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Spinach Freeze Dried Materials: Source of Nutrients for Health Benefits

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Abstract:
Spinach is recognized as a good sources of phytochemicals and nutrients. They played an important role in the prevention of human diseases and maintaining good health. Besides its nutritional composition, biomarker components of freeze dried spinach also been studied including its microbial test. Lipid (305.8 ± 0.2 mg) and protein contents (550 ± 0.6 mg) are the two major components while chlorophyll (68.3 ± 0.15 mg) is the major pigment exist in spinach freeze dried material. Antioxidant components such as vitamin E, vitamin C and β–carotene are 0.24 ± 0.1 mg, 6.4 ± 0.12 mg and 1.37 ± 0.1 mg, respectively. α-linolenic acid contributed nearly 85% of the total unsaturated fatty acid. Dietary contribution of biomarker components in the freeze-dried material are as follow; α-Linolenic acid (14-21%), Vitamin E (6-8%), Vitamin C (16%) and β-carotene (17%). Microbial test indicated that this material is of a good quality ready to eat food.

Keywords: *Freeze dried material, nutritional value, biomarker compounds, and microbial test*

1. Introduction

Plants, as food sources, are an important component in a person's daily diet. They help the human body to restore good health and prevent diseases due to their richness in phytochemicals and micronutrients. A green vegetable, such as spinach, is reported to contribute a significant amount of essential nutrients [i] while also being a good source of minerals [ii]. Phytonutrients, as well as other bioactive components, are stored in lipids which are found in the chloroplast thylakoid membrane, this is the photosynthetic membrane of a green leaf. The eating of spinach is reported to provide a significant contribution of micronutrients namely iron, vitamin A and riboflavin as it is rich in them, these levels could be increased even further if consumed more frequently and in larger amounts, especially in children [iii]. *In vivo* and *in vitro* studies of red spinach which is rich in carotene showed anticancer properties. Spinach also reported to be rich in antioxidant activity which contributed to a healthy effect when consumed [iv].

However, it is not very practical to eat large amounts of spinach in a person's daily diet; thus, it is important to identify alternative but acceptable ways for increasing the intake of such nutrients in order to prevent chronic diseases and to have a balanced diet. Thus this research suggests intake of spinach in a powder form. In addition consumer becoming increasingly aware of the health benefits of vegetables consumption. Although there are many published work on benefits of eating spinach, there is a few studies which presents compilation of nutritional data and biomarker compounds of spinach; safety information on spinach as ready to eat food also was less reported.

This study provides data on nutritional value of freeze dried spinach chloroplast including its biomarker content which is important to act as a precursor for maintaining good health. This study also reporting the safety information based on microbial test.

2. Method

2.1. Extraction from Spinach Leaf

The stem of the spinach leaves were cut off and weighed before being mixed with 0.3 M (M = molar) of the sucrose solution, at a ratio of 1:3 w/w, they were blended using a household blender for 30 seconds. Sucrose were used as osmotic agents, of which are commonly used in the isolation work of chloroplasts using buffer solutions [v]. Once blended, the slurry was filtered using cheese cloth – the remaining supernatant was then poured into a 50 mL centrifuge tube and centrifuged at 3,500 g, 5°C, for 15 minutes. Finally, the supernatant was discarded and the pellet which remained was collected for further analysis.

2.1.2. Preservation
The frozen pellets were freeze-dried (Christ, Alpha 1-4 Freeze Dryer) with a vacuum at 0.006 mbar and a condenser at -80°C.

2.2. Crude Spinach Lipid Extract
The method used for lipid extraction was adapted from Bligh and Dyer [vi]. The fresh chloroplast pellet was added to a mixture of chloroform/methanol at a ratio of 2:1, it was then vortexed for 1 minute before 0.3 mL of a saline solution (0.9% NaCl) was added. NaCl was added to facilitate the partitioning of the lipids into the organic phase [vii]. This mixture was then vortexed and left for 3 minutes before being centrifuged at 3,500 g, 5°C, for 15 minutes. The bottom layer, which contained the lipids, was transferred into a pre-weigh bottle and subsequently left in a nitrogen dryer until it was dry. After which, the lipids were weighed. The total amount of lipid was calculated using Equation 1 below.

- Equation 1:

$$Lipid\ content\ (\%) = \frac{weight\ of\ lipid\ (g)}{weight\ of\ dried\ pellet\ (g)} \times 100\%$$

When freeze-dried pellet were utilised, the procedure for lipid extraction was the same however, instead of using a vortex, a cell disruptor was used to homogenise the sample at a maximum speed for 1 minute. After which, the sample was centrifuged at 1,100 g, 4°C, for 10 minutes, three layers were then observed. The first layer contained water, methanol and soluble salts which were discarded. The second layer contained cell debris which were collected and then left to dry under a fume hood for protein analysis. Lastly, the bottom layer contained lipids that were transferred into a pre-weigh bottle and subsequently left in a nitrogen dryer until they were dry. These lipids were weighed at a later stage, and the total amount of lipid was calculated using Equation 2, below.

- Equation 2:

$$Lipid\ content(\%) = \frac{weight\ of\ lipid\ (g)}{weight\ of\ freeze\ dried\ pellet\ (g)} \times 100\%$$

2.2.1. Preservation
The dried lipids were dissolved in chloroform, at the prescribed concentration, and then stored, for use in the future, at -20°C.

2.3. Composition of the Plant Extracts Mixture Content

2.3.1. Protein
The Pierce®BCA Protein Assay Kit was used to measure the protein content of the freeze-dried chloroplasts. Bovine Serum Albumin (BSA) standards (containing BSA at 2 mg/mL in 0.9% saline and 0.05% sodium azide) were freshly prepared to obtain a set of standards for proteins. Calibration curves were then made using six different concentrations (0 to 2,000 µg/mL) of BSA. The debris from the freeze-dried sample obtained from the spinach chloroplast were then dried for 30 minutes at 60°C. The dried samples were then grounded using pestle and mortar before being added to a 1 mL solution of 2% sodium dodecyl sulphate (SDS). At this point, 100 µl of the solution was then taken and 2 mL of standard working reagent was then added. The standard working reagent was then prepared by adding 50 mL of Reagent A (in this case: sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide – NaOH) with 2 mL of Reagent B (containing 4% cupric sulphate). The samples were incubated at 60°C for 30 minutes, before being vortexed and then centrifuged at 2,000 g for 3 minutes (this step was repeated twice). After which, the supernatant were collected and diluted 100 fold with 2% of the SDS solution. The samples were then incubated with a set of protein standards for 30 minutes at 37°C. The incubated samples were left to cool for 10 minutes and absorbance was measured at 562 nm against a blank. The concentration of the protein were determined against a standard curve produced using BSA.

2.3.2. Fatty acids
The composition of the fatty acids of the freeze-dried chloroplasts were determined using gas chromatography-mass spectroscopy (GC/MS), using the Pal System CST Analytical Autosampler and DSQ and TRACE GC Ultra (Thermo Electron Corporation).The lipids extracted were solubilised in 2 mL of chloroform and 0.2 mL of trimethylsulfonium hydroxide. The mixture was left to stand for at least 15 minutes for complete conversion of the fatty acids into fatty acid methyl esters (FAMEs); at which point, 200 µl was then injected into the injector (split flow 50 mL/min-1) at 250°C.The lipids were separated by polyethylene glycol (BP20 ID 0.22 mm x 25 m) gas chromatography column (Milton Keynes, UK), at 30 mL/min nitrogen. The oven temperatures were controlled at 120°C (1 minute), they were then increased by 5°C/minute until they reached 250°C. The retention time of each peak was compared with the FAME standards (Supelco® 37-Component FAME Mix). Furthermore, identification of the peak was also verified by comparing the mass spectrum with the standard library using the Thermo Scientific™ Xcalibur™ software program.
The internal standard stock solution used in this preparation was a combination of methyl pentadecanoate and glyceryl triheptadecanoate; specifically, 0.1 g of methyl pentadecanoate and 0.1 g of glyceryl triheptadecanoate were weighed and transferred to a 10 mL volumetric flask, where they were then dissolved with chloroform. The solutions were stored at -20°C and were used

within three months. The peak areas were normalised based upon the methyl pentadecanoate and the percentage of fatty acid, as calculated using the formula below. The derivatisation efficiency was checked by comparing the glyceryl triheptadecanoate and methyl pentadecanoate peak area ratios (designed as 1:1). The percentage fatty acid component was calculated using Equation 3.

- Equation 3:

$$\%\, individual\ fatty\ acid\ = \frac{individual\ fatty\ acid\ peak\ area}{total\ fatty\ acids\ peak\ area} \times 100\%$$

2.3.3. Vitamin E

Before being vortexed, 1 mg of vitamin E lipid extract was diluted with 2 mL of methanol. The sample was filtered using a syringe filter (0.45 µm) before being centrifuged at 2,000 g for 5 minutes, at 4°C. After which, 200 µL of the sample was transferred into HPLC vials and stored at -20°C before being injecting into the HPLC systems.

The HPLC analyses for vitamin E were carried out on a Waters® 2695 Separation Module that was equipped with a Waters® 996 Photodiode Array Detector (PDA) and a JASCO Intelligent Fluorescence Detector (FP-920) using a reverse phase Nova-Pak C18 Column (3.9 x 150 mm, 4 µm, Waters).The sentry guard-column used was Nova-Pak C18, 4 µm, 3.9 x 20 mm. A dual gradient mobile phase was prepared, this was made of acetonitrile: methanol: isopropanol: aqueous acetic acid (1%) (at: 45:45:5:5, v/v) for solvent A, and acetonitrile: methanol: isopropanol (at: 25:70:5, v/v) for solvent B. The gradient started with 0% A to 60% A from 0 to 10 minutes and held there for 12 minutes before being returned to the initial conditions. The flow rate was 0.8 mL/min. The 10 µL samples were injected and detection was performed at the excitation wavelength of 298 nm and the emission wavelength of 328 nm. Calibration curves were then plotted using six different concentrations (4-100 µg/mL) of pure standards of vitamin E (for: α, β + γ and δ forms).

2.3.4. β-carotene

Before being vortexed for 1 minute, 1 mg of β-carotene lipid extract was diluted with 2 mL of diethyl ether. The sample was centrifuged at 2,000 g, at 4°C, for 5 minutes. Then, 200 µL of the sample was transferred into HPLC vials and stored at -20°C before being injecting into HPLC systems.

The HPLC analyses for β-carotene were also conducted using a Waters® 2695 Separation Module that was equipped with a Waters® 996 PDA, using a reverse phase Nova-Pak C18 Column (3.9 x 150 mm, 4 µm, Waters). The sentry guard-column used was Nova-Pak C18, 4 µm, 3.9 x 20 mm. A dual gradient mobile phase was prepared and made of two mixtures, of solvent (A) acetonitrile: water (9:1, v/v) with 0.25% triethylamine and (B) ethyl acetate with 0.25% triethylamine. The gradient started with 0% A to 60% A, from 0 to 16 minutes and continued isocratically for up to 20 minutes. The flow rate was 0.8 mL/minute and 10 µL of the samples were injected. All chromatograms were monitored at 470 nm. Calibration curves were made using six different concentrations (0-250 µg/mL) of pure standards of β-carotenes.

2.3.5. Vitamin C

Vitamin C was extracted according to the combined methods of [viii] and [ix] with a slight modification. Specifically, 0.1 g of the sample was added to 5 mL of 2% meta-phosphoric acid.The mixture was placed in a conical flask (wrapped with aluminium foil) and agitated at 100 rpm with the aid of an orbital shaker for 15 minutes at room temperature. The mixture was centrifuged at 2,000 g at, 4°C, for 5 minutes, and then filtered through Whatman No.1 filter paper, so as to obtain a clear extract. The sample was extracted in triplicate.

The HPLC analyses for vitamin C were again conducted using a Waters® 2695 Separation Module equipped with a Waters® 996 PDA, usinga reverse phase column, Synergy™ 4 µ Hydro-RP Column (150 x 4.6 mm, 4 µm, Phenomenex). The sentry guard-column used was C18, 4 µm, 3.9 x 20 mm. A dual gradient mobile phase was prepared, it was made of acetonitrile: water (50:50, v/v) for solvent A, and 0.1 M potassium acetate pH 4.9 for solvent B. The gradient started with 90% A to 10% B from 0 to 13 minutes. The flow rate was 0.8 mL/min, and 10 µL of the samples were injected. All chromatograms were monitored at 254 nm. The calibration curves were made using six different concentrations (0-100 µg/mL) of pure standards of vitamin C. The vitamin C standard was prepared by dissolving 1 mg of L-ascorbic acid in 10 mL of meta-phosphoric acid (0.3 M), resulting in an acetic acid (1.4 M) solution.

2.3.6. Microbial Analysis

Chloroplast freeze-dried materials were prepared, as were the microbiological media which were prepared in accordance with the manufacturer's instructions. The media and media supplements prepared, and the organisms targeted, included: Plate count agar (Oxoid CM0325) for general bacteria count, Bacillus cereus agar base (Oxoid CM0617) for Bacillus cereus and other pathogenic Bacillus, Listeria enrichment broth base (Oxoid, CM0862) and Fraser broth base (Oxoid, CM0895) for Listeria and Rose Bengal Chloramphenicol agar for yeasts and moulds. Violet Red Bile Glucose agar (from Lab M™) was used for Enterobacteriaceae.

Analyses were then carried out using standard methods. For Listeria, 25 g samples were diluted 1/10 in Listeria enrichment broth base broths and emulsified in a stomacher for 2 minutes. The primary enrichment was then incubated at 30°C for 24 hours, after which 0.1 mL of the incubated was sub-cultured in 10 mL of Fraser broth and incubated at 37°C for 48 hours.The secondary enrichment (Fraser) broth culture was sub-cultured after 48 hours onto Oxford agar plates. The plates were examined for the presence of typical colonies after incubation. For other tests, 1 g of the sample was dissolved in 9 mL of sterile distilled water (dH^2O). With the exception of the

test for Enterobacteriaceae, where 1 mL of the sample was plotted using the pour plate method; specifically, 0.1 mL of the sample was spread on the plates of the other media prepared with standard incubation times observed

3. Results and Discussions

3.1. Nutritional Composition of Freeze Dried Spinach Chloroplast

Table 1 shows the main analyses of the nutrients found in the freeze-dried chloroplast material. Lipids and proteins are the two dominant components in the chloroplast membrane; to illustrate, lipids were estimated at 305 mg/gram (approximately 30%) and proteins were estimated at 505 mg/gram (approximately 50%), see Table 1 for a full breakdown of the values. Other research also supports this generated data; specifically lipid content falls within a range of 30 to 40%, while the protein content ranges between 50 and 60%[x].

In terms of fatty acid composition, there are two types of fatty acid: saturated and unsaturated fatty acid. In this research, both saturated and unsaturated fatty acids were determined. Only a small amount of saturated fatty acid were present, thus within this research only unsaturated fatty acid will be reported. As expected, α-linolenic acid (ALA) was identified as the highest amount of unsaturated fatty acid in the spinach chloroplast (see Table 1). This supports [xi] research which indicated that spinach is rich in linolenic acid. The highest amount of ALA presumably comes from the glycolipid components, of MGDG and DGDG.

The analyses conducted within this research returned unexpectedly low concentrations of vitamin E and vitamin C. Specifically, only 75% and 50% of what was reported in the literature for vitamin E and vitamin C content (respectively) were found from the spinach chloroplast. With regards to vitamin E, this could be as a result of lipid oxidation which may have occurred during storage to produce lipid free radicals. In order to inhibit the peroxidation of the lipid, vitamin E as an antioxidant will interact with these produced lipid free radicals. As a consequence of lipid peroxidation, it will contribute to a reduction of vitamin E content, even though vitamin E was reported to act in the reverse of this to form its normal component after interacting with the lipid radicals [xii].

Component	Amount per g non-sugar dry solids (i.e. chloroplasts)
Total lipid	305.8 ± 0.2 mg
Total protein	550 ± 0.6 mg
Fatty acid component α-linolenic acid	291 mg 228 ± 6.6 mg
Vitamin E	0.24 ± 0.1 mg
Vitamin C	6.4 ± 0.12 mg
β-carotene	1.37 ± 0.1 mg
Chlorophyll	68.3 ± 0.15 mg

Table 1: Composition of freeze-dried spinach chloroplasts

In addition to the oxidation process, the inefficient separation of the solute, during the HPLC analyses, may also have contributed to the lower value of vitamin E. With regards to the vitamin C, as it is soluble in water, it may have leached out somewhat during the extraction, as the main solvent used was aqueous. In addition, it is likely that the blending process may have also contributed to the loss of vitamin C. In support of this, it is known that processing methods, such as washing, blanching and boiling vegetables, may reduce vitamin C content, this is as a result of its labile and water soluble properties [xii].

In addition to analysing vitamin E and vitamin C, this research also identified two pigments that appeared to significantly contribute to the properties of spinach chloroplast –both of which will now be analysed. Consequently, the chlorophyll contents were analysed – they appeared to be high in amount (see Table 1). This could be explained as a result of a minimal loss of chlorophyll content during the processing, for example during the grinding process (minimal disruption of chloroplast organelle); thus, higher values of chlorophyll content were observed.

Furthermore, spinach is dense in chlorophyll, and it is reported that a high consumption level of chlorophyll is beneficial to human health [xiv] Another pigment that is present in spinach chloroplast is that of β-carotene. β-carotene is a form of pro-vitamin A and, when consumed in high doses, can cause side effects as the human body lacks any mechanisms to break it down, unless the level of intake is carefully regulated [xiv]. β-carotene is the only pro-vitamin A determined in the spinach chloroplasts in this research[xv]. The value of β-carotene from the spinach chloroplast, extracted for the purpose of this research, demonstrated a similar value to those found in the other research [xvi]. This result was expected as a very strong orange colour was observed in the samples that were prepared for analyses.

The dietary contribution of the biomarker molecules in the freeze-dried material were calculated based on the non-sugar dry solids, as presented in Table 2.Freeze-dried materials, even in relatively small doses, can provide significant % values of the RNI values for α-linolenic acid, as well as vitamins A, C and E. It is recommended that it is more beneficial to consume the whole vegetable extract rather than just the isolated compounds of vegetables (for example carotenoid dietary supplement) as they are more effective in preventing diseases.

Component	% RNI based on 1 g non-sugar dry solids
α-Linolenic acid	14-21%
Vitamin E	6-8%
Vitamin C	16%
β-carotene	17%

Table 2: Dietary contribution of biomarker components in the freeze-dried material

3.2. Microbial Load

Five tests were conducted in order to determine viable values of bacteria in the freeze-dried spinach chloroplasts. The following tests were therefore completed: plate count agar (PCA) for the bacteria count; a test for Bacillus; a test for Enterobacteriaceae; a test for yeast and mould; and, finally a test for Listeria. The results were compared to a set of standards that were outlined by [xvii], these standards can be used to determine the quality of the chloroplast as being satisfactory, acceptable, unsatisfactory or even potentially hazardous. Four terms were used to describe the grade of the microbiological quality, including: satisfactory which is indicative of good microbiology quality, acceptable which is an index reflecting a borderline limit of microbiological quality, unsatisfactory which is indicative that further sampling may be necessary and, finally, unacceptable or potentially hazardous which is indicative that urgent attention is needed to locate the source of the problem – in which case, a detailed risk assessment would be recommended.

With regards to this research, a viable number of bacteria were counted on each of the performed tests as being satisfactory (see Table 3). The results herein indicate that the freeze-dried spinach chloroplasts were of good quality. Although growth was observed in the agar, it is normal that some contamination during the processing stage would be encountered. To illustrate, some of the media demonstrated growth of the tested microbial, as shown in Figure1.It is suggested that this was due to contamination during and/or after preparation of the materials. Practically, cross contamination in food preparation environments can occur during the processing [xviii]. In practice, it is impossible to totally prevent contamination from the environment and by the food handlers; however, it is possible to minimise the risk by practising good hygiene.

Test	Cfu/g	Conclusion
General count on plate count agar (PCA)	1.6×10^4	Satisfactory
Test for Bacillus	2.0×10^2	Satisfactory
Test for Enterobacteriaceae	5.0×10^1	Satisfactory
Yeasts and mould	1.0×10^4	Satisfactory
Listeria	Not detected	Satisfactory

Table 3: Summary of microbial analyses

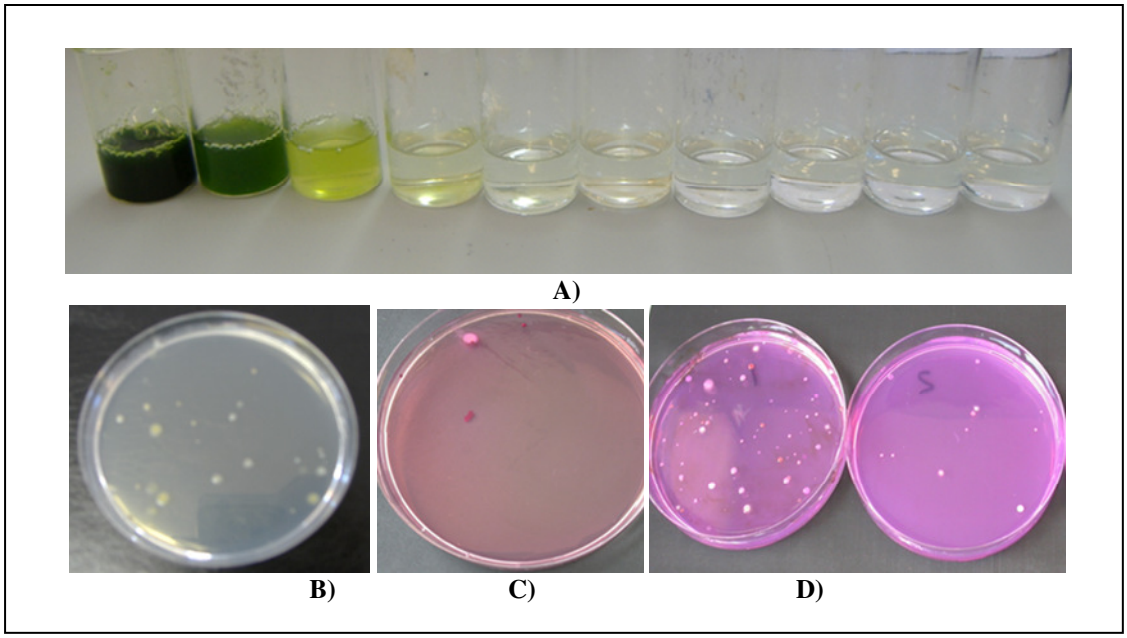

Figure 1: Panel (A) presents the serial dilution of the chloroplast, showing that the extracted material is soluble in water; Panel (B) shows growth on the plate count agar (PCA) medium; Panel (C) shows growth on the Violet Red Bile Glucose agar (VRBGA); whereas, Panel (D) shows growth on the Rose Bengal Chloramphenicol agar (RBCA) from the 10^1 (left) and 10^2 (right) dilutions

4. Conclusion

To conclude, the spinach chloroplasts extracted were nutrient dense and will ultimately potential as a functional food. Results from microbial test shows that powdered spinach is of a good quality and can be consumed – this is one of the most important requirements which need to be assessed prior to initiating *in-vivo*tests.

5. References

i. HanifR., Iqbal, Z., Iqbal, M.,Hanif, S. & Rasheed, M. (2006) Use of vegetables as nutritional food: Role in human health. Journal of Agricultural and Biol Science, 1, 18-22.
ii. Vaijayanthi Kanabur & Lalitha Reddy, R. P. (2014) Bioactive Components of Spinach and Their Effect On Some Patho Physiological Conditions: A Review. Int. J. Cur. Res. Rev., 06**,** 156.
iii. Mieke F., M., van Jaarsveld, P.J., Laubscher, R., 2007. The contribution of dark-greenleafy vegetables to total micronutrient intake of two- to five-year-old childrenin a rural setting. Water SA 33 (3 (special issue)) 407–412.
iv. Castenmiller, J.J.M. (2000). Spinach as a source of carotenoids, folate and antioxidant activity.ISBN9789058081605, pp183
v. Honda S. I., Hongladarom, T. & Laties, G. G. (1966) A new isolation medium for plant organelles. Journal of Experimental Botany, 17, 460-472.
vi. Folch, J., Lees, M. & Sloane, G. S. H. (1975) A simple method of the isolation and purification of total lipids from animal tissues. The Journal of Biological Chemistry, 226 (1), 497-509.
vii. Hincha, D. K., Oliver, E. A. & Crowe, J. H. (1998) The effects of chloroplast lipids on the stability of liposomes during freezing and drying. Biochimica et Biophysica Acta, 1368, 150-160
viii. Khalili M. A., A. H. Norhayati, M. Y. Rokiah, R. Asmah, M. Siti Muskinah & A. Abdul Manaf (2010) Determination of radical scavenging activity and Vitamin A, C and E in organically grown Red Pitaya (Hylocereus sp.). International Food Research Journal, 17**,** 405-409
ix. Abdulnabi, A. A., Emhemed, A. H., Husssein G. D. & Biacs P. A. (1996) Determination of antioxidant vitamins in tomatoes. Food Chemistry, 60, 207-212.
x. Jacob and Miller Jacob, J. S. and Miller, K. R. (1986) the effects of galactolipids depletion on the structure of a photosynthetic membrane. J. Cell Bio., 103: 1337-1347.
xi. Poincelot, R. P. (1974) an Improved Method for the Isolation of Spinach Chloroplast Envelope Membranes. Plant Physiol., 54**,** 780-783.
xii. Packer, K. J. & Rees, C. (1972) Pulsed NMR studies of restricted diffusion. I. droplet size distribution in emulsions. Journal of Colloid and Interface Science, 40 (2), 206-218.
xiii. Babalola O. O., Tugbobo O. S. & Daramola A. S. (2010) Effect of processing on the vitamin C content of seven Nigerian green leafy vegetables. Advance Journal of Food Science and Technology, 2, 303-305.
xiv. Kamat J.P., Boloor K.K., Devasagayam, T.P.A and Venkatachalam, S.R., 2000. Antioxidant properties of Asparagus racemosus against damage induced by ɣ-radiationin rat liver mitochondria. Journal of Ethmopharmacology, 71(3), 425-435
xv. Simonich MT, Egner PA, Roebuck BD et al., (2007) Natural chlorophyll inhibits aflatoxin B1-induced multi-organ carcinogenesis in the rat. Carcinogenesis 28:1294–1302
xvi. Rao, AV and Rao, LG (2007). Carotenoids and human health. Pharmacological Research Volume 55, Issue 3, Pages 207–216
xvii. Gilbert M., C. Wilhelm & M. Richter (2000) Bio-optical modelling of oxygen evolution using in vivo fluorescence: Comparison of measured and calculated photosynthesis/irradiance (P-I) curves in four representative phytoplankton species. Journal of Plant Physiology., 157**,** 307-314.
xviii. Yoda and Uchimura (2006).An outbreak of Campylobacter jejuni food poisoning caused by secondary contamination in cooking practice at a high school. Japanese Journal of Infectious Diseases., 59**,** 408-409.