industrial population in India, 30.9 percent of men and 32.8 per cent of women were reported to have abdominal obesity.

iii. Prevalence of obesity at national level

Girdhar *et al.*, (2016) studied the prevalence of obesity in India. According to the statistics, percentage of women who are overweight or obese is highest in Punjab (29.9%), followed by Kerala (28.1%) and Delhi (26.4%). Selvaraj and Sivaprakasam, (2013) mentioned that prevalence of obesity in India is up to 50% in women and 32.2% among men in the upper strata of the society. In Delhi, the prevalence of obesity is 33.4% among urban women and 21.3% in men. Kumar *et al.*, (2014) in a study on the socio- demographic correlation on overweight and obesity among adults in rural Meerut found that out of 1382 individuals 244 (17.7%) had a BMI ≥ 25 . Prevalence of obesity in females (22%) was found to be more than males (13.8%). The prevalence of overweight and obesity is increasing in rural adult population. Rohilla *et al.*, (2014) reported that the problem of being overweight and obesity is confined not only to adults but also being reported among the children and adolescents in the developed countries during the past two decades.

iv. Prevalence of obesity and overweight in Tamilnadu

Gupta *et al.*, (2011), in an observational cross-sectional study in the Salem town of Tamilnadu found that out of 263 respondents, 35.7% were females and 64.3% were males. Females had a significantly higher BMI value than the males. 63.9% of the subjects were found to be overweight (obese or pre-obese) and 19.0% were obese. According to NFHS-3, (2005) the prevalence of overweight in Tamil Nadu is 19.8 per cent among men and 24.4 per cent among women and the rates are high among women when compared to men. The trend is similar to Indian scenario where the prevalence is more among women particularly in urbans than the rurals (Parimalavalli *et al.*, 2009). Shajithanoop *et al.*, (2013) in a study conducted at Tamilnadu examined that the mean BMI in boys ($16.7 \pm 2.8 \text{ kg/m}^2$) was lower than that of girls ($19.1 \pm 3.3 \text{ kg/m}^2$). Prasad *et al.*, (2017) in a study conducted at Namakkal district obesity was found to be predominant among women (62%) than

men (59.7%) and overweight was found to be predominant among men (14.28%) than women (13.11%).

D. Complications of overweight and obesity

i. Cardiovascular diseases and obesity

Obesity is a common risk factor for several subtypes of cardiovascular diseases such as Coronary Heart Disease (CHD), stroke, and heart failure (Zalesin *et al.*, 2011, Pandey *et al.*, 2015 and Aune *et al.*, 2016) . Lu *et al.*, (2014) in a meta-analysis indicated that most of the associations of obesity with CHD and stroke may be mediated by hypertension, dyslipidemia and diabetes mellitus. Mechanisms other than hypertension, dyslipidemia, and diabetes mellitus, such as excess metabolic demand and direct adverse effects of adiposity on the myocardium, play an important role in the development of heart failure among persons with excess weight (Abel *et al.*, 2008 and Alpert *et al.*, 2014).

Del *et al.*, (2015), Kenchaiah *et al.*, (2002), Morkedal *et al.*, (2014) and Wannamethee *et al.*, (2011) indicated that at least half of the association between obesity and CHD and three-quarters of the association between obesity and stroke are likely explained by hypertension, dyslipidemia, and hyperglycemia. The relatively limited prospective data regarding an independent link between obesity and incident heart failure, however, suggest a persistent risk association after accounting for traditional CVD mediators. Laboratory and clinical data suggest that there exist a direct link between obesity and myocardial injury that may predispose to fibrosis, myocardial dysfunction, and future heart failure (Garg *et al.*, 2005) and Ndumele *et al.*, (2014). Lavie *et al.*, (2013) mentioned in their study that Several processes are hypothesized to contribute to myocardial injury and dysfunction among persons with obesity, including increased metabolic demand, the paracrine effects of adipose tissue and increased myocardial triglyceride accumulation leading to myocardial damage and potential apoptosis.

ii. Obesity and insulin resistance

Obesity and Type 2 Diabetes Mellitus are serious health concerns. The global epidemic of obesity and Type 2 diabetes (T2D) is worsening (WHO, 2013). Updated WHO reports mention that 347 million people worldwide have Diabetes Mellitus (DM) of which Type 2 diabetes comprises the vast majority (90%) (WHO., 2013 and Danaei *et al.*, 2011). Daousi *et al.*, (2006) reported that 86% of adults with Type 2 diabetes are overweight or obese; 52% have obesity and 8.1% have morbid obesity. Lutsey *et al.*, (2010) proposed that factors other than fat mass per se also influence risk of development of T2D. It is clear that fat distribution is relevant to the risk of developing T2D, with central (visceral) adiposity conferring the greatest risk. Waist circumference, a useful clinical surrogate measure of visceral fat, is a useful predictor for subsequent development of T2D. An increase in waist circumference is associated with an increase in the risk of developing T2D. Hart *et al.*, (2007) mentioned that overweight and obesity accounts for a major proportion of T2D and that prevention of obesity would reduce the incidence of T2D.

Nguyen, (2011) and colleagues explored the relationship between obesity and T2D in a US adult population based on findings from the National Health and Nutrition Examination Survey, 1999–2006. Amongst the 21,205 surveyed adults, 2,894 (13.6%) had T2D. Amongst those with T2D, 80.3% were overweight and 49.1% were obese, the prevalence of T2D increasing according to severity of obesity. Pajunen *et al.*, (2013), found that abdominal diameter and BMI measurement also predict development of T2D. Duration of abdominal obesity is associated with risk for development of T2D independent of severity of abdominal adiposity (Reis *et al.*, 2013). These observations implicate central (visceral) adiposity as an important fat depot for T2D development.

iii. Overweight and development of cancer

Wolin *et al.*, (2010) found that about 20% of all types of cancers are caused by over weight. The International Agency for Research into Cancer (IARC) (International Agency for Research on Cancer, 2002) and the World Cancer

Research Fund (WRCF, 2007) reports showed that common cancers in obese people are predominantly endometrial, esophageal adenocarcinoma, colorectal, postmenopausal breast, prostate and renal. Less common malignancies associated with obesity are malignant melanoma, thyroid cancers (Kitahara *et al.*, 2011), and leukemia, non-Hodgkin's lymphoma and multiple myeloma (Lichtman, 2010). Jagers *et al.*, (2009) found a positive multivariable-adjusted association between cancer mortality and abdominal obesity that increases the risk of cancer mortality up to 24%. Spindler, (2005) proved that excess calorie intake will lead to increased cancer incidence and that positive energy balance promotes cancer cell proliferation and tumour development and long-term calorie intake leads to a reduction in cancer occurrence and extended life in rodents. McMillan *et al.*, (2006) mentioned in a study that high calorie diet like alcohol, animal fats etc will lead to health hazards like cancer.

iv. Malnutrition of obesity

Kaidar *et al.*, (2008) and Strohmayer *et al.*, (2010) found that vitamin D insufficiency is mainly caused because of obesity and also reported that the prevalence of vitamin D insufficiency (defined as <30 mg/dL) in obese individuals ranges from 80–90%. Pittas *et al.*, (2007) showed that subjects with impaired fasting glucose showed stability in fasting glucose levels after three years of daily supplementation of 700 IU of cholecalciferol compared to an increase in fasting glucose in the placebo group. Aasheim *et al.*, (2008) reported that obese patients had significantly lower mean serum concentrations of vitamins A, B-6, C, 25-hydroxyvitamin D, and lipid-standardized vitamin E. Flancbaum *et al.*, (2006) also reported the deficiency of iron in 44% adults prior to bariatric surgery which suggests that obesity is a causative factor for iron deficiency.

Gemmel *et al.*, (2009) reported a low vitamin B12 status in 18 % of obese individuals before the bariatric surgery and also folic acid deficiency was common among the highly obese individuals. Stavra and Xanthakos, (2009) in their study reported that micronutrients like iron, and vitamins D, B12, E and C were deficient among the study participants who are overweight and obese. Mandy *et al.*, (2014)

reported a lower vitamin B₁₂ status among the substantial number of obese adolescents at risk of type 2 diabetes identified, and the potential severity of B₁₂ deficiency indicates that the association between obesity, insulin resistance, metformin use and low B₁₂ status. MacFarlane *et al.*, (2011) and Pinhas-Hamiel *et al.*, (2006) indicated that low B₁₂ concentrations may be associated with obesity during childhood. Pinhas-Hamiel *et al.* reported a greater than 4-fold increased risk of low vitamin B₁₂ status in obese compared to normal weight Israeli children and adolescents.

E. Importance of vitamin B₁₂ in nutrition and its association with diet

i. Functions of vitamin B₁₂

Vitamin B₁₂, otherwise known as cobalamin, is a water-soluble vitamin containing cobalt, a compound essential for maintaining the health of all cells because it is needed for production of DNA and RNA, the body's genetic material (online resource). Candela *and* Meiner, (2004) and NIH (2014) mentioned that vitamin B₁₂ is especially important in maintaining the health of the body's nerve and blood cells. Specifically, it is crucial for maintaining healthy nerve cells and for supporting bone marrow production of red blood cells (RBCs). Methionine synthase is essential for the synthesis of purines and pyrimidines. The reaction depends on methyl cobalamin as a co-factor and is also dependent on folate, in which the methyl group of methyl tetra hydrofolate is transferred to homocysteine to form methionine and tetrahydrofolate (Gibson, 2005). Methylmalonyl CoA mutase converts methylmalonyl CoA to succinyl CoA, with 5-deoxy adenosyl cobalamin required as a cofactor. It is a defect in this reaction, and the subsequent accumulation of methylmalonyl CoA is thought to be responsible for the neurological effects in vitamin B₁₂ deficiency.

ii. Food sources of vitamin B₁₂

Leary and Samman, (2010) in a review mentioned that Vitamin B₁₂ is synthesised by certain bacteria in the gastrointestinal tract of animals and is then absorbed by the host animal. Vitamin B₁₂ is concentrated in animal tissues, hence,

vitamin B₁₂ is found only in foods of animal origin . Foods that are high in vitamin B₁₂ (µg/100g) include: liver (26–58), beef and lamb (1–3), chicken (trace-1), eggs (1–2.5) and dairy foods (0.3–2.4). *Watanabe et al., (2013)* reported that vitamin B₁₂ is synthesized only by certain bacteria, and it is primarily concentrated in the bodies of predators located higher in the food chain . Vitamin B₁₂ is well-known to be the sole vitamin that is absent from plant-derived food sources. Foods (meat, milk, eggs, fish and shellfish) derived from animals are the major dietary sources of Vitamin B₁₂. Korean centenarians were obtaining approximately 30% of their dietary vitamin B₁₂ from foods of plant origin (*Fabian and Elmadfa, 2004*). *Kwak et al., (2008)* reported that the consumption of algae like laver by Koreans is responsible for the normal vitamin B₁₂ status among them. *Watanabe et al., (2002)* also reported the role of different algae in preventing the vitamin B₁₂ deficiency.

iii. Prevalence of vitamin B₁₂ deficiency

Pawlak et al., (2014) reported the deficiency prevalence of vitamin B₁₂ among infants reached 45%. The deficiency among the children and adolescents ranged from 0 to 33.3%. Deficiency among pregnant women ranged from 17 to 39%, dependent on the trimester. Adult and elderly individuals had a deficiency range of 0–86.5%. Higher deficiency prevalence was reported in vegans than in other vegetarians. A study by *Finkelstein et al., (2017)* reported the vitamin B₁₂ status of south Indian pregnant women and their baby is lower. At baseline (<14 weeks of gestation), 51% of mothers were vitamin B₁₂ deficient (vitamin B₁₂<150 pmol/l) and 43% had impaired vitamin B₁₂ status (vitamin B₁₂<150 pmol/l and methyl malonic acid >0.26 µmol/l); 44% of infants were vitamin B₁₂ deficient at 6 weeks of age. *Mittal et al., (2017)* reported the prevalence of B₁₂ deficiency in infants was found to be 57%. Forty-six per cent of mothers were deficient and a positive correlation between the B₁₂ levels of the infants and their mothers. Particularly there is a high prevalence of vitamin B₁₂ deficiency in Indian infants and their mothers.

iv. Complications of vitamin B₁₂ deficiency

Watkins and Rosenblatt and Thompson *et al.*, (2009) found that neural tube defect including spina bifida, anencephaly and encephalocele in vitamin B₁₂ deficiency as vitamin B₁₂ act as a cofactor for methionine synthase in the folate cycle. Mobasheri *et al.*, (2010) reported neural tube defects in infants due to the vitamin B₁₂ deficiency of mothers. Smith and Refsum, (2009) observed cognitive decline in vitamin B₁₂ deficiency. Raised methyl malonic acid concentrations are associated with cognitive decline and Alzheimer's Disease (Lewis *et al.*, 2005). Hin *et al.*, (2006) and Garrod *et al.*, (2008) mentioned that raised methylmalonic acid leads to the cognitive decline among the elderly. In older people with low vitamin B₁₂ status, a high serum folate concentration was associated with increased odds of cognitive impairment, but in subjects with normal vitamin B₁₂ status, high serum folate was found to be protective against cognitive impairment (Morris *et al.*, 2007).

v. Vegetarianism and vitamin B₁₂ deficiency

Recently, the vegetarian eating style has increased in popularity, with 10% of the population opting to exclude animal foods from their diet (Eurispes, 2016). Allen, (2008) mentioned that vitamin B₁₂ deficiency is common among vegetarians and is the result of a very low intake of animal foods. Herrmann *et al.*, (2003), reported that consumption of milk products is not sufficient to maintain a normal vitamin B₁₂ status among vegetarians. Vegetarians and vegans are at high risk for developing a vitamin B₁₂ deficiency because they do not ingest foods of animal origin, such as meat and dairy products, which contain vitamin B₁₂. It was once thought that only strict vegans lacked adequate intake of vitamin B₁₂ containing foods, however, it is now known that deficiencies can be found among persons observing all types of vegetarian diets, including lacto-ovo-vegetarians (Pawlak *et al.*, 2014).

III. METHODOLOGY

The methodology followed for the present study entitled “**Effect of *Chlorella vulgaris* in improving the vitamin B₁₂ status among the vegetarian obese and overweight young adult women**” is presented as follows:

- A. Selection and procurement of sample
- B. Analysis of proximate principles, phytochemicals, antimicrobial activity and toxic substances in *Chlorella vulgaris*
- C. Conduct of supplementation study
 - i. Selection of area and participants
 - ii. Formulation of questionnaire and eliciting the background information
 - iii. Imparting nutrition education to the selected participants
 - iv. Anthropometric assessment of participants
 - v. Conduct of supplementation study
- D. Statistical analysis and interpretation of data

A. Selection and procurement of sample

Sample selected for the study is *Chlorella vulgaris*. *Chlorella vulgaris* is a eukaryotic, unicellular green algae. *Chlorella vulgaris* is estimated to have been on earth for more than 2.5 billion years (Sacasa, 2013). The sample is selected based upon the previous research which have been done on the vitamin B₁₂ content. No fungi, plants or animals (including humans) are capable of producing vitamin B₁₂. Only bacteria and archaea have the enzymes needed for its synthesis (Linus Pauling Institute., 2014). Plant sources have very little amount of vitamin B₁₂, so vegetarians are more likely to suffer from vitamin B₁₂ deficiency (Kam *et al.*, 2014). Vegetarians are usually deficient in serum vitamin B₁₂ because their only source of vitamin B₁₂ is dairy products and studies also proved that dairy products doesn't meet the daily requirement of vitamin B₁₂ (Herrmann *et al.*, 2003). The daily requirements of vitamin

B₁₂ is 2.4 µg (WHO, 2005). *Chlorella vulgaris* contain 29.87±2µg/100g and 26.84±2µg/100 g dry weight of vitamin B₁₂ in the form of methylcobalamin which is an active vitamin B₁₂ (Kumudha *et al.*, 2015). Among the different species of *Chlorella*, *Chlorella pyrenoidosa* and *Chlorella vulgaris* seems to contain highest vitamin B₁₂ content. According to the ease and access of *Chlorella vulgaris*, this species was chosen for the present study.

Chlorella vulgaris was procured in the form of dry powder from Divy Agro Industries, New Delhi. Divy Agro Industries produces the best quality *Chlorella vulgaris* with affordable price. This is a sun dried form of *Chlorella vulgaris* and should be stored in a cool and dry place (Plate 1).

B. Analysis of proximate principles, phytochemicals, antimicrobial activity and toxic substances in *Chlorella vulgaris*

Chlorella vulgaris was analysed for the following, namely, proximate principles including ash, protein, fibre, total carbohydrate, starch, vitamin C, calcium, phosphorous, iron, and vitamin B₁₂, (Plate 1 and 2) phytochemicals like tannin, terpenoids, phenol, saponins, quinone, glycosides, coumarin, sterol, flavonoid and alkaloid and antimicrobial activity and toxic substances.

Proximate principles

Proximate analysis are the procedures which are followed for compounds occurring naturally in animal and vegetable tissues and separable by analytical methods, the proximate principles of food are protein, fat, carbohydrates, mineral salts, and water (Merriam Webster, *medical dictionary*, 2018). The principle and procedure followed for estimation of proximate principles are:

Ash: By continuous heating, the substance gets charred which can be used for the determination of minerals present (AOAC, 1990).

Protein: The given sample is digested with concentrated sulphuric acid in a macrokjeldahl flask when nitrogen gets converted to ammonium sulphate.

Ammonia is liberated by the action of strong alkali in a macrokjeldahl steam distillation apparatus. This nitrogenous substance is converted to ammonium borate by absorbing 2% boric acid and is titrated against N/70 H₂SO₄. The volume of acid required to bring the test sample to the colour of the blank gives the acid equivalent to the ammonia (AOAC, 1990).

Fibre: The term "crude fibre" ordinarily meant in agriculture and food analysis is the organic residue consisting largely of cellulose, that is left after other carbohydrates and proteins have been removed by successive treatment with boiling acids and alkalies. The crude fibre obtained in this way is not cellulose but contains distinct properties of hemicellulose and nitrogenous substances. These however are not sufficient to prevent the results from being reasonably accurate and comparable (AOAC, 1990).

Total carbohydrate: In hot acidic medium, glucose is dehydrated to hydroxymethyl furfural. This forms a green colored product with phenol and has absorption maximum at 490nm (Dubois *et al.*, 1953 and Krishnaveni *et al.*, 1984).

Starch: The sample is treated with 80% alcohol to remove sugar and then starch is extracted with perchloric acid. In hot acidic medium, starch is hydrolysed to glucose and dehydrated to hydroxyl methyl perforate. This compound forms a green colour product with anthrone (AOAC, 1990).

Vitamin C: Vitamin C is a good reducing agent and it reduces the dye 2,6 dichlorophenol indophenol. In this reaction, the ascorbic acid itself is oxidised to dehydro ascorbic acid. In the absence of interfering substances, the capacity of an extract of the sample to reduce a standard solution of the dye as determined by titration is directly proportional to the vitamin C content. Oxalic acid is not only used to reduce the pH of the extracting medium, thereby establishing vitamin C but also form complexes with metals (AOAC, 1990).

Calcium: Calcium is determined by precipitating it as calcium oxalate and titrating the oxalate solution in dilute sulphuric acid against standard potassium permanganate (AOAC, 1990).

Phosphorous: When the ash solution is treated with ammonium molybdate, phosphomolybdic acid is formed. Phosphomolybdic acid is reduced by the addition of 1,2,4 amino naphthol sulphonic acid reagent to produce a blue colour which is apparently a mixture of oxides of molybdenum. The intensity of the colour developed is the measure of phosphorus present (AOAC, 1990).

Iron: The food sample is oxidized with ignition or oxidation. Iron as ferric iron reacts with ammonium thiocyanate or with potassium thiocyanate to give ferric thiocyanate which is red in colour. The colour which is a measure of the concentration is measured colorimetrically (AOAC, 1990).

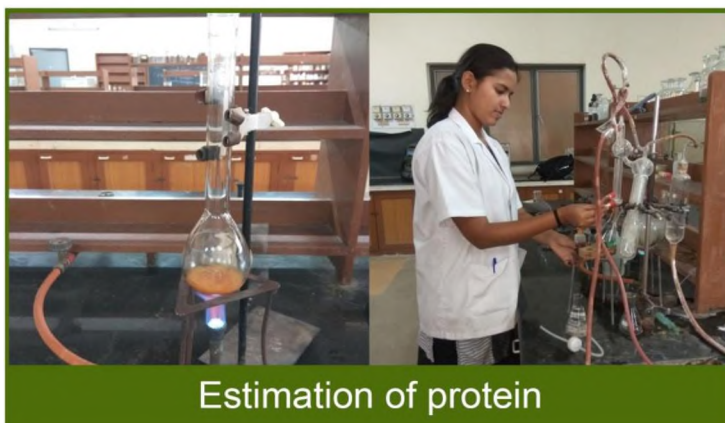
Vitamin B₁₂ : Decomposition of vitamin B₁₂ by HNO₃ followed by subsequent formation of a stable colored complex (λ max 435 nm) between the liberated cobalt ion and Nitroso-R-salt (Ahmed *et al.*, 2003) (Plate 2 and 3).



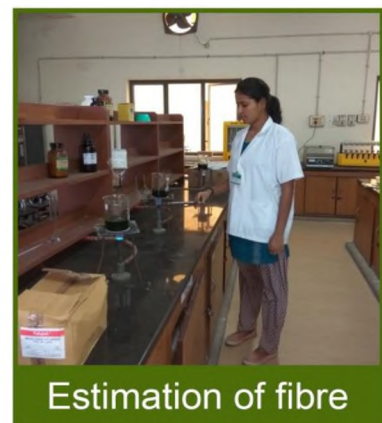
Sun dried Chlorella vulgaris powder



Estimation of ash



Estimation of protein



Estimation of fibre

Plate:1

Sun dried chlorella powder and analysis of proximate principles

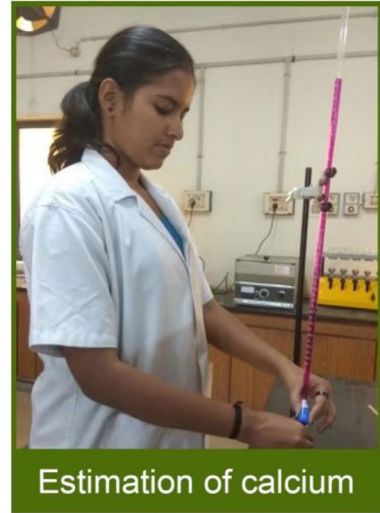


Plate: 2
Analysis of proximate principles

Phytochemical analysis

Plants are composed entirely of chemicals of various kinds (Andrew, 2017). Phytochemicals (from Greek *phyto*, meaning "plant") are chemicals produced by plants through primary or secondary metabolism (Russel *et al.*, 2007 and Jeffrey *et al.*, 1999). The phytochemical analysis of the medicinal plants are important and have commercial interest in both research institutes and pharmaceutical companies for the manufacturing of the new drugs for treatment of various diseases (Wadood *et al.*, 2013).

Preparation of phytochemical extracts

a. Aqueous extract

The pulverized material was used to prepare an infusion in hot (95°C) distilled water. The infusion was left overnight under refrigeration (4°C) to prevent any possible contamination. After 24 hours, the extracts was kept in a rotary shaker at 100 rpm for one hour and filtered with Whatman No.1 filter paper (Doughari , 2012).The aqueous extract of the *Chlorella vulgaris* was prepared using the above standard method.

b. Solvent extract

The pulverized material was mixed with sufficient quantity of solvents viz., methanol and ethanol. It was kept in rotary shaker at 100 rpm overnight and filtered with Whatman No.1 filter paper (Doughari *et al.*, 2012).The methanol and ethanol extracts of *Chlorella vulgaris* was prepared using the above standard method.

The phytochemical analysis was done with aqueous and solvent extracts (methanol and ethanol) of *Chlorella vulgaris* (Plate 3). The phytochemicals analysed are:

Tannins: About 0.5g of the dried power was boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride was added and was observed for brownish green or a blue black coloration (AOAC, 2005).

Terpenoids: Ten milliliter aqueous extract was placed in a small beaker and evaporated to dryness. The residue was dissolved in 0.5 ml each of acetic anhydride and chloroform. The solution was transferred into a dry test tube and concentrated sulphuric acid was added. Brownish red or violet rings at the zone of the contact with the supernatant and green or violet coloration denoted the presence of sterols and triterpenes (AOAC, 2005).

Phenol (Ferric chloride test): To 2ml of plant extract, 2ml of distilled water followed by 10 % FeCl₃ solution was added. Bluish black colour indicates the presence of phenol (Kumar *and* Yadav, 2015).

Saponins: 0.5 g extract were dissolved in 10ml of distilled water in a test tube was stoppered with a cork and shaken vigorously for about 30 seconds. The test tube was allowed to stand in a vertical position and observed over a 30 minutes period of time. If a honey comb froth above the surface of liquid persists after 30 minutes the sample is suspected to contain saponin (AOAC, 2005).

Quinone: The test solution was treated with a few drops of concentrated sulphuric acid or aqueous sodium hydroxide solution. Colour formation indicates the presence of quinone compound (AOAC, 2005).

Glycosides: 0.5g extract of sample was dissolved in 1 ml water and then aqueous sodium hydroxide was added. Formation of yellow color indicated the presence of glycosides (AOAC, 2005).

Coumarin: To 2 ml of the test solution, a few drops of alcoholic sodium hydroxide were added. Appearance of yellow colour indicates the presence of coumarin (AOAC, 2005).

Sterol (Sulphuric acid Test): To the plant extracts 2 ml of chloroform was added. 2 ml of conc. H₂SO₄ was added by the sides of the test tube and observed for red colour at the lower chloroform layer (Kumar *and* Yadav, 2015).

Flavonoid (Shindo's test): To the test solution, a few magnesium turnings and a few drops of concentrated hydrochloric acid were added and boiled for five minutes.

Appearance of red or orange red colour indicates the presence of flavonoids (AOAC, 2005).

Alkaloids: One ml of aqueous extract was stirred and placed in 1% aqueous hydrochloric acid on a steam bath. Then, 1 ml of the filtrate was treated with Dragendorff's and Mayer's reagent. Turbidity or precipitation with this reagent was considered as evidence for the presence of alkaloids (AOAC, 2005).

Antimicrobial activity

Principle: Paper discs impregnated with specific antibiotics or the test substances were placed on the surface of the Muller Hinton Agar or Rose Bengal chloramphenicol inoculated with the target organisms. The plates were incubated and the zones of inhibition around each disc was measured (Plate 3).

Reagents

- a. **Muller Hinton Agar Medium (MHA):** The medium was prepared by dissolving 33.9 g of Muller Hinton Agar Medium along with 1 gram of agar agar in 1000 ml of distilled water. The dissolved medium was autoclaved at 15 lbs pressure at 121°C for 15 min (pH 7.3). The autoclaved medium was cooled, mixed well and poured onto 100 mm petriplates (25 ml/plate).
- b. **Rose Bengal Chloramphenicol (RBC):** The medium was prepared by dissolving 32.15 g of Rose Bengal Chloramphenicol medium along with 1 gram of agar agar in 1000 ml of distilled water. The dissolved medium was autoclaved at 15 lbs pressure at 121°C for 15 min (pH 7.3). The autoclaved medium was cooled, mixed well and poured onto 100 mm petriplates (25 ml/plate).

Procedure

- Fresh culture to be tested was completely swabbed on the surface of the agar plates.
- The provided antibiotic discs were placed on the surface with the help of sterile forceps. Care was taken to ensure that the zone formation to an antibiotic did not interfere with another.

- The plates were incubated at 37° C for 24 hours for antibacterial test and for antifungal test, the plates are kept in the room temperature.
- Zone of inhibition was measured with the help of a meter scale and compared with the given standard chart (Bauer *et al.*, 1966) (Plate 3).

All the tests was done in triplicates in order to remove any deviations and to have concordant values in the result.

Analysis of toxic substances

Chlorella vulgaris is analysed for determination of toxic substances. The toxic substances analysed are mycotoxins such as *Aflatoxin* and *Ochratoxin* the common mycotoxins found in food (Samuel *et al.*, 2016).

a. Determination of Aflatoxins

Principle of LC/MS: HPLC, with the detection power of mass spectrometry. Even with a very sophisticated MS instrument, HPLC is still useful to remove the interferences from the sample that would impact the ionisation. Interface that will eliminate the solvent and generate gas phase ions, then transferred to the optics of the mass spectrometer (FSSAI, 2012).

b. Determination of Ochratoxin

Principle: Test portion is extracted by blending with acetonitrile–wa-ter. The extract is cleaned up by passing through an immunoaffinity column. Ochratoxin A (OTA) is eluted with methanol, further purified and identified by LC, and quantified by fluorescence (Entwisle *et al.*, 2000).



3 extracts of *Chlorella vulgaris*



Extracts in shaker system



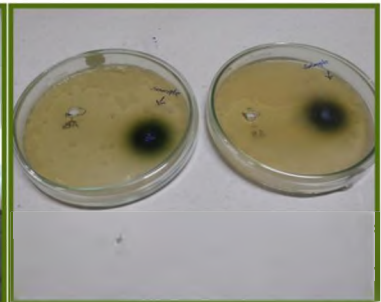
Pytochemical screening of Aqueous, methanolic and ethanolic extracts



Medium



Preparation of petriplates



Antibacterial activity



Antifungal activity

Plate: 3

Phytochemical analysis and antimicrobial activity of *Chlorella vulgaris*

C. Conduct of supplementation study

i. Selection of area and participants

The area selected for the study was Coimbatore city which is known as the education hub. Selected areas included are Chinthamani nagar, Raja Annamalai street of Saibaba Colony and Ramalingam colony. Chinthamani Nagar, is an area in Saibaba Colony, Coimbatore where majority of the population are vegetarians.

Participants selected for the study are young adult women of age group 18-24 years since vitamin B₁₂ is particularly an important vitamin for women of child bearing age. Only vegetarians are selected and non vegetarians are excluded as vitamin B₁₂ deficiency is prevalent among vegetarians only. Women with normal weight are excluded from the study. And only the obese and overweight individuals are selected for the study as the vitamin B₁₂ deficiency is more common among obese and overweight individuals. The samples for the study are selected by purposive sampling method. A purposive sample is a non probability sample that is selected based on characteristics of a population and the objective of the study. Purposive sampling is also known as judgemental, selective or objective sampling (Crossman, 2017).

A total of 40 vegetarian young adult women of age 18-24 years, who are either overweight or obese were selected for the study. Hence, the investigator selected purposive sampling technique to select the samples for the study with the following inclusion and exclusion criteria:

Inclusion criteria: Young adult women

- of age 18-24 years
- who are vegetarian
- who are either overweight or obese
- who are willing to co-operate in the study

Exclusion criteria: Young adult women

- less than 18 and more than 24 years
- who are non vegetarians
- who are normal weight and underweight

ii. **Formulation of questionnaire and eliciting the background information**

A questionnaire is a list of questions that several people are asked so that information can be collected about something (Cambridge.org. dictionary). The tool selected for this study was a close ended questionnaire, which was formulated by the investigator herself for primary data collection. Any question which can be answered with a simple "yes" or "no", or with a specific piece of information is said to be the close ended questions (yourdictionary.com, 2017).

The questionnaire was divided into four sections , namely socio economic background, physical activity pattern, dietary and food consumption pattern and nutrition knowledge. A structured and reviewed questionnaire was used to collect background information and assess the nutritional knowledge of the target population (Appendix I).

Data was collected in two phases namely pre awareness and post awareness phase by administering questionnaire personally to the individual respondent by the investigator herself. Primary data on socio economic background, physical activity pattern, dietary and food consumption pattern, nutrition knowledge was collected using close ended questionnaire, prior to imparting nutrition education. Post evaluation was done by administering the same questionnaire to the participants consisting of questions on various aspects of nutrition education given and also the same questions which are used to assess the nutritional knowledge.

In the study, the socio economic status was measured by education and income level. Physical activity pattern was assessed by the activity in which the participants are involved . Nutritional status was assessed by dietary and food consumption pattern whereas nutrition knowledge status for both pre awareness and post awareness was measured by giving points based on correctly answered questions . Each question answered correctly was attributed one point. Wrong answers did not receive any score.

iii. Imparting nutrition education to the selected respondents

Nutrition education was imparted to the 40 adult young women who are the participants of the supplementation study using two ways - power point presentation for every 15 days for 3 times and whatsapp messages everyday for a period of 50 days. The nutrition education was imparted using power point presentation as the tool using the laptop. Once in every 15 days one main topic was covered with 10 slides . The slides are prepared by the investigator. The another way of giving education was using whatsapp as the tool, using the android mobile phone service of instant messaging system. Each day one message was sent, consisting of 450-500 character including special character and spaces. The messages are tailored by the investigator. The investigator sends message between 8 and 9 am in the morning according to the time preference of the respondents (Plate 4).

The information obtained during the pre awareness phase was utilized for preparing the content where questions relevant to general as well as on vitamin B₁₂ and its importance was answered. Since the time period of nutrition education was 50 days, three power point presentations (every 15 days for 3 times) and a total of 50 messages was framed for e-nutrition education. Each message was drafted within 450-550 characters, notations and spaces. Words were chosen carefully to make the meaning of the message understandable for the respondents in simple way as possible. The contents for the PPT and Whatsapp messages were taken from articles and journals.

iv. Anthropometric assessment of participants

Anthropometric measurements are body measurements and provide information on body muscles mass and fat reserves. A variety of anthropometric measurements can be made either by covering the whole body or parts of the body (Srilakshmi, 2012).

The anthropometric measurements which are measured in the study are: weight, height, BMI, waist circumference, hip circumference, WHR and WtHR (Plate 5).

Weight: Human body weight refers to a person's mass or weight. Body weight is measured in kilograms, a measure of mass, throughout the world (https://en.wikipedia.org/wiki/Human_body_weight). The weight of the participants are taken using a weighing machine. The weighing machine is placed on firm flooring. The heavy clothing, shoes or chappal of the participants was removed and the participants are made to stand with bare feet in the center of the scale, and the weight is recorded to the nearest decimal fraction.

Height: The height of a person or thing is their size or length from the bottom to the top (<https://www.collinsdictionary.com/dictionary/english/height>). To measure the height, the participants were asked to stand with barefoot with minimum clothing and unbraided hair that interferes with the measurement. The height was taken on flooring that is not carpeted and against a flat surface such as a wall with no molding. It was made sure by the investigator that the legs are straight, arms at sides and shoulders are level. The measurement was taken while the participant stands with head, shoulders, buttocks and heels touching the flat surface (wall). And a headpiece was used to form a right angle with the wall and lowered the headpiece until it firmly touches the crown of head. It was made sure by the investigator that the eyes are at the same level as the headpiece. And the investigator made a mark lightly where the bottom of the headpiece met the wall. A metal tape was used to measure from the base on the floor to the marked measurement on the wall to get the height measurement. The height was accurately recorded to the nearest 0.1 centimeter.

Body Mass Index: The BMI or Body Mass Index (BMI) is a simple index of weight-for-height that is commonly used to classify underweight, overweight and obesity in adults. It is defined as the weight in kilograms divided by the square of the height in metres (kg/m^2) (WHO, 2006). The body mass index of the participants was calculated using the weight and height which was measured.

$$\text{BMI} = \frac{\text{Weight (kg)}}{\text{Height (m}^2\text{)}}$$

Waist circumference: Waist circumference is a measurement taken around the abdomen at the level of the umbilicus (belly button) (Frey, 2017). Waist circumference should be measured at the midpoint between the lower margin of the least palpable rib and the top of the iliac crest, using a stretch resistant tape that provides a constant 100 g tension (WHO, 2008). To measure the waist circumference the participant was asked to stand with feet close together, arms at the side and body weight evenly distributed, and with little clothing. During measurement, the participant was relaxed, and the measurement was taken at the end of a normal expiration. Each measurement was repeated twice; and if the measurements was within 1 cm of one another, the average was calculated. If the difference between the two measurements exceeded 1 cm, the two measurements was repeated until the accurate value was obtained . The value above 88 cm is considered as a higher waist circumference for women (NHLBI, 2000).

Hip circumference: A person's hip circumference is measured at the level of maximum posterior extension of the buttocks (Australian institute of health and welfare, 2005). The criteria followed for measuring the waist circumference was followed in measuring the hip circumference also.

Waist Hip Ratio (WHR): The Waist Hip Ratio (WHR) is the ratio of waist circumference to hip circumference (Hara, 2013). WHR is used as a measurement of obesity which in turn is a possible indicator of health conditions. WHO states that abdominal obesity is defined as a waist-hip ratio above 0.9 for males and above 0.8 for females (WHO, 2008) as the cut off values. WHR of the participants was calculated using the measured waist circumference and hip circumference.

Waist-to-Height Ratio (WtHR): A person's waist-to-height ratio (WtHR), also called waist-to-stature ratio (WSR), is defined as their waist circumference divided by their height, both measured in the same units. The WtHR is a measure of the distribution of body fat. Higher values of WtHR indicate higher risk of obesity-related cardiovascular diseases; it is correlated with abdominal obesity (Lee *et al.*, 2008).The

WtHR of all the participants was calculated using the measured waist circumference and height. A WHtR cut off of 0.5 can be used in different sex and ethnic groups and that the same cutoff can be applied in children and adults (Ashwell *et al.*, 2012).

v. Conduct of supplementation study

The study is an experimental placebo study ; and therefore the subjects are divided into two groups, namely the experimental group and placebo group with the proportion of 1:1 ratio.

A sample size of 40 respondents was included in this study. Thus a total of 20 respondents were included each in the experimental group and the same number of participants in placebo group. The participants are grouped according to their willingness to take either *Chlorella vulgaris* juice or curd. Informed consent was obtained from all the participants regarding their willingness for the supplementation and for biochemical analysis.

Biochemical assessment

Biochemical assay was done for all the participants of the study to test the hemoglobin status and vitamin B₁₂ status. For women, 12.0 to 15.5 grams per deciliter is the normal range of blood hemoglobin (<https://www.mayoclinic.org/tests-procedures/hemoglobin-test/about/pac-20385075>) and the vitamin B₁₂ reference range is 211-911 pg/ml.

The biochemical assay of blood hemoglobin and vitamin B₁₂ was taken twice namely before supplementation and after supplementation of 50 days to see the effect of supplementation. Two ml of blood was collected from the participants before and after supplementation for the biochemical assay.

To draw blood, participants were asked to relax themselves to locate the vein easily. The palm of the participant was faced up. Then the vein was located and then the area was cleaned with an alcohol swab. A tourniquet was tied in the arm to help blood collect. The needle was positioned at a 15 degree angle and stick it into the skin and blood was collected in two tubes, one for hemoglobin and another for

vitamin B₁₂. The blood sample was collected from the participants with the help of a trained lab technician (Plate 5).

Heamoglobin: The heamoglobin was detected with SLS- Heamoglobin method. This method uses Sodium Lauryl Sulfate (SLS), a surfactant. Presumably SLS will convert hemoglobin into methemoglobin in the order of oxyhemoglobin, hemochrome and methemoglobin, and its oxidative activity. Therefore, unlike others, this method does not need oxidative reagents and does not generate toxic wastes such as KCN and NaN₃ which cause environmental pollution. The proposed method is applicable for the manual and Hemalog-8/90 procedures often used in clinical laboratories (Oshiro *et al.*, 1982).

Vitamin B₁₂: Serum Vitamin B₁₂ was detected using fully automated bidirectionally interfaced chemi luminescent immunoassay. In general, luminescence is the emission of visible or near-visible ($\lambda = 300\text{--}800\text{ nm}$) radiation which is generated when an electron transitions from an excited state to ground state. The resultant potential energy in the atom gets released in the form of light. In spectrophotometry, luminescence has an advantage over absorbance in that the former is an absolute measure whereas the latter is relative (Cinquanta *et al.*, 2017).

24 hour dietary recall

24 hour dietary recall of the participants is taken for a the period of three days. A 24-hour diet recall is a dietary assessment tool that consists of a structured interview in which participants are asked to recall all foods and drinks they have consumed in the previous 24 hours and is self-administered (Russel, 2006). In the interview, participants are asked to describe the foods and drinks they have consumed in the previous 24 hours; the participants are asked to provide more detail than what was initially provided. The open-ended nature of the interview was intended to help produce the most detailed description of foods and drinks consumed over the previous 24 hours. Details might include time of day, source of food and portion size of food. A 24-hour diet recall is typically completed in 20–60 minutes .

Supplementation to the selected participants

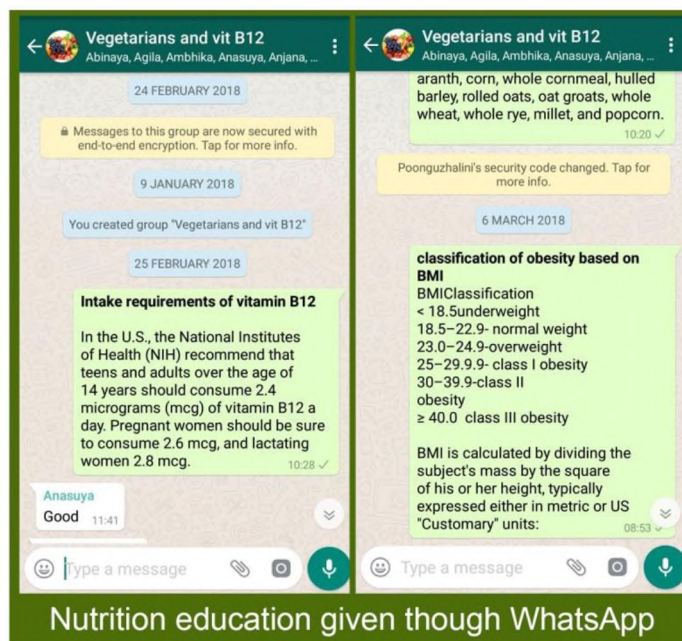
A supplementation study was conducted for the period of 50 days. The supplementation given for experimental group and placebo group were *Chlorella vulgaris* juice and curd respectively. *Chlorella vulgaris* juice was prepared by adding three grams chlorella in 200 ml of water containing 25 g of palm jaggery / person. Three grams of *Chlorella* contains about 120% of the recommended daily amount of vitamin B₁₂ (<https://www.echlorial.fr/blog/video-chlorella-definition-proprietes-sante>). The *Chlorella vulgaris* juice was given to experimental group (N=20) for a period of 50 days.

The *Chlorella vulgaris* was given in the form of juice as it was easy to prepare and consumption was also easy as it is sweet in taste. Since the participants are overweight and obese table sugar was not used due to the empty calories in sugar. Jaggery is sugarcane based natural sweetener made by the concentration of sugarcane juice without any use of chemicals. It contains the natural sources of minerals and vitamins inherently present in sugarcane juice and it is one of the most wholesome and healthy sugars in the world (Nath *et al.*, 2015). Jaggery has proved itself better as compared to white sugar (Shrivastav, 2016).

To the placebo group 200 ml of curd was given. The curd was given for placebo group as curd is also rich in vitamin B₁₂ and milk product seem to be the only source of vitamin B₁₂ for vegetarians . 200 ml of curd contain 0.6 µg of vitamin B₁₂ (Canadian nutrient file, 2015). Curd was supplemented to the placebo group for the duration of 50 days as that of the experimental group (Plate 6).



Nutrition education given through PPT



Nutrition education given though WhatsApp



Measurement of weight and height

Plate: 4

Nutrition education and anthropometric measurements



Plate: 5
Anthropometric measurements and collection of blood sample



Measured chlorella vulgaris



Chlorella vulgaris juice



Participants receiving Chlorella vulgaris juice



Participants receiving Curd

Plate:6
Supplementation to the experimental and placebo group

D. Statistical analysis and interpretation of data

The collected data were consolidated, tabulated and analysed statically to assess the effectiveness of *Chlorella vulgaris* in improving the vitamin B₁₂ among the vegetarian obese and overweight young adult women.

The study was presented before the Institutional Human Ethics Committee of Avinashilingam Institute of Home and Higher Education for Women and approval was obtained. The IHEC approval number is AUW/ IHEC/ FSN-17-18/XPD/03 (Appendix II).

The research design used in the study is presented in Figure I and conduct of supplementation study is presented in Figure II.

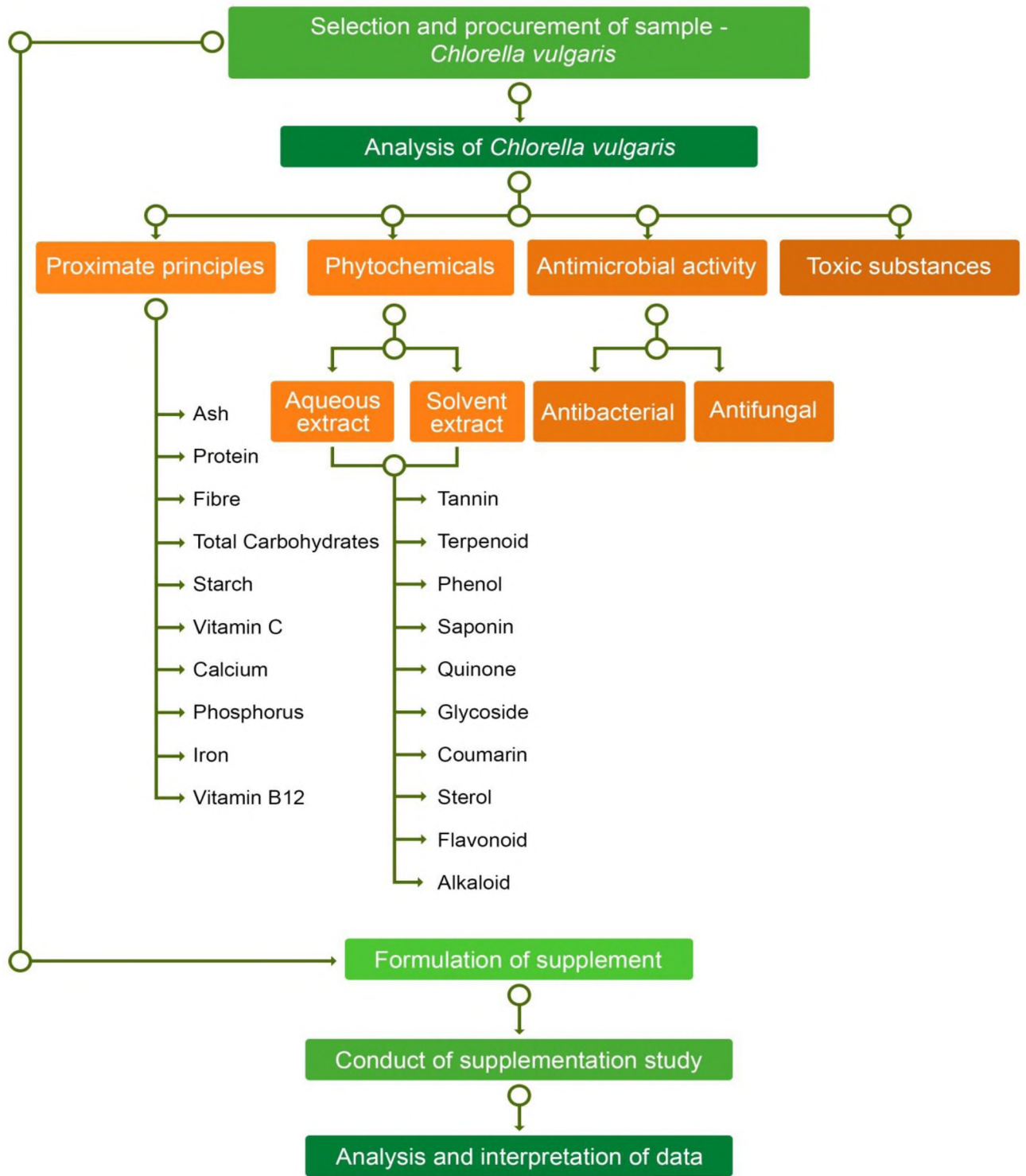


FIGURE 1
Research design

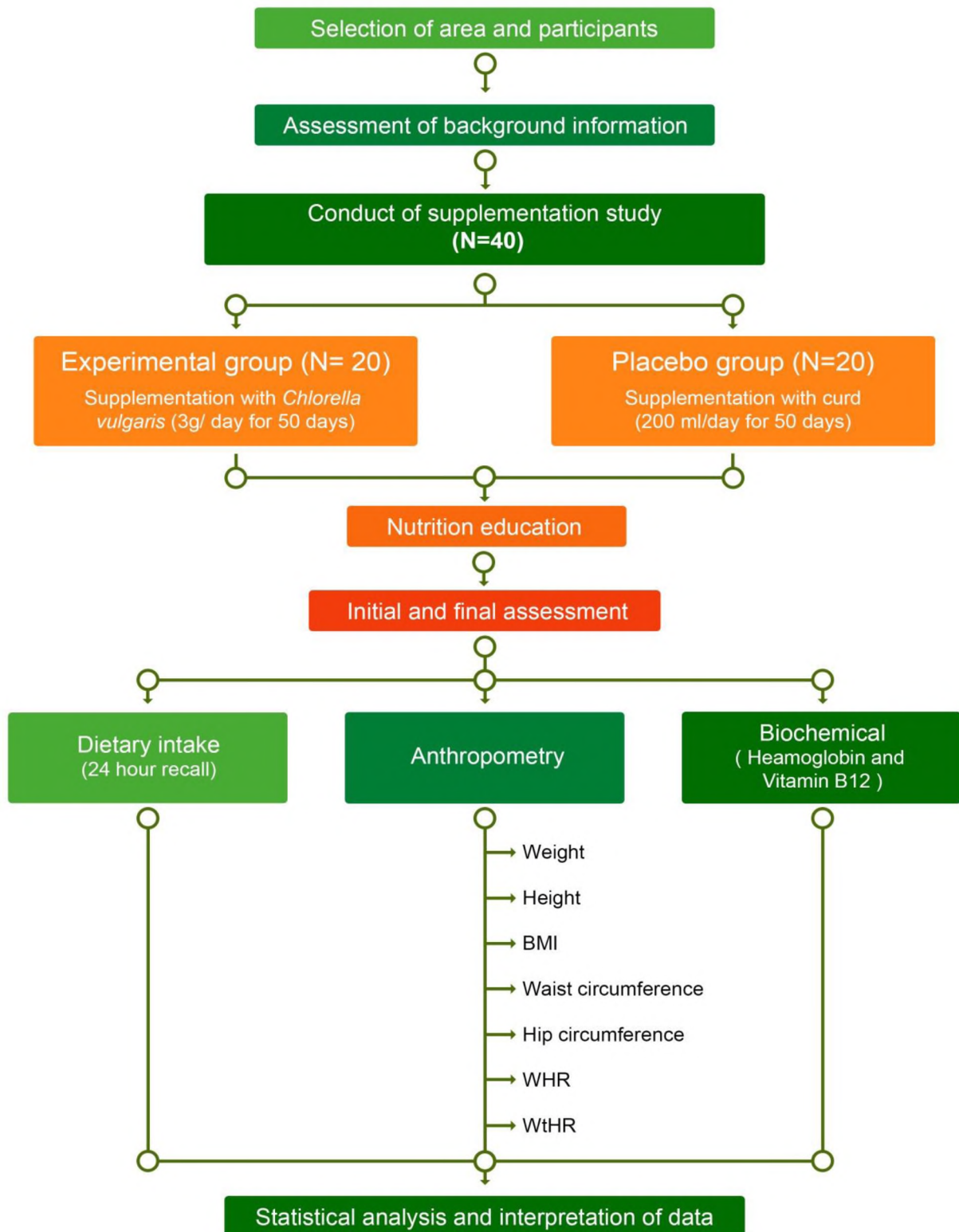


FIGURE 2
Conduct of supplementation study

IV. RESULTS AND DISCUSSION

The results of the present study titled as “ **Effect of *Chlorella vulgaris* in improving the vitamin B₁₂ status among the vegetarian obese and overweight young adult women**”, is presented and discussed under the following headings.

- A. Proximate composition, phytochemicals, antimicrobial activity and toxic substances in *Chlorella vulgaris*
- B. Background information of the young adult women
- C. Dietary intake of young adult women
- D. Impact of nutrition intervention on nutritional knowledge of young adult women
- E. Anthropometric measurements of the young adult women
- F. Effect of supplementation of *Chlorella vulgaris* on the vitamin B₁₂ status of the young adult women

A. Proximate composition, phytochemicals, antimicrobial activity and toxic substances in *Chlorella vulgaris*

i. Proximate composition of *Chlorella vulgaris*

The proximate principles of *Chlorella vulgaris* namely ash, protein, fibre, total carbohydrate, starch, calcium, phosphorus, iron, vitamin B₁₂ and vitamin C was analysed using standard procedures.

Table I reveals the mean proximate composition of dried *Chlorella vulgaris* powder.

TABLE I
PROXIMATE COMPOSITION OF *CHLORELLA VULGARIS*

Proximate principles	Composition/ 100 g
Ash (g)	9.0±1.0
Protein (g)	49.58±0.72
Fibre (g)	10.73±0.11
Total carbohydrate (g)	38.8±0.58
Starch (g)	2.42±0.05
Calcium (mg)	552±4
Phosphorus (mg)	1066.60±23.09
Iron (g)	23.80±0.23
Vitamin B ₁₂ (µg)	28.80±2.0
Vitamin C (mg)	54.58±1.01

Each value represents the mean ± SD of three determinations on dry weight (DW) basis.

From the table it is evident that mean ash content of the *Chlorella vulgaris* is 9 g. The macronutrient composition of *Chlorella vulgaris* constitute highest composition of protein (49.58g), followed by the total carbohydrate (38.8 g), fibre (10.73 g) and starch (2.42 g). And among the micronutrients analysed the highest contribution is by iron, 23.8 g which is phosphorus (1066.6 mg), calcium (552 mg), vitamin C (54.58 mg) and the least is contributed by the vitamin B₁₂, 28.8 µg.

The protein and fibre content is correlated with the findings of Ramraj *et al.*, (2016) with 48.8 g and 17.8 g respectively. In the present study, the total carbohydrate content of *Chlorella vulgaris* is 38.8 g which is lower than it's protein content and the starch contribute to 2.42 g /100 g dry weight. Similarly Habib *et al.*, (2003) analysed for carbohydrate in *Chlorella vulgaris* (30.8 g) and starch 17 per cent of the total nutrients (Bruno *et al.*, 2012). The vitamin B₁₂ content present in *Chlorella vulgaris* is 28.8 µg / 100 g dry weight and vitamin C contributes to 54.58 mg. The vitamin B₁₂ in *Chlorella vulgaris* was 29.87 g (Kumudha *et al.*, 2015) and 161 mg vitamin C (Klinghartacedemy.com). Kent *et al.*, (2015) found out the ash content of *Chlorella vulgaris* as 5.71 g which is nearer to the present analysis. The ash content is generally recognized as a measure of quality for the assessment of the functional properties of food (Hoffman *et al.*, 2002). This in turn, indicated a high content of

minerals such as phosphorus (1066.6 mg/100 g dry weight), followed by calcium (552 mg/100 g dry weight) and iron (23.8 g/100 g dry weight). The findings were similar to that of the Radhakrishnan *et al.*, (2017) as 1761.5 mg, 593.7 mg and 259.1 mg respectively but the iron content is lower in the present study.

ii. Phytochemicals of *Chlorella vulgaris*

Chlorella vulgaris was analysed for the phytochemicals namely tannin, terpenoid, phenol, saponin, quinone, glycoside, coumarin, sterol, flavonoid and alkaloid in aqueous, methanol and ethanol extract.

Table II shows the phytochemicals present in *Chlorella vulgaris*.

TABLE II
PHYTOCHEMICALS OF *CHLORELLA VULGARIS*

Phytochemicals	Aqueous extract	Methanolic extract	Ethanol extract
Tannin	+++	++	+
Terpenoid	-	+	-
Phenol	+++	++	++
Saponin	+++	+	++
Quinone	+	-	-
Glycoside	+	+	+
Coumarin	+++	-	-
Sterol	+	++	+
Flavonoid	+++	+	-
Alkaloid	-	-	-

+++ : Highly present, ++ : moderately present, + : Low, - : absent

The present study revealed that the aqueous, methanolic and ethanolic extracts of *Chlorella vulgaris* contain tannin, terpenoid, phenol, saponin, quinone, glycoside, coumarin, sterol and flavonoid.

The aqueous extract of *Chlorella vulgaris* showed the presence of tannin, phenol, saponin, quinone, glycoside, coumarin, sterol and flavonoid. Terpenoid and

alkaloid was absent in aqueous extract of *Chlorella vulgaris*. Tannin, phenol, saponin, coumarin and flavonoid was highly present in aqueous extract and the presence of quinone, glycoside and sterol was found to be low.

The methanolic extract of the *Chlorella vulgaris* showed the presence of tannin, terpenoid, phenol, saponin, glycoside, sterol and flavonoid. Quinone, coumarin and alkaloid was absent in methanolic extract of *Chlorella vulgaris*. Tannin, phenol and sterol was moderately present and the presence of terpenoid, saponin, glycoside and flavonoid was found to be low. Tannin, phenol, saponin, glycoside and sterol was found to be present in ethanolic extract of *Chlorella vulgaris*. Phenol and saponin was moderately present in ethanolic extract and the presence of tannin, glycoside and sterol was found to be low. Terpenoid, quinone, flavonoid and alkaloid was absent in ethanolic extract of *Chlorella vulgaris*.

The present study showed that the phytochemicals in *Chlorella vulgaris* is highly found in the aqueous extract followed by methanolic and ethanolic extract. And majority of phytochemicals is found in aqueous extract. Similarly Adhoni *et al.*, (2016) analysed *Chlorella vulgaris* for its phytochemical content and found that alkaloids was absent in all the three extracts namely aqueous, methanol and ethanol. Tannins, glycosides, saponin and sterol is found to be present in all the three extracts of *Chlorella vulgaris*. Terpenoid is found to be present only in methanolic extract. Geetha *et al.*, (2010) found the presence of quinones and coumarin in aqueous extracts of *Chlorella vulgaris*.

iii. Antimicrobial activity of *Chlorella vulgaris*

Antimicrobial activity of *Chlorella vulgaris* was analysed against bacteria and fungi. The activity of *Chlorella vulgaris* was compared with a control having antibacterial and antifungal activity.

Table III shows the antimicrobial (antibacterial and antifungal) activity of *Chlorella vulgaris*.

TABLE III

ANTIMICROBIAL ACTIVITY OF *CHLORELLA VULGARIS*

Name of the organism	Zone of inhibition (mm)	
	Control	Aqueous extract
Bacteria		
<i>E.coli</i>	13	8
<i>Staphylococcus aureus</i>	12	10
Fungi		
<i>Aspergillus niger</i>	6	9
<i>Aspergillus flavus</i>	8	13

The above table presents the antimicrobial activity namely antibacterial and antifungal activity of *Chlorella vulgaris*. The bacteria selected are *E.coli* (Gram -ve) and *Staphylococcus aureus* (gram +ve). The aqueous extract of *Chlorella vulgaris* was found to contain a zone of inhibition of 8 mm against *E.coli* and 10 mm against *Staphylococcus aureus*. The control showed a higher zone of inhibition than the *Chlorella vulgaris* extract. The antifungal activity of aqueous extract of *Chlorella vulgaris* was tested with fungi namely *Aspergillus niger* and *Aspergillus flavus* and showed a zone of inhibition of 9 mm and 13 mm respectively. The present study showed a higher antifungal activity of *Chlorella vulgaris* than the control. The control showed the antifungal activity of 6 mm and 8 mm for *Aspergillus niger* and *Aspergillus flavus* respectively.

Similarly the findings of Adhoni *et al.*, (2016) is well correlated with the present study of antibacterial activity of *Chlorella vulgaris* that is 10 mm zone of inhibition for both *E.coli* and *Staphylococcus aureus*. Adhoni *et al.*, (2016) reported the antifungal activity of *Chlorella vulgaris* (aqueous extract) against *Aspergillus niger* with a zone of inhibition of 6 mm. In another study by Syed *et al.*, (2015) the ethanolic extract of the *Chlorella vulgaris* showed highest antibacterial activity against *E.coli* with a zone of inhibition of 15 mm. In the present study, it can be concluded that *Chlorella vulgaris* shows higher antifungal activity than the antibacterial activity.

iv. Toxic substance in *Chlorella vulgaris*

Table IV presents the toxic substance (mycotoxins) composition in *Chlorella vulgaris*.

TABLE IV
TOXIC SUBSTANCE IN *CHLORELLA VULGARIS*

Mycotoxins	Composition
Aflatoxin	14.0 ppb*
Ochratoxin	BDL**

The above values are approximately to 100 g, ppb*- parts per billion, BDL**- Below Detectable Level

The above table shows the composition of mycotoxins present in *Chlorella vulgaris* namely the aflatoxin and ochratoxin. The aflatoxin and ochratoxin are the common toxins found in food. The aflatoxin content of *Chlorella vulgaris* is 14 ppb and ochratoxin is found below detectable level. The US Food and Drug Administration (US FDA) has established maximum allowable levels of total aflatoxin in food at 20 µg/kg (Gupta, 2011). The aflatoxin content in *Chlorella vulgaris* are below the reference range and it is safe to consume the product.

B. Background information of the young adult women

i. Socio economic status of the young adult women

Table V reveals the socio economic status of the young adult women who participated in the supplementation study.

TABLE V
SOCIO ECONOMIC STATUS OF THE YOUNG ADULT WOMEN
N =40

Age (in years)	Number
18	7
19	10
20	11
21	6
22	4
23	1
24	1
Total	40
Place of residence	
Home	8
Hostel	24
Paying Guest	5
Rent House	3
Total	40
Type of family	
Joint family	10
Nuclear family	30
Total	40
Designation	
Student	34
working	6
Total	40
*Income level of the participants	
Low income (<Rs. 5000)	8
Middle income (Rs. 5000-20,000)	27
High income (>Rs. 20,000)	5
Total	40

*(HUDCO, 2010)

Among the 40 young adult women, seven were belonged to the age group of 18 years; 10 of them are of 19 years age, 11 in the the age group of 20 years, six in the age group of 21 , four in the age group of 22 years, each one in both 23 and 24 years age group. And it is evident that majority of the selected participants were in the age group of 20.

Regarding the place of residence eight members resided in their homes, 25 stayed in hostels, five were staying as paying guest and three lived in rented houses. With regard to the type of family, 30 of the young adult women belonged to nuclear family and remaining ten were from joint families.

The above table shows that 34 of the selected young adult women are students and only six of them are working and they are working in IT companies, schools and tailoring shop. The income level of the participants reveal that eight young adult women belonged to the low income group, followed by 27, belonging to the middle income group and this contribute the highest number and five of them were in high income group.

ii. Physical activity pattern of the young adult women

Table VI shows the physical activity pattern of the young adult women.

TABLE VI
PHYSICAL ACTIVITY PATTERN OF THE YOUNG ADULT WOMEN
N =40

Regularity Of Exercise	Number
Yes	15
No	25
Total	40
Type of exercise	
Walking/jogging	10
Playing games	2
Yoga	2
Aerobics	1
Total	15
Time spent for daily exercise	
30 minutes	6
1 hour	5
Above 1 hour	0
Weekly twice or thrice	4
Total	15

Fifteen young adult women had the habit of exercising regularly and 25 did not have the habit of exercising regularly. Out of 15 young adult women, 10 did walking/jogging, two did playing games, and the same number (two) did yoga and only one performed aerobics. Time spent for daily exercises vary from 30 minutes ,

1 hour, above one hour and weekly twice or thrice. Six of them did spent 30 minutes doing their regular physical activity, five of them spent one hour doing physical activity, 4 of them did physical activity only twice or thrice in a week and none of them performed physical activity above one hour.

iii. Dietary and food consumption pattern

Meal pattern and beverage consumption pattern

Table VII gives the meal pattern and beverage consumption pattern of the young adult women.

TABLE VII
MEAL PATTERN AND BEVERAGE CONSUMPTION PATTERN

N =40	
Meal pattern	Number
2 meal	5
3 meal	27
4 meal	8
Total	40
Beverage consumption pattern	
Tea	16
coffee	10
milk	8
others	6
Total	40

Among the 40 young adult women, five followed a two meal pattern, 27 followed a three meal pattern and eight followed four meal pattern. Sixteen of the young adult women used to consume tea, 10 consumed coffee, followed by eight of them consuming milk and six consuming other beverages.

Meal skipping pattern

Table VIII shows the meal skipping pattern followed by the young adult women.

TABLE VIII
MEAL SKIPPING PATTERN OF THE YOUNG ADULT WOMEN

Meal skipping pattern	Number
Yes	18
No	22
Total	40
Breakfast	9
Lunch	1
Dinner	8
Total	18
Reason for skipping meal	
Time Constraints	9
Fasting	5
Others	4
Total	18
Frequency of skipping meal	
Daily	5
Once/Twice a week	8
Occasionally	5
Total	18

Eighteen of the young adult women skipped their meals and the rest 22 regularly consumed their meals. Out of 18 young adult women who skipped their meals, nine skipped their breakfast, one skipped their lunch and the remaining participants skipped their dinner. The reason for skipping meals by the young adult women were due to time constraints (nine), fasting (five) and other reason contribute four. The frequency of skipping meals daily is by five of the young adult women followed by eight of occasional skipping and the remaining eight skipped their meals once or thrice a week.

Habit of eating outside foods

Table IX presents the eating out habit of the young adult women

TABLE IX
HABIT OF EATING OUTSIDE FOODS
N =40

Habit of eating outside foods	Number
Yes	30
No	10
Total	40
Preferred places for eating out	
Restaurant	13
Cafeteria	2
Fast food shops	9
Street vendors	6
Total	30

Among 40 young adult women, majority (30) had a habit of eating outside foods and only 10 did not have the habit of eating outside foods. The preferred places for eating out were restaurant (thirteen), cafeteria (two), fast food shops (nine) and street vendors (six).

C. Dietary intake of young adult women

Dietary intake of the participants of the supplementation study was obtained through 24 hour dietary recall. The various nutrients calculated are energy, protein, fat, carbohydrate, iron, calcium, β carotene, folic acid, vitamin C, fiber and vitamin B₁₂.

Table X and Figure 3 and 4 presents the dietary intake of young adult women.

TABLE X
DIETARY INTAKE OF YOUNG ADULT WOMEN

N =40

Nutritional parameters	RDA	Experimental group		Placebo group	
		Actual	±RDA	Actual	±RDA
Energy (Kcal)	1900	2074.92 ±299.43	+174.92	2043.46 ±267.86	+143.46
Protein (g)	55	52.22 ±10.81	-2.78	49.62 ±11.63	-5.38
Fat (g)	25	50.18 ±13.11	+25.18	52.60 ±8.84	+27.6
Carbohydrate (g)	360	359.04 ±59.64	+0.96	345.77 ±57.29	-14.23
Iron (Mg)	21	13.56 ±4.56	-7.44	13.64 ±5.8	-7.36
Calcium (mg)	600	415.86 ±168.88	-184.14	415.32 ±170.29	-184.68
β Carotene (µg)	4800	1231.26 ±580.67	-3568.74	1269.12 ±569.89	-3530.88
Folic Acid (mg)	200	86.60 ±41.30	-113.4	98.04 ±55.98	-101.96
Vitamin C (mg)	40	51.20 ±25.17	+11.2	54.57 ±23.89	+14.57
Fiber (g)	30	8.46 ±3.59	-21.54	7.33 ±2.70	-22.67
Vitamin B ₁₂ (µg)	2.4	0.34 ±0.34	-2.06	0.34 ±0.30	-2.06

The above table shows the dietary intake of both experimental and placebo group obtained through the 24 hour recall method. The energy intake of experimental group and placebo group is 2074.92 kcal and 2043.46 kcal respectively. The protein intake of experimental group is 52.22 g and that of the placebo group is 49.62 g. Intake of visible fat among the experimental and placebo group is 50.18 g and 52.60 g respectively. The carbohydrate intake of experimental group is 359.04 g and placebo group is consuming slightly lesser carbohydrate (345.77 g) than the experimental group.

The minerals and vitamin namely, iron and calcium intake of experimental group and placebo group is almost similar. The iron intake is 13.56 mg and 13.64 mg and the calcium intake is 415.86 mg and 415.32 mg respectively among experimental and placebo group. The β carotene, folic acid and vitamin C intake in experimental group is lesser than the placebo group. The intake of β carotene, folic acid and vitamin C among the experimental group is 1231.26 μ g and 1269.12 μ g respectively among the experimental and placebo group. Folic acid intake is 86.60 mg and 98.04 mg among the experimental and placebo group respectively. And that of the vitamin C is 51.20 and 54.57 mg in experimental and placebo group respectively. The fiber intake of the experimental group is higher than the placebo group. The fiber intake of the experimental group is 8.46 g and that of the placebo group is 7.33 g. The vitamin B₁₂ intake of both experimental and placebo group is same 0.34 μ g.

Both experimental and placebo group is consuming a higher calorie , visible fat and vitamin C than the Recommended Dietary Intake (RDA) and except this all other nutrients are lesser than the RDA. The calorie intake is +174.92 kcal and +143.46 kcal than the RDA among the experimental and placebo group respectively. The fat intake of the placebo group and experimental is +27.6 g and +25.18 g than the RDA. The vitamin C intake is +14.57 mg and +11.2 mg than the RDA among the placebo and experimental group. The protein intake of placebo group is -5.38 g lesser than the RDA and the protein intake of experimental group is slightly lesser than the RDA (-2.78 g). The carbohydrate intake of experimental group is higher than the RDA (+0.96g) and that of the placebo group is lesser than than the RDA (-14.23 g). Iron deficit among experimental and placebo group is almost similar.

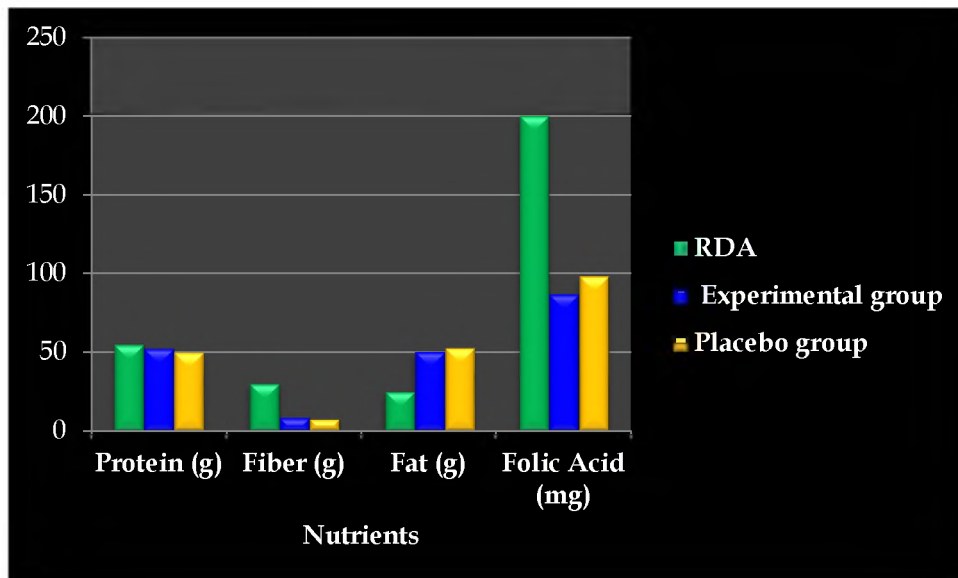
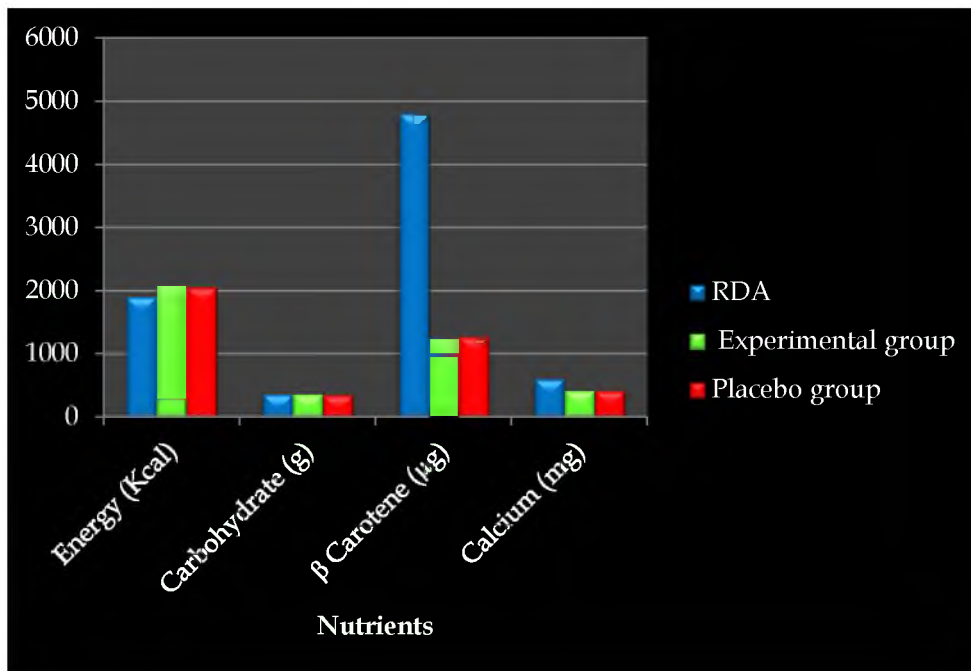


Figure 3
Dietary intake of the participants

For the experimental group the iron deficit is -7.44 mg and that of the placebo group is -7.36 mg. Comparing to the experimental group the placebo group is taking higher iron in their diet. The calcium intake of both experimental and placebo group is lesser than the RDA and the deficit is almost similar (-184.14 mg and -184.68 mg respectively). The β carotene intake is highly lesser than the RDA in both experimental and placebo group. The deficit is -3568.74 μg and -3530.88 μg in experimental and placebo group respectively. The folic acid intake is below half of the daily requirement in both experimental and placebo group. The deficit is higher in experimental group (-113.4 mg) than the placebo group (-101.96 mg). The RDA for fibre is 30 g/day (NIN, 2009), but both the experimental and placebo group is consuming below one third of their daily requirement. The deficit of vitamin B₁₂ is same in both experimental and placebo group (-2.06 μg).

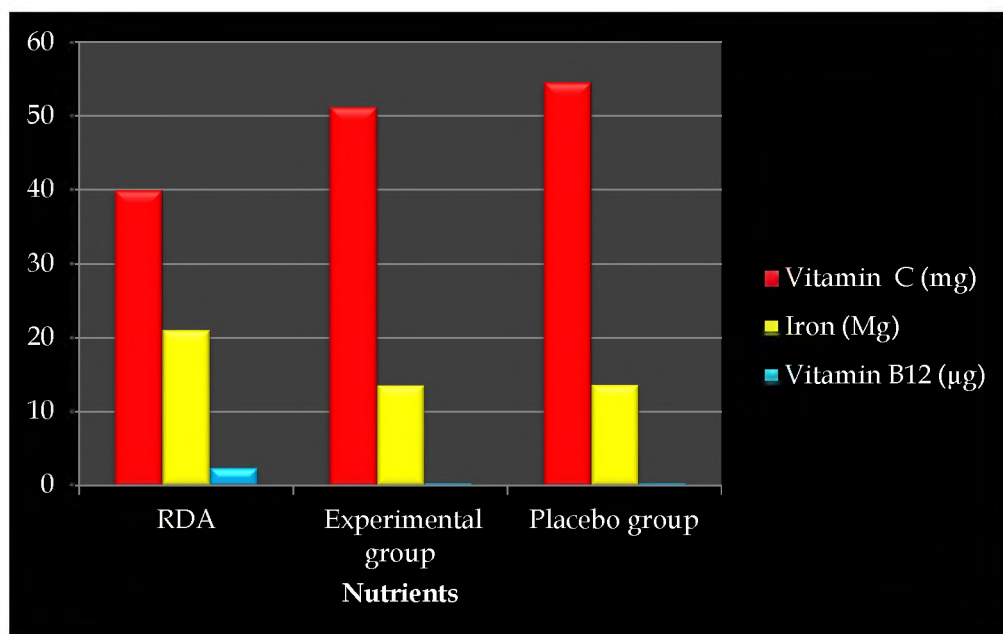


Figure 4

Dietary intake of participants

D. Impact of nutrition intervention on nutritional knowledge of young adult women

Nutrition knowledge of the participants was assessed before giving nutrition education through questionnaire and nutrition education regarding the importance of vitamin B₁₂ was given through Whatsapp and power point presentation for a period of 50 days.

The input of nutrition education is tabulated in Table XI among the participants.

TABLE XI
NUTRITION KNOWLEDGE BEFORE AND AFTER NUTRITION EDUCATION

N =40

Question / statements	Nutrition knowledge	
	Before	After
Awareness on types of vitamins	22	32
Vitamin B ₁₂ fat/water soluble	10	30
Two vitamin B ₁₂ rich foods	8	32
Is vitamin B ₁₂ deficient among vegetarians	2	36
Is vitamin B ₁₂ lower among obese and overweight persons	1	31
Fat soluble vitamin	8	28
Vitamin B ₁₂ produced by plants, true/false	12	28
Vitamin B ₁₂ works closely with	3	23
Function of vitamin B ₁₂ except	4	26
Symptoms of vitamin B ₁₂ deficiency, except	3	28
Expand BMI	12	23
Normal BMI	0	21
Hormone responsible for vitamin B ₁₂ deficiency among obese	3	35
Reliable plant source of vitamin B ₁₂	2	33
Normal blood vitamin B ₁₂	1	19
Compound raised in blood in vitamin B ₁₂ deficiency	4	36
Requirement of vitamin B ₁₂ / day for adults	2	18
BMI of obese	4	23
Vitamin B ₁₂ in 250 ml milk	2	10
Two methods to avoid obesity	3	22
Mean±SD	5.30±5.33	26.70±6.81

The table shows the score obtained by the participants before and after the nutrition education. Each correctly answered question was given one point and there were totally 20 questions which was given through the questionnaire. For all the questions, the score increased after nutrition education. The range of scores before nutrition education is between 0 and 22 and after nutrition education the range of scores is between 10 and 36. This shows that more number of people answered the question after attending the nutrition education. i.e. after nutrition education the participants were aware about the concepts, health benefits, nutritional importance and consequences of vitamin B₁₂, obesity, vegetarian foods etc.

The mean score of the participants was found to be 5.30 before nutrition intervention that increased to 26.70 after the intervention. There is a great difference in the mean score of the participants before and after nutrition education. It was interesting to note that nutrition score increased after intervention. These results are on par with the findings of Yatnatti (2009), Wall et al.,(2012) and Usharani (2013) who reported the changes in food choice of the participants after their nutrition intervention.

E. Anthropometric measurements of the young adult women

i. Changes in anthropometric measurements before and after supplementation

Table XII shows the anthropometric measurements of the participant's before and after the supplementation study.

TABLE XII
CHANGES IN ANTHROPOMETRIC MEASUREMENTS
BEFORE AND AFTER THE SUPPLEMENTATION

N =40

Variables	Experimental group		Placebo group	
	Initial	Final	Initial	Final
Weight (kg)	64.75±7.81	64.32±7.81	62.75±7.08	62.75±7.05
Height (cm)	155.45±5.79	155.45±5.79	152.85±4.22	152.85±4.22
BMI (kg/m ²)	26.77±2.43	26.58±2.40	27.01±1.87	27.01±1.89
Waist circumference (cm)	93.40±6.65	92.79±6.85	87.75±4.39	87.75±4.44
Hip circumference (cm)	104.65±5.25	104.19±5.19	100.7±4.06	100.6±4.12

Table XII represents the direct measure anthropometric variables of experimental and placebo group before and after the supplementation period. The value of height and weight of both the groups were quite similar before supplementation. The mean value for height of experimental and placebo group were 155.45 cm and 152.85 cm respectively. The mean value for weight of experimental and placebo group were 64.75 kg and 62.75 kg respectively before supplementation. As experimental and placebo group had quite similar weight and height, the BMI of experimental and placebo group is almost same. The BMI of both groups were 26.77±2.43 and 27.01±1.87 as mean and SD respectively and only slight difference was found. The mean waist circumference and hip circumference of the experimental group is higher (93.4 cm and 104.65 cm respectively) when compared to the waist circumference and hip circumference of the placebo group (87.75 cm and 100.7 cm respectively). Snehathalatha *et al.*, (2003) reported that the normal waist circumference for women is 80 cm. This shows that both the experimental and placebo are above their cut off values.

After the supplementation the mean weight of the experimental group is slightly reduced than the initial weight. The weight after supplementation period is

64.32 kg. This shows the mean decrease in difference of 0.43 kg after the supplementation. There is no change in the weight after the supplementation period in placebo group. Because of the change in body weight of the experimental group there is a slight reduction in the BMI. The BMI changed from 26.77 kg/m² to 26.58 kg/m² so the mean difference is 0.19. But there is no change in the BMI of the placebo group as there is no change in the weight of the placebo group after supplementation. The waist circumference of the experimental group is slightly decreased (0.61 cm). There is no change in the waist circumference of the placebo group after supplementation. There is a difference in the hip circumference of both experimental and placebo group after supplementation. The hip circumference is changed from 104.65 cm to 104.19 cm and in placebo group the value decreased from 100.7 cm to 100.6 cm.

ii. Derived body composition variables

Table XIII reveals the derived body composition variables of the experimental and placebo group.

TABLE XIII
DERIVED BODY COMPOSITION VARIABLES

N =40

Variable	Cut off value	Experimental group		Placebo group	
		Initial	Final	Initial	Final
WHR	0.81	0.88±0.04	0.88±0.04	0.86±0.02	0.86±0.03
WtHR	0.5	0.60±0.04	0.59±0.05	0.57±0.02	0.57±0.02

The mean waist hip ratio before supplementation for the experimental group is 0.88 and that for placebo group is 0.86. The values are almost similar. This shows that both experimental and placebo group are above their normal cut off value for WHR. Snehalatha *et al.*, (2003) reported that the normal cut off value for WHR for women is 0.81. Fox *et al.*, (2007) mentioned that WHR is the better indicator of obesity than all other anthropometric measurements. Lucy, (2010) reported that the WtHR above 0.5 increase the health risk and it is the perfect indicator of obesity. In

the present study the WtHR of experimental group is 0.60 and the placebo group is 0.57. This clearly states that experimental group is in higher risk when compared to the placebo group. WtHR is more sensitive than BMI as an early warning of health risks (Ashwell and Hsies, 2009).

After supplementation there was no change in the waist hip ratio of both experimental and placebo group. In the case of waist to height ratio there is a slight reduction in the experimental group after the supplementation and there is no change in WtHR among the placebo group.

iii. Classification of BMI

Table VII presents the Classification of the study participants according to their BMI.

TABLE XIV
CLASSIFICATION OF PARTICIPANTS BASED ON BMI N =40

Classification	BMI	N	Mean±SD
overweight	23-24.9	5	24.32±0.23
obesity grade-I	25-29.9	31	26.64±1.12
obesity grade-II	30-39.9	4	31.99±1.08

From the total of 40 participants, five participants were overweight and four participants belonged to grade II obesity and remaining 31 belonged to grade I obesity. This shows that most of the participants are in the category obesity grade I. The revised guidelines categorize overweight as a BMI of 23.0 – 24.9 and obesity as BMI ≥ 25 for Indians (Aziz *et al.*, 2014).

F. Effect of supplementation of *Chlorella vulgaris* on the vitamin B₁₂ status of the young adult women

Supplementation for experimental group with *Chlorella vulgaris* juice and placebo group with curd was given for a period of 50 days. Table XV and Figure 5 presents the effect of supplementation of *Chlorella vulgaris* on the vitamin B₁₂ status of the young adult women.

TABLE XV

EFFECT OF SUPPLEMENTATION OF *CHLORELLA VULGARIS* ON THE VITAMIN B₁₂ STATUS OF THE YOUNG ADULT WOMEN

Biochemical parameters	Experimental group		Mean difference	't' value	Placebo group		Mean difference	't' value
	Initial	Final			Initial	Final		
Vitamin B ₁₂	227.50 ±76.16	338.60 ±75.26	+111.1	9.268**	238.90 ±52.71	241.60 ±51.71	+2.70	1.4 ^{NS}
Heamoglobin	11.61 ±0.77	12.24 ±0.87	+0.63	4.709**	11.98 ±0.74	12.04 ±0.74	+0.60	7.48 ^{NS}

** - significant at 1% level, NS not significant

The mean value of vitamin B₁₂ in the experimental group before supplementation is 227.50 pg/ml and after the supplementation the mean value increased to 338.60 pg/ml. Hence the mean increase in difference was 111.1 pg/ml. At the end of 50 days of supplementation, the mean value of vitamin B₁₂ among the experimental group showed a significant difference at 1% level statistically. The mean value of placebo group before supplementation is 238.90 pg/ml and after supplementation the value increased to 241.60 pg/ml, the mean increase in difference was 2.70. The difference between initial and final was found to be not significant statistically.

The mean haemoglobin value of experimental group before supplementation was 11.61 g/dl and the value found to be significant at 1% level after the supplementation (t value=4.709). Mean difference after the supplementation is 0.63 g/dl. The haemoglobin before supplementation in the placebo group is 11.98 g/dl and the mean value after supplementation is 12.04 g/dl. Value showed no significant difference (t value=0.60, not significant) statistically.

The formulated supplement was found to have an impact in the vitamin B₁₂ status of vegetarian obese and overweight young adult women and hence the objective is fulfilled. The null hypothesis of the study "There is no effect on supplementing *Chlorella vulgaris* in improving the vitamin B₁₂ status among obese and overweight young adult women" is disproved.

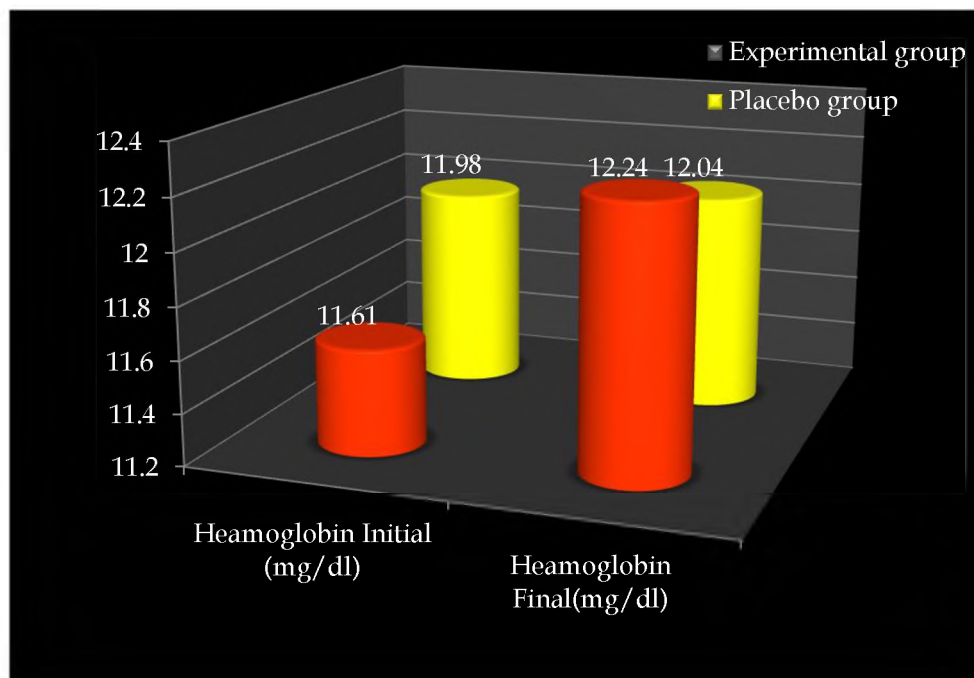
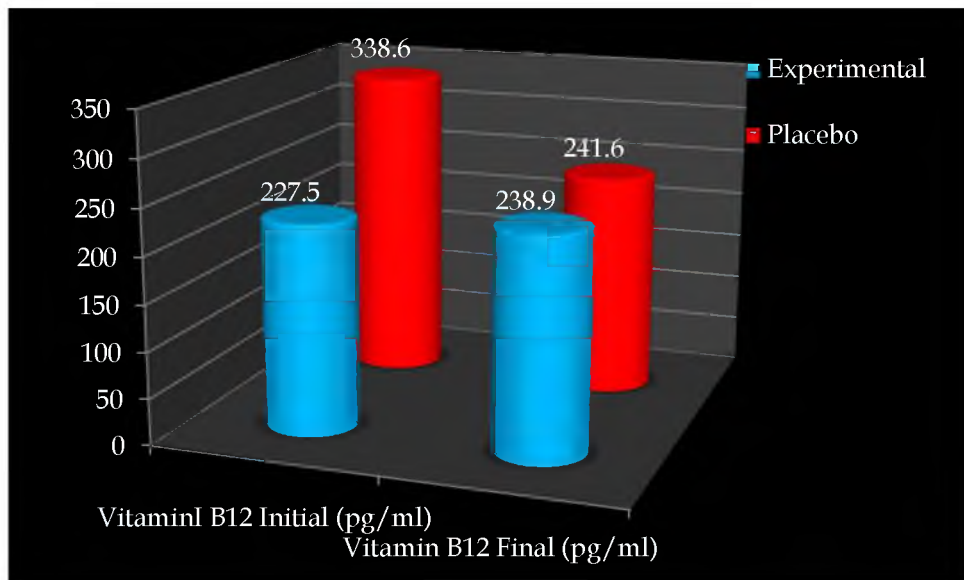


Figure 5

Effect of supplementation of *Chlorella vulgaris* on the vitamin B₁₂ and Haemoglobin status of the young adult women

V. SUMMARY AND CONCLUSION

Nutrition and health are two sides of the same coin and therefore are inseparable. Overall obesity rates increase through adulthood. Some countries have strong cultural or religious traditions that promote vegetarianism, such as in India, while in other countries secular ethical concerns dominate, including animal rights and environmental protection, along with health concerns. Vitamin B₁₂ is absolutely critical for good mental and physical health. It is essential for the DNA (cell's genetic material) and works with other B vitamins to form all types of blood cells. Compared with lacto ovo vegetarians and omnivores, vegans typically have lower plasma vitamin B₁₂ concentrations, higher prevalence of vitamin B₁₂ deficiency, and higher concentrations of plasma homocysteine. Elevated homocysteine in blood indicates a lower serum vitamin B₁₂ levels in most cases and elevated homocysteine has been considered as a risk factor for CVD. Absolute vitamin B₁₂ deficiency was found in 6% patients in India, borderline vitamin B₁₂ deficiency in 9%, absolute folic acid deficiency in 26% and combined vitamin B₁₂ and folic acid deficiency in 10% patients. Vitamin B₁₂ is naturally found in animal products, including fish, meat, poultry, eggs, milk, and milk products. Vitamin B₁₂ is generally not present in plant foods. Obesity and overweight leads to further B₁₂ deficiency. Young adults are highly prone to obesity and vitamin B₁₂ is very much important for woman of child bearing age. Nutritional needs should be met primarily from foods. Foods in nutrient-dense forms contain essential vitamins and minerals and also dietary fiber and other naturally occurring substances that may have positive health effects. An alternative to meat source for vitamin B₁₂ is algae and *Chlorella vulgaris* is a rich source of vitamin B₁₂. *Chlorella vulgaris* being an algae is categorised under vegetarian food but not commonly used for consumption.

The present study entitled “ **Effect of *Chlorella vulgaris* in improving the vitamin B₁₂ status among the vegetarian obese and overweight young adult women**” was aimed at finding the effect of *Chlorella vulgaris* supplementation in improving the vitamin B₁₂ status of the vegetarian obese and overweight young adult women. The *Chlorella vulgaris* was used in the form of dry powder. Analysis of

proximate principles, phytochemical analysis, antimicrobial activity and a test for detection of toxic substance if any was conducted in the dried powder of *Chlorella vulgaris*. 40 vegetarian young adult women who are either obese or overweight were selected for the study and their background information was elucidated using a questionnaire. Nutrition knowledge of among the participants was tested using a questionnaire containing 20 questions and after testing their nutrition knowledge, nutrition education was given through two modes: one is through powerpoint presentation and another through Whatsapp instant messaging services for a period of 50 days. Anthropometric measurements namely weight, height, BMI, waist circumference, hip circumference and waist to height ratio of the participants was measured and the dietary intake of the participants was calculated using 3 days 24 hour recall method. Biochemical tests was conducted to measure the vitamin B₁₂ and haemoglobin status of the study participants. The participants was divided into experimental and placebo group according to their willingness to take *Chlorella vulgaris* juice and curd respectively. And a supplementation study for a period of 50 days was conducted with *Chlorella vulgaris* juice for experimental group and curd for placebo group. At the end of the supplementation study, post supplementation biochemical tests as well as anthropometric measurements was done to check the effect of *Chlorella vulgaris* in improving the vitamin B₁₂ status and to check if there is any effect in anthropometry among the study participants. The study protocol was approved by the Institutional Ethical Committee of Avinashilingam and the approval number was AUW/ IHEC/ FSN-17-18/XPD/03.

The salient findings of the study are summarized below:

- Mean ash content of the *Chlorella vulgaris* is 9 g. The macronutrient composition of *Chlorella vulgaris* constitute highest composition of protein (49.58g), followed by the total carbohydrate (38.8 g), fibre (10.73 g) and starch (2.42 g). And among the micronutrients analysed the highest contribution is by iron, 23.8 g which is phosphorus (1066.6 mg), calcium (552 mg), vitamin C (54.58 mg) and the least is contributed by the vitamin B₁₂, 28.8 µg.
- The aqueous extract of *Chlorella vulgaris* showed the presence of tannin, phenol, saponin, quinone, glycoside, coumarin, sterol and flavonoid. Terpenoid and

alkaloid was absent in aqueous extract of *Chlorella vulgaris*. Tannin, phenol, saponin, coumarin and flavonoid was highly present in aqueous extract and the presence of quinone, glycoside and sterol was found to be low. The methanolic extract of the *Chlorella vulgaris* showed the presence of tannin, terpenoid, phenol, saponin, glycoside, sterol and flavonoid. Quinone, coumarin and alkaloid was absent in methanolic extract of *Chlorella vulgaris*. Tannin, phenol and sterol was moderately present and the presence of terpenoid, saponin, glycoside and flavonoid was found to be low. Tannin, phenol, saponin, glycoside and sterol was found to be present in ethanolic extract of *Chlorella vulgaris*. Phenol and saponin was moderately present in ethanolic extract and the presence of tannin, glycoside and sterol was found to be low. Terpenoid, quinone, flavonoid and alkaloid was absent in ethanolic extract of *Chlorella vulgaris*.

- Antimicrobial activity namely antibacterial and antifungal activity of *Chlorella vulgaris* was conducted. The bacteria selected are *E.coli* (Gram -ve) and *Staphylococcus aureus* (gram +ve). The aqueous extract of *Chlorella vulgaris* was found to contain a zone of inhibition of 8 mm against *E.coli* and 10 mm against *Staphylococcus aureus*. The control showed a higher zone of inhibition than the *Chlorella vulgaris* extract. The antifungal activity of aqueous extract of *Chlorella vulgaris* was tested with fungi like *Aspergillus niger* and *Aspergillus flavus* and shows a zone of inhibition of 9 mm and 13 mm respectively. The present study showed a higher antifungal activity of *Chlorella vulgaris* than the control. The control showed the antifungal activity of 6 mm and 8 mm for *Aspergillus niger* and *Aspergillus flavus* respectively.
- The aflatoxin and ochratoxin are the common toxins found in food. The aflatoxin content of *Chlorella vulgaris* is 14 ppb and ochratoxin is found below detectable level. The aflatoxin content in *Chlorella vulgaris* are below the reference range and it is safe to consume the product.
- Among the 40 young adult women, seven were belonging to the age group of 18 years; 10 of them are 19 years of age, 11 in the the age group of 20 years, six in the age group of 21 , four in the age group of 22 years, each one in both 23 and 24 years age group. And it is evident that majority of the selected participants

were in the age group of 20. Regarding to the place of residence eight members resided in their homes, 25 stayed in hostels, five were staying as paying guest and three lived in rented houses. With regard to the type of family, 30 of the young adult women belonged to nuclear family and remaining ten were from joint families.

- Thirty four of the selected young adult women are students and only six of them are working and they are working in IT companies, schools and tailoring shop. The income level of the participants reveal the eight young adult women belonged to the low income group, followed by 27, belonging to the middle income group and this contribute the highest number and five of them were in high income group.
- Fifteen young adult women had the habit of exercising regularly and 25 did not have the habit of exercising regularly. Out of 15 young adult women, 10 did walking/jogging, two did playing games, and the same number (two) did yoga and only one performed aerobics. Time spent for daily exercises vary from 30 minutes , 1 hour, above one hour and weekly twice or thrice. Six of them did spent 30 minutes doing their regular physical activity, five of them spent one hour doing physical activity, 4 of them did physical activity only twice or thrice in a week and none of them performed physical activity above one hour.
- Among the 40 young adult women, five followed a two meal pattern, 27 followed a three meal pattern and eight followed four meal pattern. Sixteen of the young adult women used to consume tea, 10 consumed coffee, followed by eight of them consuming milk and six consuming other beverages.
- Eighteen of the young adult women skipped their meals and the rest 22 regularly consumed their meals. Out of 18 young adult women who skipped their meals, nine skipped their breakfast, one skipped their lunch and the remaining skipped their dinner. The reason for skipping meals by the young adult women were due to time constraints (nine), fasting (five) and other reason contribute four. The frequency of skipping meals daily is by five of the young adult women followed by eight of occasional skipping and the remaining eight skipped their meals once or thrice a weak.

- Among 40 young adult women, majority (30) had a habit of eating outside foods and only 10 did not have the habit of eating outside foods. The preferred places for eating out were restaurant (thirteen), cafeteria (two), fast food shops (nine) and street vendors (six).
- The energy intake of experimental group and placebo group is 2074.92 kcal and 2043.46 kcal respectively. The protein intake of experimental group is 52.22 g and that of the placebo group is 49.62 g. Intake of visible fat among the experimental and placebo group is 50.18 g and 52.60 g respectively. The carbohydrate intake of experimental group is 359.04 g and placebo group is consuming slightly lesser carbohydrate (345.77 g) than the experimental group.
- The minerals and vitamin namely, iron and calcium intake of experimental group and placebo group is almost similar. The iron intake is 13.56 mg and 13.64 mg and the calcium intake is 415.86 mg and 415.32 mg respectively among experimental and placebo group. The β carotene, folic acid and vitamin C intake in experimental group is lesser than the placebo group. The intake of β carotene, folic acid and vitamin C among the experimental group is 1231.26 μ g and 1269.12 μ g respectively among the experimental and placebo group. Folic acid intake is 86.60 mg and 98.04 mg among the experimental and placebo group respectively. And that of the vitamin C is 51.20 and 54.57 mg in experimental and placebo group respectively. The fiber intake of the experimental group is higher than the placebo group. The fiber intake of the experimental group is 8.46 g and that of the placebo group is 7.33 g. The vitamin B₁₂ intake of both experimental and placebo group is same 0.34 μ g.
- Both experimental and placebo group is consuming a higher calorie, visible fat and vitamin C than the Recommended Dietary Intake (RDA) and except this all other nutrients are lesser than the RDA. The calorie intake is +174.92 kcal and +143.46 kcal than the RDA among the experimental and placebo group respectively. The fat intake of the placebo group and experimental is +27.6 g and +25.18 g than the RDA. The vitamin C intake is +14.57 mg and +11.2 mg than the RDA among the placebo and experimental group. The protein intake of placebo group is -5.38 g lesser than the RDA and the protein intake of

experimental group is slightly lesser than the RDA (-2.78 g). The carbohydrate intake of experimental group is higher than the RDA (+0.96g) and that of the placebo group is lesser than than the RDA (-14.23 g). Iron deficit among experimental and placebo group is almost similar. For the experimental group the iron deficit is -7.44 mg and that of the placebo group is -7.36 mg. Comparing to the experimental group the placebo group is taking higher iron in their diet. The calcium intake of both experimental and placebo group is lesser than the RDA and the deficit is almost similar (-184.14 mg and -184.68 mg respectively). The β carotene intake is highly lesser than the RDA in both experimental and placebo group. The deficit is -3568.74 μ g and -3530.88 μ g in experimental and placebo group respectively. The folic acid intake is below half of the daily requirement in both experimental and placebo group. The deficit is higher in experimental group (-113.4 mg) than the placebo group (-101.96 mg). Both the experimental and placebo group is consuming below one third of their daily requirement. The deficit of vitamin B₁₂ is same in both experimental and placebo group (-2.06 μ g).

- The range of scores before nutrition education is between 0-22 and after nutrition education the range of scores is between 10-36. This shows more number of people answered the question after imparting the nutrition education. i.e. after nutrition education the participants were aware about the concepts, health benefits, nutritional importance and consequences of vitamin B₁₂, obesity, vegetarian foods etc. The mean score of the participants was found to be 5.30 before nutrition intervention that increased to 26.70 after the intervention. There is a great difference in the mean score of the participants before and after nutrition education. It was interesting to note that nutrition score increased after intervention.
- The value of height and weight of both the groups were quite similar before supplementation. The mean value for height of experimental and placebo group were 155.45 cm and 152.85 cm respectively. The mean value for weight of experimental and placebo group were 64.75 kg and 62.75 kg respectively before supplementation. As experimental and placebo group had quite similar weight

and height, the BMI of experimental and placebo group is almost same. The BMI of both group was 26.77 ± 2.43 and 27.01 ± 1.87 as mean and SD respectively and only slight difference was found. The mean waist circumference and hip circumference of the experimental group is higher (93.4 cm and 104.65 cm respectively) when compared to the waist circumference and hip circumference of the placebo group (87.75 cm and 100.7 cm respectively). Snehalatha et al., (2003) reported that the normal waist circumference for women is 80 cm. This shows that both the experimental and placebo are above their cut off values.

- After the supplementation the mean weight of the experimental group is slightly reduced than the initial weight. The weight after supplementation period is 64.32 kg. This shows the mean decrease in difference of 0.43 kg after the supplementation. There is no change in the weight after the supplementation period in placebo group. Because of the change in body weight of the experimental group there is a slight reduction in the BMI. The BMI changed from 26.77 kg/m^2 to 26.58 kg/m^2 so the mean difference is 0.19. But there is no change in the BMI of the placebo group as there is no change in the weight of the placebo group after supplementation. The waist circumference of the experimental group is slightly decreased (0.61 cm). there is no change in the waist circumference of the placebo group after supplementation. There is a difference in the hip circumference of both experimental and placebo group after supplementation. The hip circumference is changed from 104.65 cm to 104.19 cm and in placebo group the value decreased from 100.7 cm to 100.6 cm.
- The mean waist hip ratio before supplementation for the experimental group is 0.88 and that for placebo group is 0.86. After the supplementation there was no change in the waist hip ratio of both experimental and placebo group. In the case of waist to height ratio there is a slight reduction in the experimental group after the supplementation and there is no change in WtHR among the placebo group.
- From the total of 40 participants, five participants are overweight and four participants beyond to grade II obesity and remaining 31 beyond to grade I obesity. This shows that most of the participants are in the category obesity grade I.

- The mean value of vitamin B₁₂ experimental group before supplementation is 227.50 pg/ml and after the supplementation the mean value increased to 338.60 pg/ml. Hence the mean increase in difference was 111.1 pg/ml. At the end of 50 days of supplementation the mean value of vitamin B₁₂ among the experimental group showed a significant difference at 1% level statistically. The mean value of placebo group before supplementation is 238.90 pg/ml and after supplementation the value increased to 241.60 pg/ml, the mean increase in difference was 2.70. The difference between initial and final was found to be not significant statistically. The mean haemoglobin value of experimental group before supplementation was 11.61 g/dl and the value found to be significant at 1% level after the supplementation (t value=4.709). Mean difference after the supplementation is 0.63 g/dl. The haemoglobin before supplementation in the placebo group is 11.98 g/dl and the mean value after supplementation is 12.04 g/dl. And the value showed no significant difference (t value=0.60, not significant). The formulated supplement was found to have an impact in the vitamin B₁₂ status of vegetarian obese and overweight young adult women and hence the objective is fulfilled.

In conclusion, the study revealed that there is an effect of supplementation of *Chlorella vulgaris* in improving the vitamin B₁₂ status among the obese and overweight young adult women. The *Chlorella vulgaris* juice can be easily prepared by just mixing with water and if necessary the sweetener like jaggery can be added. And this will be an effective way for preventing the vitamin B₁₂ deficiency especially among the child bearing age group. A normal vitamin B₁₂ status prevents high circulating homocysteine in the blood due to elevated methylmalonic acid and reduces the cardiovascular diseases especially among the obese. Hence *Chlorella vulgaris* will be an alternative source of vitamin B₁₂ among the vegetarians.

Recommendations for further research:

- ✓ Studies on reducing the homocysteine level in blood using *Chlorella vulgaris*
- ✓ Development of food products using *Chlorella vulgaris* for ease of consumption
- ✓ Development of RTS food using *Chlorella vulgaris*
- ✓ Antioxidant and bioactive components in *Chlorella vulgaris* products
- ✓ Conduct of shelf life study on *Chlorella vulgaris* grown in different waters

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APPENDIX I

Questionnaire to elicit details on Socioeconomic background and Nutrition Knowledge

1. Name:
2. Age:
3. Date of birth:
4. Place of residence
a) Home b) Hostel c) Paying guest d) Rent house
5. Address for communication:

Mobile number:
6. Type of family: a) Nuclear b) Joint
7. Designation :
8. Marital status
a) Married b) Unmarried
9. Income : Low/ Middle/ High

Physical activity pattern

Do you exercise regularly? Yes/No

If yes, the kind of exercise

- Walking/jogging
- Playing games
- Yoga
- Aerobics

Duration:

30 minutes/ 1 hour/ >1 Hour/ weekly twice or thrice

Dietary and food consumption pattern

1. Type of meal pattern followed (tick the appropriate one)

Meal pattern	Breakfast	Lunch	Evening	Dinner
2 meal				
3 meal				
4 meal				

2. Do you consume any beverages?

- Tea
- Coffee
- Milk
- Others

3. Do you skip any meals ? Yes/ No

If yes, Breakfast /Lunch /Dinner

4. Mention the reason for skipping meals?

- a) Time constraints
- b) Fasting
- c) Others specify

5. Frequency with which you skip meal?

- a) Daily
- b) Once or thrice a week
- c) Occasionally

6. Do you eat outside? Yes/ No

If yes, where?

- A) Restaurant
- b) Cafeteria
- c) Fast food shops
- d) Street vendors

Nutrition knowledge assessment

Questionnaire to evaluate the nutrition knowledge on the importance of vitamin B₁₂

1. Do you know that vitamins are of two forms-water soluble and fat soluble?

Yes	No
-----	----

2. Vitamin B₁₂ is fat soluble / water soluble?

3. Can you name any two vitamin B₁₂ rich foods?

- a)
- b)

4. Do you know that vitamin B12 is deficient among vegetarians?

Yes	No
-----	----

5. Do you know the vitamin B12 status is lower among obese and overweight persons?

Yes	No
-----	----

6. Pick up the fat soluble vitamin from the following:

- Vitamin B₁₂
- Vitamin A
- Folic acid
- vitamin c

7. Vitamin B12 is produced by plants? True/False
8. Vitamin B12 works very closely with which of the following vitamin?
 - Vitamin D
 - Vitamin C
 - Folic acid
 - Biotin
9. Which of the following is not the function of vitamin B12?
 - Calcium absorption
 - Formation of genetic material
 - Formation of red blood cells
 - Boost immunity
10. Symptoms of vitamin B12 deficiency, except?
 - Extreme tiredness
 - Depression
 - Muscle pain
 - Vision problems
11. Expand BMI?
 - Body Mass Index
 - Basal Metabolic Index
 - Basal Metabolic Impedance
 - Bone Mass Index
12. Normal BMI of an individual?
 - 18.5-22.9
 - 23-24.9
 - 25-29.9
 - 30-39.9
13. Obesity causes vitamin b12 deficiency through increased production of ----- hormone?
 - Ghrelin
 - Leptin
 - Aldosterone
 - Growth hormone
14. Which is the reliable plant source of Vitamin B₁₂ ?
 - Spirulina
 - Chlorella vulgaris
 - Barley grass
 - Nori
15. Normal level of vitamin B12 in blood?
 - 200-600 pg/ml
 - 300-600 pg/ml
 - 211-911 pg/ml
 - 320-700 pg/ml
16. Which of the following compound raised in blood under vitamin B₁₂ deficiency?
 - Heamoglobin
 - Homocysteine
 - WBC
 - Platelets
17. Requirement of vitamin B₁₂ / day for adults?
 - 3.0 µg
 - 2.4 µg
 - 4.0 µg
 - 2.9 µg
18. The person is said to be obese when the BMI is above -----
 - < 18.5
 - >25
 - 18.5-22.9
 - 23-25
19. How much vitamin B12 is present in 250 ml of milk?
 - 6 µg
 - 2 µg
 - 1 µg
 - 10 µg
20. List out 2 methods to avoid obesity?

APPENDIX II

Ethical clearance

INSTITUTIONAL HUMAN ETHICS COMMITTEE



Avinashilingam

Institute for Home Science and Higher Education for Women

University

(Estd. U/s 3 of UGC Act 1956)

Chairman

Dr. S. Ramalingam
Principal, PSG Institute
of Medical Sciences
& Research, Coimbatore

Member Secretary

Dr.S.Uma Mageshwari
Associate Professor,
Department of Food Service
Management & Dietetics

Members

Dr. S. Kowsalya
Dr.P.R.Padma
Mr. K.Arulmoli (Legal Expert)
Dr. N.S. Rohini
Dr.A. Saraswathy
Mrs. V. Mangayarkarasi
Dr.Subhashini K. Sripathi
Mrs. S. Radha Devi
Dr.G.Victoria Naomi
Dr. Judith Justin
Dr.AnithaSubash

19th March 2018

To
Ms. Deepika.M
Department of Food Science and Nutrition
Avinashilingam Institute for Home Science and
Higher Education for Women
Coimbatore – 641 043

Dear Deepika,

Ref: Your proposal No. IHEC/17-18/FSN/03 “Effect of *Chlorella vulgaris* in Improving the Vitamin B12 Status among the Vegetarian Obese and Overweight Young Adult Women” submitted for approval of the IHEC on 14th December.

The Institutional Human Ethics Committee of our University hereby grants approval to your research proposal No. IHEC/17-18/FSN/03 “Effect of *Chlorella vulgaris* in Improving the Vitamin B12 Status among the Vegetarian Obese and Overweight Young Adult Women” submitted by you. The Approval number for the same is AUW/ IHEC/ FSN -17-18/XPD/03.

We wish you all the best in your research endeavours.

Regards,

S. Uma Mageshwari
Dr.S.Uma Mageshwari
Member Secretary

