

Development of a nutraceutical product as a supplement during pregnancy

*Dissertation submitted in partial fulfillment of the requirements for the
Degree of*

Bachelor of Technology

In

Biotechnology

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DECLARATION BY THE SCHOLAR

I hereby declare that the work reported in the B-Tech thesis entitled “**Development of a nutraceutical product as a supplement during pregnancy**” submitted at **Jaypee University of Information Technology, Wagnaghat India**, is an authentic record of my work carried out under the supervision of **Dr. Udaya Banu**. I have not submitted this work elsewhere for any other degree or diploma.

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SUPERVISOR'S CERTIFICATE

This is to certify that the work titled “**Development of a nutraceutical product as a supplement during pregnancy**”, submitted by **Abha Sharma** and **Shraddha Sinha** for end semester (Even) of Bachelor of Technology in Biotechnology Engineering to Jaypee University of Information Technology, Waknaghat, Solan has been carried out under my supervision. This work has not been submitted partially or fully to any other university or Institute for the award of this or any other degree or diploma.

Signature of Supervisor:

Name of Supervisor: Dr. Udayabanu

Designation: Assistant Professor- Senior grade

Date:

Acknowledgement

The success and final outcome of this project required a lot of guidance and assistance from many people and I am extremely privileged to have got this all along the completion of my project. All that I have done is only due to such supervision and assistance and I would not forget to thank them.

I owe my deep gratitude to our mentor and project guide Dr. Udayabanu, who took keen interest on our project work and guided us all along, till the completion of our project work by providing all the necessary information for developing a good system. I am also thankful to Lab staff of Biotechnology department for their continuous support and encouragement. Also, I would like to thank the officials of Jaypee University of Information Technology (JUIT), Wagnaghat for their help and cooperation.

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Abstract

Nutrition during pregnancy is very important. There are several essential nutrients and micro nutrients that regulate the healthy growth of the fetus as well as keeps the pregnant woman fit. While in the state of pregnancy, a woman is generally prescribed to have iron, calcium, zinc, folic acid, DHA, Vitamin C and Vitamin D. Therefore, in this current study that was carried in Jaypee University of Information Technology, Waknaghat in the biotechnology department we focused on the development of a nutraceutical product for a pregnant woman that will be used as a supplement during pregnancy. Based on the literature review, we identified that flax seeds (DHA, Iron, Zinc), asparagus (folic acid), mushroom (Vitamin D) and *Cissus quadrangularis* (Vitamin C, Calcium) were the plants we can use for our study as they had all the desired components that we were looking for. Further qualitative and quantitative tests were performed and data was recorded. Qualitative tests were performed so that there is an assurance of several micronutrients present in the sample. Once it was done, samples were subjected to quantitative analysis through HPLC and Atomic Absorption Spectroscopy. Data was recorded and further subjected for the formation of a desired ratio.

CHAPTER 1- INTRODUCTION

Nutrition during pregnancy is important to ensure the healthy growth of the fetus.

Several micronutrients are important for the health of the developing fetus.

The effectively existing sources are for the most part creature sources and the items accessible in the market are artificially integrated. Along these lines, we need to supplant that item with a natural item to limit the symptoms.

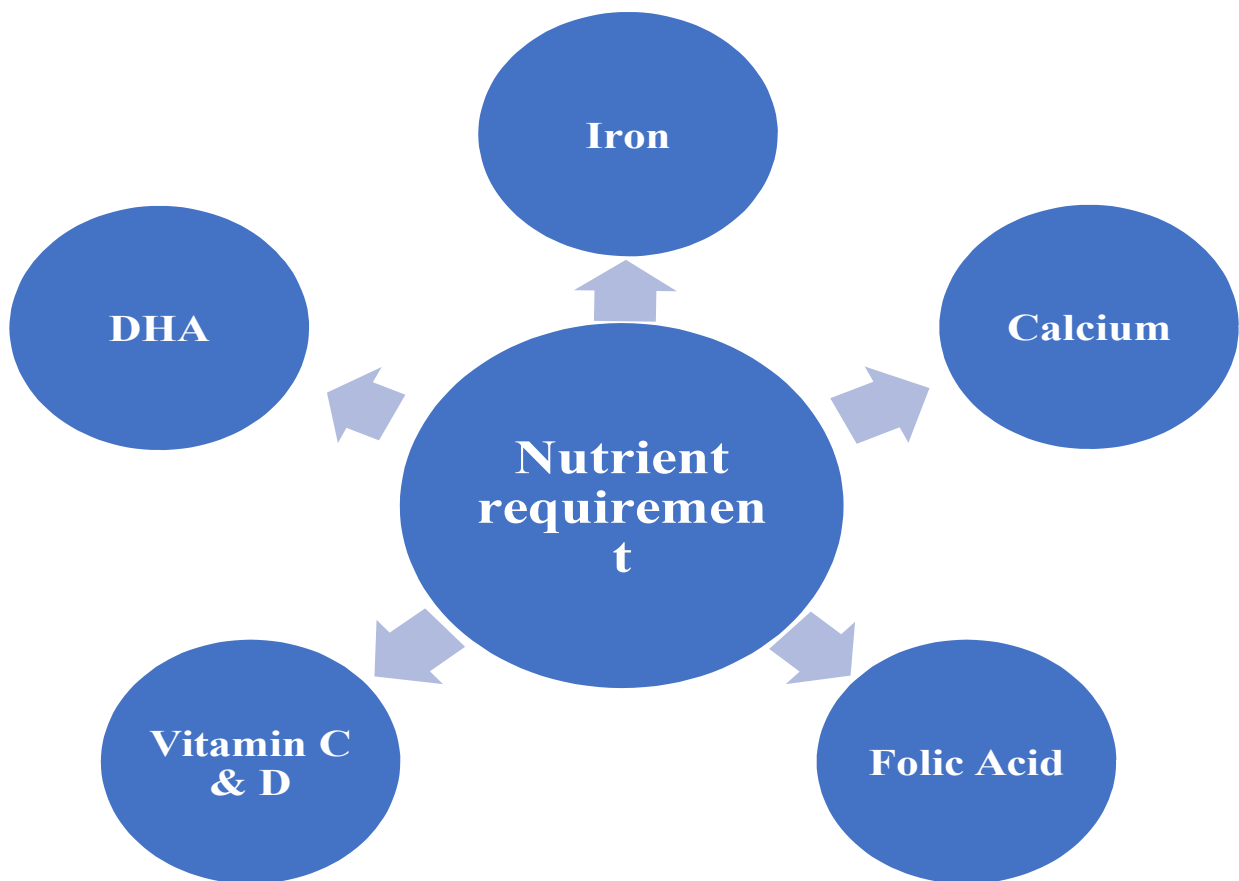


Fig: Important micronutrients that are required during pregnancy.

Their defined roles during pregnancy are as follows:

1- Folic Acid- Folic acid admission in pregnant ladies has appeared to diminish the danger of fetal neural tube deformities, for example, spina bifida (i.e.-birth imperfection where there is inadequate closing of spine and backbone around the spinal cord). Folic acid is useful for a pregnant woman as it works with nutrient B12 to frame healthy red platelets. Not having enough folic acid can prompt paleness and anemia.

2- DHA- Utilization of DHA is significant in light of the fact that newborn children can't produce DHA proficiently and must get it from lady through placenta amid pregnancy and in breast milk after birth. DHA helps in building infant's cerebrum, sensory system, and eyes. Infants get DHA from breast milk or from baby formula enhanced with DHA. It's important to the point that regardless of whether a mother doesn't expend much DHA, her body will utilize its own supply of DHA to give it to her developing infant amid growth and afterward through breast milk after birth.

3- Calcium- It is required for the development of bones. Likewise, it keeps teeth and bones sound. Calcium contributes in regulating muscular function, especially contraction and relaxation. Great measure of calcium in the body likewise improves heart work guideline. Calcium is likewise connected with improving sensory system message transmission or synaptic procedures and catalyst work. Besides, solid, circulatory and sensory system works additionally advantage from adequate measure of calcium amid this stage.

4- Iron- It diminishes the danger of maternal anemia and furthermore helps in the improvement of blood-iron dimensions. Pregnant ladies need more iron than non-pregnant ladies to help with expanded blood volume, to accommodate the infant's iron needs and to prepare the body for the birth.

5- Vitamin C- It advances the development of collagen which is the essential part of connective tissues. This aids in the sound development of infant's organs. A few analysts trust that nutrient C insufficiencies in infants can hinder mental advancement. Otherwise called ascorbic acid, nutrient C is basic for tissue repair, wound recuperating, bone development and fixation, and

healthy skin. Nutrient C enables our body to battle contaminations and goes about as a cancer prevention agent, shielding cells from harm. It will likewise help in the absorption of iron, particularly from vegetarian sources.

6- Vitamin D- It is required to keep up appropriate dimensions of calcium and phosphorus and furthermore diminishes complications like gestational diabetes (i.e.-glucose levels become excessively high) and preclampsia (i.e.-pregnancy intricacy that causes hypertension and kidney harm). Nutrient D insufficiency amid pregnancy is the beginning for a large group of future dangers for the child, particularly impact on neurodevelopment and resistant framework.

Note: Their levels have to be maintained quantitatively before the formation of a nutraceutical according to the minimal dosage requirement as their overdoses can be lethal both for mother and fetus.

CHAPTER 2- REVIEW OF LITERATURE

Literature Review:

Nutraceutical: A food stuff that is held to provide health or medical benefits in addition to its basic nutritional value. The term “Nutraceutical” was coined from “Nutrition” & “Pharmaceutical” in 1989 by Stephen DeFelice, MD, Founder and Chairman of the Foundation for Innovation in Medicine (FIM).

Other words used in the context: Dietary supplementation, Functional, Multi-functional Foods, etc.

Minerals and vitamins essential in pregnancy:

1- Folic Acid- Folic acid intake in pregnant women has shown to decrease the risk of fetal neural tube defects such as spina bifidia (i.e.- birth defect where there is incomplete closing of backbone and membranes around the spinal cord). These defects affect baby’s brain & spinal cord. Helps in RBC formation, formation of genetic material of cell, very much essential during pregnancy.

2- DHA- Consumption of DHA is important because developing infants cannot produce DHA efficiently and must receive it from woman through placenta during pregnancy and in breast milk after birth. Reduce CVD & improve mental, visual function. Helps in development of baby’s eyes & brain.

3- Calcium-It is required for the development of bones. Also, it is essential for bone and teeth, maintaining bone strength, nerve, muscle and glandular function, blood clotting. Required to build baby bones& regulates your body’s use of fluids.

4- Iron-It reduces the risk of maternal anemia and also helps in the improvement of blood-iron levels. Helps in energy production, Hb, oxygen transport.

5- Vitamin C-It promotes the formation of collagen which is the primary component of connective tissues. This helps in the healthy growth and development of baby’s organs.

6- Vitamin D-It is required to maintain proper levels of calcium and phosphorus and also helps to reduce complications like gestational diabetes (i.e- blood sugar levels become too high) and preclampsia (i.e.- pregnancy complication that causes high blood pressure and kidney

damage). Essential for formation of bones and teeth, helps the body to absorb and use calcium. Required to prevent low birth weight.

7-Zinc: Essential for cell reproduction, for development in Neonates, wound healing, production of sperm and testosterone hormone.

Note: Their levels have to be maintained quantitatively before the formation of a nutraceutical according to the minimal dosage requirement as their overdoses can be lethal both for mother and fetus.

Components Taken:

Cissus quadrangularis:



overview: It is a perennial plant. By and large found in parts of Asia and Africa. It is normally utilized as a therapeutic plant in different parts of Thailand because of its gainful wellbeing benefits. Cissus quadrangularis is utilized for obesity, diabetes, a bunch of coronary illness hazard factors called "metabolic disorder," and high cholesterol. It has additionally been utilized for bone cracks, gout, rheumatoid joint pain, hypersensitivities, loss of hunger (anorexia), feeble bones (osteoporosis), scurvy, malignancy, steamed stomach, hemorrhoids, peptic ulcer disease(PUD), difficult menstrual periods, asthma, seizures, jungle fever, wound mending, and torment. Cissus quadrangularis is additionally utilized in weight training supplements as an option in contrast to anabolic steroids.

Uses:

Bone breaks: Early research demonstrates that taking Cissus quadrangularis dried herb concentrates can diminish agony and swelling and improve the rate of healing in individuals with different sorts of bone cracks.

Hemorrhoids: Early research demonstrates that taking *Cissus quadrangularis* orally for 1-2 weeks, or applying a cream containing *Cissus quadrangularis* to hemorrhoids for multi week, does not improve hemorrhoid side effects.

Joint agony: Early research demonstrates that taking a *Cissus quadrangularis* item for about two months diminishes torment and firmness in men with joint torment brought about by exercise.

Stoutness and weight reduction: Early research demonstrates that taking *Cissus quadrangularis* extricates, alone or with different fixings, lessens weight in certain individuals who are large and overweight. Be that as it may, this exploration is commonly low quality.

Bone imperfections brought about by gum sickness. Early research demonstrates that adding *Cissus quadrangularis* to a material called hydroxyapatite, which is utilized in dentistry to treat tissue misfortune brought about by gum malady, does not improve tissue regrowth in individuals with explicit bone imperfections called periodontal intrabony abandons.

Allergies.

Asthma.

Bodybuilding.

Cancer.

Diabetes.

Gout.

Heart disease risk factors that occur together (metabolic syndrome).

High cholesterol.

Loss of appetite (anorexia).

Low bone mass (osteopenia).

Malaria.

Osteoporosis.

Pain.

Painful menstrual periods.

Peptic ulcer disease (PUD).

Rheumatoid arthritis.

Scurvy.

Seizures.

Upset stomach.

Wound healing.

Other conditions

Constituents:

Two new iridoids 6-O-[2,3-dimethoxy]-trans-cinnamoyl catalpol

6-O-meta-methoxy-benzoyl catalpol

iridoid picroside 1

two stilbenes quadrangularin A

also, pallidol

quercetin

quercitrin

β -sitosterol

β -sitosterol glycoside

Side-effects:

Cissus quadrangularis is Possibly safe when taken by mouth for short period of time (for up to 6-10 weeks). *Cissus quadrangularis* can cause reactions, for example, migraine, intestinal gas, dry mouth, loose bowels, and a sleeping disorder. However, there isn't sufficient data to know how regularly these reactions may happen.

The long-term effects of *Cissus quadrangularis* isn't known.

Precautions and Warnings:

Pregnancy: There isn't sufficient dependable data about the security of taking *Cissus quadrangularis* on the off chance that you are pregnant.

Diabetes: *Cissus quadrangularis* may bring down glucose. Taking *Cissus quadrangularis* alongside meds for diabetes may bring down glucose to an extreme level. Watch for indications of low glucose (hypoglycemia) and check your glucose levels intently, in case you have diabetes.

Medical procedure: *Cissus quadrangularis* may bring down glucose and could meddle with glucose control amid and after surgeries. Quit utilizing *Cissus quadrangularis* somewhere around about fourteen days before a planned medical procedure.

Asparagus:



Overview:

During past, asparagus was used as a sexual enhancer. This succulent, exquisite vegetable contains an invigorating mix of supplements that assists energy, clean the urinary tract and neutralizes ammonia, which can cause weakness and sexual disengagement.

High in nutrient K and folate (nutrient B9), asparagus is amazingly well-balanced, even among supplement rich vegetables. "Asparagus is high in mitigating the inflammation," stated nutritionist Laura Flores. It likewise "gives a wide variance of anti-oxidants supplements, including nutrient C, beta-carotene, nutrient E, and the minerals zinc, manganese and selenium."

Uses:

Asparagus has more than 1 gram of dissolvable fiber per glass, which brings down the danger of coronary illness, and the amino-acid asparagine helps flush of excessive salt from your body. In conclusion, asparagus has astounding mitigating effects on inflammation and elevated amounts of cancer prevention agents, the two of which may help lessen the danger of coronary illness.

Regulating glucose level:

Nutrient B6 may influence glucose levels and exhorts alert for individuals who have diabetes or low glucose. Anyhow, healthy individuals can profit by asparagus' ability to manage it.

Menacing the danger of sort 2 diabetes:

Similarly, as with coronary illness, danger of sort 2 diabetes elevates with inflammation and oxidative pressure. As a result, asparagus' amazing anti-inflammatory properties and elevated amounts of cancer prevention agents make it a decent preventive foodstuff. A recent report in the British Journal of Nutrition proposed that asparagus' capacity to improve insulin production and improve beta-cell work, helps bring down the danger of sort 2 diabetes.

Anti-aging benefits:

The cancer prevention agent glutathione is thought to moderate the aging procedure. Furthermore, the folate that asparagus gives works with B12 to prevent cognitive decrease.

Skin:

One more astonishing thing about glutathione: it shields the skin from sun harm. A 2014 examination distributed in Clinical, Cosmetic, and Investigational Dermatology studied sound grown-up ladies aged 30-50, who put a glutathione moisturizer to the half portion of their faces and an inactive cream to the next half for 10 weeks. The glutathione side of the face was much more moisturized than the other half, less wrinkle development and smoother skin.

Anticipating kidney stones:

Asparagus can go about as a characteristic diuretic. This can help reduce the collection of excessive salts, making it particularly useful for individuals having disorders like edema and hypertension. It additionally helps flush out toxic substances from kidneys and reduce chances of kidney stones.

Healthy Pregnancy:

Flores noticed asparagus' critical measure of folate, which she said "is significant for ladies of childbearing age to consume on a daily basis." Folate can diminish the danger of neural-tube defects in hatchlings, so it is fundamental that moms to-be, get enough of it.

Stomach related wellbeing:

"Asparagus is known to help balance out the digestion process because of the high levels of fibers and proteins," said Flores. "Both help in food transportation via gut and give alleviation from uneasiness caused during digestion."

Side-effects:

"There are no dangerous reactions of eating an excessive amount of asparagus," said Flores, "however there might be some symptoms, for example, gas, and a perceptible smell to the pee."

It is also possible to have an asparagus sensitivity, in this case you should probably not consume it, she said. Individuals who are hypersensitive to the members of the lily family, for example, onions, garlic, and chives, are bound to be adversely affected by asparagus. Side effects incorporate a runny nose, hives, breathing problems, and puffiness or swelling around the mouth and lips.

Mushroom:



overview:

Mushroom is a fungi that a few people portray as "extreme" and "woody" with an unpleasant taste. The fruiting body (over the ground part) and mycelium are utilized as prescription.

Mushroom is utilized for boosting the immune; viral contaminations, for example, this season's cold virus (flu), swine influenza, and avian influenza; lung conditions including asthma and bronchitis; coronary illness and contributing conditions, for example, hypertension and elevated cholesterol; kidney malady; malignant growth; and liver ailment. It is likewise utilized for HIV/AIDS, height ailment, interminable weakness disorder (CFS), inconvenience dozing (sleep deprivation), stomach ulcers, harming, and herpes torment. Different utilizations incorporate lessening pressure and anticipating weakness.

Uses:

Early research proposes that consuming mushroom on a daily basis for a year diminishes the quantity of tumors in individuals with colorectal adenomas.

Blocked arteries:

Early research recommends that taking a mushroom item (Ganopoly) decreases side effects of blocked arteries, including chest agony and shortness of breath.

Diabetes:

Early research proposes that consuming mushroom item (Ganopoly) for 12 weeks lessens hemoglobin but not glucose levels in individuals with sort 2 diabetes.

Hepatitis B:

Early research shows that taking a particular mushroom item (Ganopoly) for 12 weeks decreases the amount of the hepatitis B infection coursing in the body. This item likewise appears to improve liver capacity in individuals with this condition.

Elevated cholesterol levels:

Early research shows that consuming mushroom every day for 12 weeks does not influence cholesterol levels in individuals with hypertension and additionally elevated cholesterol.

Hypertension:

There is conflicting proof about the impacts of mushroom on hypertension. Early research shows that intake of mushroom on a daily basis for 12 weeks does not bring down circulatory strain in individuals with marginally high blood pressure. In any case, other research shows that intake of mushroom brings down pulse in individuals with serious hypertension.

Lung cancer:

Early research shows that consuming mushroom does not affect lung tumors. As a result, it appears to improve immune function in individuals with lung cancer.

Shingles-related agony:

A few people report that heated water concentrates of mushroom diminishes pain when ordinary treatment does not work.

Viral diseases.

Prostate malignant growth.

Kidney malfunction.

Liver illness.

HIV illness.

Weakness.

sleep deprivation.

Stomach ulcers.

Different conditions.

Side-effects:

Mushroom is safe for use when taken orally for as long as one year.

Mushroom is safe when taken as powder for over one month. Utilization of powdered mushroom is associated with harmful impacts for the liver.

Mushroom can likewise cause opposite reactions including dryness of the mouth, throat, and nasal irritation, stomach upset, nosebleed, and grisly stools. Inhalation of spores can trigger sensitivities.

Flax seeds:



overview:

Flax is a nourishment and fiber crop that develops in Europe and the Mediterranean. Flaxseeds are the brilliant yellow to ruddy darker seeds of flax. These seeds contain phytoestrogens, which are like the hormone estrogen. Flaxseed has been taken up as a sustenance or utilized as a drug since 5000 BC.

Individuals consume flaxseed for blockage, colon harm because of abuse of diuretics, diarrhea, aggravation of the covering of the internal organ (diverticulitis), inflamed colon, bruises in the lining of the digestive organ (ulcerative colitis), irritation in the lining of the stomach (gastritis), and irritation of the small intestine (enteritis).

Individuals take flaxseed orally for heart disease and blood vessels, including coronary illness, high triglyceride levels, elevated cholesterol, "solidifying of the supply routes" (atherosclerosis), hypertension, coronary course infection, and metabolic disorder.

Uses:

Diabetes:

Taking flaxseed may improve glucose control in individuals with sort 2 diabetes. Advantages appear to be most prominent with whole flaxseed and when utilized for 12 weeks. Flaxseed appears to work best in individuals with sort 2 diabetes that is difficult to control.

Rise in cholesterol level:

Research demonstrates that different flaxseed mixtures, including ground flaxseed, defatted flaxseed, flaxseed concentrate, and flaxseed bread and biscuits, appear to diminish absolute cholesterol level by 5% to 15% and LDL cholesterol by 8% to 18% in individuals with typical cholesterol levels, just as in men and pre-menopausal ladies with elevated cholesterol. Nonetheless, there is some clashing proof. Some exploration demonstrates that flaxseed does not improve LDL cholesterol levels in postmenopausal ladies with ordinary or elevated cholesterol. It additionally does not appear to diminish absolute cholesterol or LDL cholesterol in individuals with somewhat elevated cholesterol compared with following a cholesterol-bringing down diet. Likewise taking flaxseed on a daily basis for about a month in biscuits and breads does not reduce total or LDL cholesterol in kids with a family history of elevated cholesterol. The distinctions in adequacy may be identified with the type of flaxseed utilized just as varieties in the severity of cholesterol levels in the general population considered.

Hypertension:

Research recommends that taking flaxseed can decrease circulatory strain. Likewise, eating processed flaxseed in bread on a daily basis for a half year appears to decrease circulatory strain in individuals with narrowed veins and hypertension.

Bosom pain (mastalgia):

Research demonstrates that eating a flaxseed biscuit on a daily basis for 3 months or taking flaxseed powder every day for 2 months decreases bosom pain associated with the beginning of the menstrual cycle.

Immune system disorder (foundational lupus erythematosus, SLE):

Taking entire or ground flaxseed orally appears to improve kidney's working in individuals with SLE.

Weight reduction:

Taking no less than 30 grams of flaxseed for each day for 12 weeks appears to help manage body weight, BMI, and waist size in grown-ups. Flaxseed appears to work best in individuals who are tolerably obese before treatment.

Side-effects:

Flaxseed is considered safe for most grown-ups when taken orally. Adding flaxseed to the eating routine may expand the quantity of solid discharges every day. It may also cause gastrointestinal (GI) reactions, for example, swelling, gas, stomach torment, clogging, looseness of the bowels, stomachache, and sickness. Higher dosages are probably going to cause more GI reactions.

There is concern that taking a lot of flaxseed could obstruct the digestion tracts because of the bulk-forming laxative effects of flaxseed. Flaxseed ought to be taken with a lot of water to keep this from occurring.

Lignans are the synthetic compounds in flaxseed that are believed to be responsible for a large number of the impacts. Some clinical research demonstrates that a particular flaxseed lignan extract can be securely utilized for as long as 12 weeks.

Amount required on daily basis in pregnant women:

Iron: 30 mg/day.

Vitamin C: 85 mg/day.

Calories: Additional 300.

Vitamin D: 10 mg/day.

Calcium: 1200 mg/day.

Folate: 600-800 mg/day.

DHA: 200 mg/day.

Qualitative tests:

Iron: Hydrogen sulphide test.

Folic acid: High performance liquid chromatography.

Vitamin D/C: High performance liquid chromatography.

Calcium: Cobalt Nitrate test, Flame test.

Quantitative tests:

Iron: Inductively Coupled Plasma-Atomic Emission Spectroscopy.

Calcium: Inductively Coupled Plasma-Atomic Emission Spectroscopy.

Vitamin D: High Performance Liquid Chromatography.

Folic acid: High Performance Liquid Chromatography.

DHA: Gas chromatography with flame ionization detector.

- Identification/ Selection of organic (natural sources) rich in Iron, Calcium, Folic Acid, DHA, Vitamin C and D.
- To estimate qualitatively and quantitatively the various nutrients present.
- Development of a nutraceutical product to supplement minimal daily nutritional requirement of a pregnant women.

CHAPTER 4- Materials Required and Methodology:

4.1- Materials Required:

1- The basic glassware that was used during the experiments:

Flasks, tarsons, funnel, closed jars, dropper, distilled water, milli q water, crucibles, Whatman Filter paper, beakers, Millipore filter paper (syringe filter).

2- Chemicals Required:

Sulphuric acid, 5N Nitric Acid, Sodium phosphate monobasic, Sodium phosphate dibasic, 2-mercapto ethanol, methanol, sodium hydroxide.

3- Instruments used:

Oven, incubator, weighing balance, muffle furnace, desiccator, centrifuge, vortex shaker, chromatograph.

4- Plants used:

Asparagus, *Cissus quadrangularis*.

5-Seeds used:

Flax seeds

6- Fungi used:

Mushroom

4.2- Methodology:

1- Collection of plants and organic matter.

- Different organic matter and plants were collected according to the required properties.
- It was then washed separately with milli q water.
- Wet matter's weight is done on weighing balance and values are noted.
- Each matter is further kept in an oven at 60 degrees Celsius for 2 days for drying.
- Dried matter weight is noted.
- It is then crushed using a mixer into a fine powder.

2- Elemental Analysis

2.1: Ash Value

- Crucibles were taken and washed properly with milli q water.
- It was then washed with 1-2mL of sulphuric acid.
- It was then placed in an incubator for drying for 1 hour.
- After that, the crucibles are moved to the desiccator for 2-3 hours.
- Then, the weight of crucibles is done before adding the sample.
- After addition of samples, the overall weight of crucible is done.
- The crucibles are then placed in the muffle furnace at 500 degree Celsius for 3 hours.
- After 3 hours, switch off the muffle furnace and let the matter to cool down.

2.2: Chemical Preparation. (Preparation of 5N Nitric acid)

- $\text{Molarity} = \text{Normality} \times \text{n-factor}$ [n-factor of nitric acid= 1]

Therefore, $\text{Molarity} = \text{Normality}$

- $\text{Mass of pure Nitric Acid} = 5 \text{ moles/L} \times 1\text{L} \times 63\text{g/mol}$
 $= 315 \text{ grams.}$
- $\text{Mass of 70\% HNO}_3 \text{ needed} = 315\text{g}/0.70$

= 450g in 1L. Therefore, 45g in 100mL.

3- Sample Preparation.

A- Sample preparation for atomic absorption spectroscopy.

- After cooling, spill a few drops of demineralized water (milli q), until wet.
- Dissolve in 5mL 5N Nitric Acid, put in a 100mL volumetric flask.
- Rinse crucible porcelain 3 times each flushing with 10mL of milli q water.
- Dilute with milli q water until the marking line and shake until homogeneous mixture was filtered, discard the first 10mL filtrate.

B- Sample preparation for chromatography analysis.

- Firstly, the samples were extracted with 50mL of 0.1 mol/L phosphate buffer (pH-7.0).
- Then 0.1% (V/V) of 2-mercaptoethanol was added.
- The mixtures were then shaken for 30 minutes each in a vortex shaker.
- It was then centrifuged at 3500 rpm for 15 minutes.
- It was then filtered through a syringe filter before chromatography analysis.

4- Purification.

- Whatman filter paper was used for the purification.
- Filter paper is placed in funnel and it is filtered thoroughly.
- Accommodate the subsequent filtrate in glass bottle.
- It is then stored and will be used for quantitative analysis.

5- Qualitative Analysis tests.

A- Qualitative test for iron.

- All the plant samples (mushroom, flax seeds, asparagus, *cissus quadrangularis*) were taken separately in a powdered form.
- Samples were then dipped in water and organic solvents and kept overnight so that the minerals are dissolved properly in water for further testing.
- Next day, the solution / samples were taken in a test tube.
- Then, to a drop of unknown solution several drops of ammonium thiocyanate (NH_4SCN) was added.
- Bloody red color was then appeared which confirmed the presence of iron.

B- Qualitative test for Calcium (Flame test)

- The compound/ metal salt was taken and is mixed with few drops of concentrated hydrochloric acid.
- This sample is then heated strongly in a Bunsen flame on the end of a clean wire or loop.
- After some time, it will show some characteristic color.
- Here, in case of calcium, a white colored precipitate is observed.

C- Qualitative test for DHA

- A few drops of phenolphthalein solution is taken in a test tube.
- One or two drops of very dilute alkali solution is added to it, just sufficient to give the solution a pink color.
- Now a few drops of the mixture is added and shaken well.
- The color will disappear as the alkali is neutralized by the free fatty acids present in the oil. This will confirm the presence of DHA.

D- Qualitative test for Zinc.

- Sample mixture is taken.
- Dilute sodium hydroxide solution is added to the solution where the ions are suspected.
- It was then stirred a bit.
- After that, a white precipitate of zinc hydroxide appeared.
- It confirms the presence of zinc in the solution.

6- Quantitative Analysis tests.

A- Samples quantified through inductively coupled plasma atomic emission spectroscopy.

- Iron
- Calcium

B- Samples quantified through high performance liquid chromatography.

- Vitamin D
- Folic Acid

C- Samples quantified through gas chromatography with flame ionization detector.

- DHA.

Chapter 5- Results and Discussions:

Elemental analysis was conducted to calculate the ash value that will be further used in quantification through atomic emission spectroscopy. The ash values thus recorded for different components are as follows:

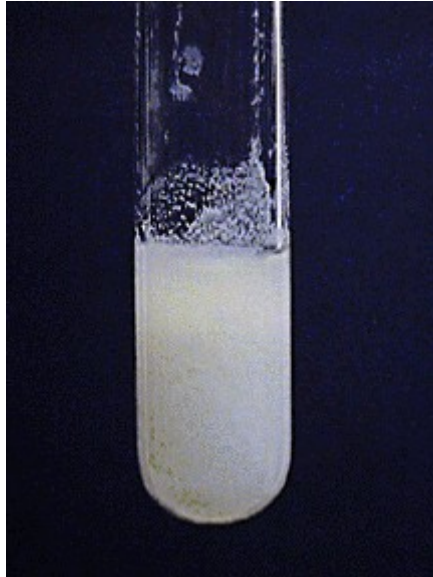
- 10 g of each components was weighed.
- *Cissus quadrangularis*:
Crucible 1 weight = 22.64 g.
Total Weight = Crucible + CQ = 32.7 g.
After ash formation,
weight of crucible + CQ = 24.17 g.
CQ = 24.17 - 22.64
= 1.53 g.
- Mushroom:
Crucible 2 weight = 19.7 g.
Total Weight = Crucible + Mushroom = 29.7 g.
After ash formation,
weight of crucible + Mushroom = 21.23 g
Mushroom = 21.23 - 19.7
= 1.53 g.
- Flax seeds:
Crucible 3 weight = 22.55 g.
Total Weight = Crucible + Flax seeds = 32.63 g.
After ash formation,
weight of crucible + Flax seeds = 22.86 g.
Flax seeds = 22.86 - 22.55
= 0.31 g
- Asparagus:
Crucible 4 weight = 22.03 g.
Total Weight = Crucible + Asparagus = 32.08 g.
After ash formation,
weight of crucible + Asparagus = 22.33 g
Asparagus = 22.33 - 22.00 = 0.30 g

Qualitative test results.

- Bloody red color appeared confirmed the presence of iron in flax seeds the most.



- Formation of white color precipitate confirmed the presence of calcium in the solution of *cissus quadrangularis* the most.



- Disappearance of color from the solution confirms the presence of DHA in the solution of flax seeds.
- Appearance of white precipitate of zinc hydroxide confirms the presence of zinc mostly in flax seeds.



Quantitative test results.

- Vitamin D analysis by HPLC:

Control = 31.8 micro g /100 g.

2 hours exposure = 2126.99 micro g/ 100g.

30 mins exposure = 1390.50 micro g/ 100g of dried mushroom.

Chapter 6-References:

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