# ‘Folic Acid Supplement’

Shubham S. Pawar[1], Manasi D. Suryawanshi[1], Vrushali B. Baviskar[1], Asmita K. More[1], Satyam B. More[1], R. Y. Ghegde[2]

1. Students, GES’s Sir. Dr. M. S. Gosavi College of Pharmaceutical Education and Research, Nashik, Maharashtra, India-05.
2. Assistant Professor Pharmacognosy, Department of Pharmacognosy, GES’s Sir Dr. M. S. Gosavi College of Pharmaceutical Education and Research, Nashik, Maharashtra, India-05.

Corresponding author – *Shubham S. Pawar[1], Manasi D. Suryawanshi[1]
shubhampawar4354@gmail.com[1], suryawanshimanasi19@gmail.com[1]

Address - GES’s Sir. Dr. M. S. Gosavi College of Pharmaceutical Education and Research, Nashik, Maharashtra, India-05.

## Abstract

*The vitamin B9, abundantly found in food, vitamin supplements and fortified like products, provided through such supplements and/or the stable form folate, plays important role in the synthesis of DNA and RNA, principal material of cell nucleus and metabolism of amino acid for cell division, as a paramount concern of folic acid deficiency could lead to serious metabolic manifestations like preterm delivery, infant low birth weight and fetal growth retardation especially in children and women across the globe, herein the majority of population takes diets with low concentration of vitamins and minerals; thus the reduced intake of folic acid further causes the elevated homocysteine level develops pregnancy complications like placental abruption and preeclampsia lead to low birth weight and gestation period confers poor pregnancy. The present study includes the systematic review of overview of folic acid, its deficiency disorders and its role in cell cycle and its metabolism; methods of extraction and determination of folic acid and the folic acid rich herbal plants overview to find out the natural remedy in future, if any.*

**Keywords – Folic acid, Synthesis of DNA & RNA, Pregnant**

# 1. INTRODUCTION

The B vitamin folate, commonly known as vitamin B-9, is found naturally in some foods. Manufacturers add folic acid as a type of folate to vitamin supplements and fortified meals. One of the B vitamins is folate, also referred to as vitamin B9 and folacin. Because it is more stable during processing and storage, manufactured folic acid, which is transformed into folate by the body, is used as a dietary supplement and in food fortification. The body needs folate to synthesize DNA and RNA and to metabolize the amino acids needed for cell division. As folate cannot be produced by humans, it must be obtained through diet, making it an essential nutrient. Many foods naturally contain it[1,2].

**1.1 Requirement table of folic acid**

| **Category** | **Folate (folic acid) Recommended Dietary Allowance (RDA) For children 1, only an adequate intake (Al) is available** |
|---|---|
| 0-60 Months | 65 micrograms/day Adequate |
| 7-12 Months | 80 micrograms/day Adequate |
| 1-3 Years | 150 micrograms/day |
| 4-8 Years | 200 micrograms/day |
| 9-13 Years | 300 micrograms/day |
| 140Years and up | 400 micrograms/day |
| Pregnant women | 600 micrograms/day |
| Breastfeeding Woman | 500 micrograms/day |

**1.2 Deficiency of folic acid**

The symptoms of folate deficiency are often subtle which includes as follows: Fatigue, Gray hair, Mouth sores, Tongue swelling, Growth problems, Peripheral neuropathy (the result of damage to one or groups of nerve)[2].

**1.3 Disease**

Conditions that affect absorption in the gastrointestinal tract can cause folate deficiencies. They include:

- Crohn’s disease
- Celia’s disease
- Certain types of cancer

- Several kidney problems that require dialysis

### 1.4 Available medicine in market

| Allopathic formulation | Herbal formulation |
|---|---|
| Folic acid systemic | 5-HTP |
| Deplin | Azo-Cranberry |
| I-methyl folate | Evening Primrose |
| Folacin-800 | Valerian Root |
| FA-8 | Ginkgo Biloba |
| Elfolate | Sambucol |
| XaQuil XR | Ginger Root |
| Denovo | Venastat |
| | Ellura |
| | TheraCran HP |
| | OranMagic Rx |

### 1.5 Natural supplements provide Folic acid

- Broccoli
- Brussels sprouts
- Leafy green vegetables such as Cabbage, Spring greens and Spinach
- Chickpeas and kidney beans
- Sunflower seeds
- Peanuts
- Avocados
- Asparagus.

## 2. Overview of Folic Acid

### 2.1 Chemical and physical data of folic acid

- Molecular formula: $C_{19}H_{19}N_7O_6$
- Molar mass: 441.401 - 441.404 $g/mol^{-1}$
- Density: 1.6±0.1 $g/cm^3$
- IUPAC Name: (2S)-[4-[(2-amino-4-hydroroxypteridin-6-yl) methylamino] benzo amido] glutamic acid
- Category: Hematinic
- Melting point: 250˚C (482˚F)
- Solubility in water: 1.6 mg/L
- Description: A yellow to yellowish-orange, crystalline power, odorless or almost odorless.
- Structure:

**2.2 Pharmacokinetic data**:

- Bioavailability: 50-100 %
- Metabolism: Liver
- Excretion: Urine

**2.3 Mechanism of Action:**

Folic acid is a crucial vitamin for the production of proteins and nucleic acids (DNA and RNA). All cells require folic acid for development, which is produced by bacteria from the substrate para-amino-benzoic acid (PABA). Mammalian cells are either diffused or transported with folic acid (as a vitamin in meals). By diffusion or active transport, folic acid cannot penetrate bacterial cell walls, nevertheless, bacteria must therefore produce folic acid from PABA. As a substrate for the enzyme dihydropteroate synthase, which converts PABA into dihydropteroic acid, the direct precursor of folic acid, sulfonamides compete with PABA to have an effect. Bacteriostatic agents are sulfonamides.

Bacteriostatic medications such as trimethoprim, ormetoprim, and pyrimethamine prevent the dihydrofolate reductase activity required for the production of purine and pyrimidine nucleotides. Similar to sulfonamides, they also prevent the synthesis of folic acid, but at a different stage of the metabolic process. Mammalian cells do not contain dihydropteroate synthase, however pyrimethamine and trimethoprim are more effective against the dihydrofolate reductase of parasites than it is against the mammalian enzyme [4].

**2.4 Deficiencies of folic acid:**

Folate deficiency:

Folate, or folic acid is a type of B vitamin. It helps to:

- Make DNA
- Repair DNA
- Produce red blood cells

You could develop a folate shortage if you don't get enough folate in your diet. Citrus juices and dark green vegetables are two examples of meals and beverages that are particularly effective providers of folate. Within a few weeks, a shortage might result from eating insufficient amounts of folate. A illness or genetic mutation that stops your body from absorbing or converting folate to its useable form may also result in deficiency. Anemia may result from a folate deficit. Too few RBCs are present when you have anemia. Because RBCs deliver the oxygen, anemia can deprive your tissues of the oxygen they require. This might interfere with how they work. Folate is crucial for those who have uteruses that are ready to bear children. During pregnancy, a folate shortage can result in abnormal births. Most people consume adequate folate through their diets. In order to prevent deficiency, folic acid, a synthetic form of folate, is currently added to a lot of foods. However, it is advised that women who may get pregnant take supplements.

The symptoms of folate deficiency are often subtle. They include: (Fatigue, Gray hair, Mouth sores, Tongue swelling, Growth problems, Peripheral neuropathy (the result of damage to one or more groups of nerves)[5]
The symptoms of anemia that occur due to folate deficiency include:

- Persistent fatigue
- Weakness
- Lethargy
- Pale skin
- Shortness of breath
- Irritability [6]

**Causes of folate deficiency**

A water-soluble vitamin is folate. It doesn't get stored in your fat cells and dissolves in water. This means that since your body cannot build a reserve, you must continue taking folate. Vitamins that are water soluble are excreted in excess by people in their urine.
The causes of folate deficiency include:

1. Diet:
   The main factor contributing to a lack of folate is a diet that is low in fresh fruits, vegetables, and fortified cereals. Additionally, vitamins can occasionally be destroyed by overcooking meals. If you don't consume enough meals high in folate, your body's levels of the vitamin can drop in as little as a few weeks.
2. Diseases: Conditions that affect absorption in the gastrointestinal tract can cause folate deficiency. They include:
   - Crohn’s disease
   - Celiac disease
   - Certain types of cancers
   - Severe kidney problems that require dialysis
3. Genetics:
   Some persons have a genetic mutation that prevents their body from effectively converting dietary or supplementary folate to methyl-folate, which is folate's useable form.
4. Medication side effects: Certain medications can cause folate deficiency. These include:
   - Phenytoin (Dilantin)
   - Trimethoprim-sulfamethoxazole Trusted Source
   - Methotrexate
   - Sulfasalazine
5. Excessive alcohol intake: Alcohol prevents the body from absorbing folate. Additionally, it enhances the excretion of folate in urine.
6. Risk factors for folate deficiency:

Factors that may increase the likelihood of having a folate deficiency include:

- Heavy alcohol abuse
- Pregnancy
- Being of childbearing age
- Eating overcooked foods
- Consuming a vitamin-poor diet
- Medical conditions, such as sickle cell disease
- Low socioeconomic status
- Elderly people living in institutions
- A genetic polymorphism in the MTHFR gene
- Malabsorption syndromes, such as celiac disease and inflammatory bowel disease
- Certain medications

7. Pregnancy complication:

During pregnancy, folic acid is very crucial. Folic acid can aid in the prevention of some birth problems in infants known as neural tube defects. Because they might harm the brain or spinal cord, neural tube abnormalities are dangerous. They consist of:

- **Spina Bifida**: When a baby's spinal cord or brain don't fully develop in the pregnancy, this syndrome develops. Spina bifida babies frequently suffer paralysis and other physical impairments, and they frequently need surgery.

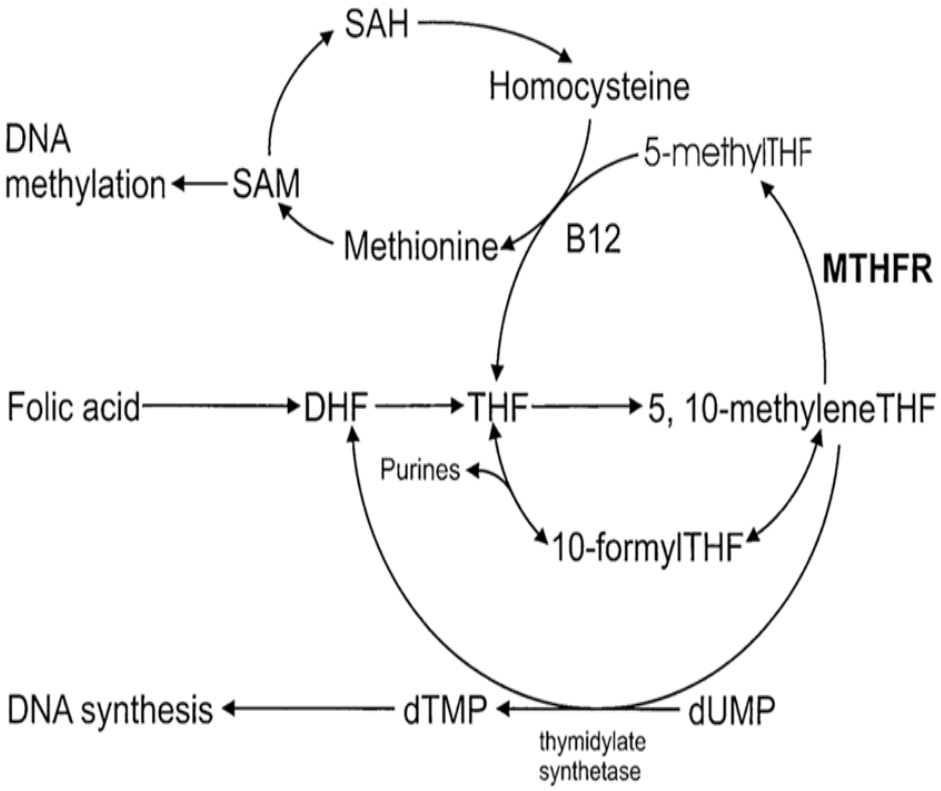


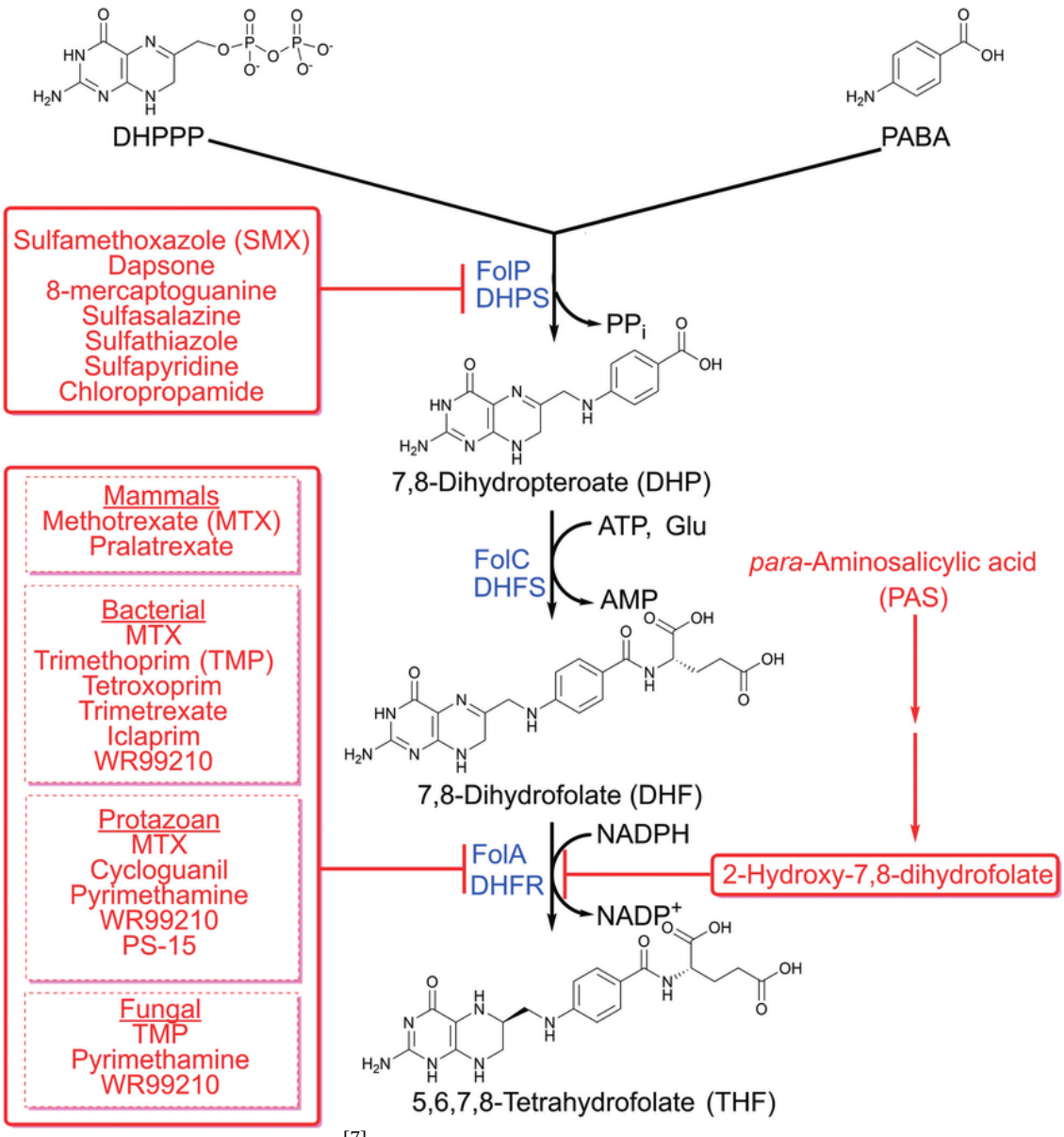
Fig. 2.5 Metabolic pathway of folic acid [8]

- **Anencephaly:** The brain and skull of a developing child fail to fully develop in the womb, in this syndrome. The majority of anencephaly newborns are still born or pass away soon after birth.

**Complications of folate deficiency**

For RBCs to be produced normally, folate is necessary. A deficiency's complications could include:

- Megaloblastic anemia is characterized by larger-than-normal and incompletely formed RBCs.
- Low amounts of platelets and white blood cells.
- Neural tube defects are severe birth anomalies in the growing fetus's spinal cord and brain.




- Peripheral neuropathy.[7]

Fig.2.6 Biosynthesis of folic acid [9,10]

## 3. Herbal sources of folic acid:

### 3.1 Spinach:

Biological Source: *Spinacia oleracea*

A leafy green blooming plant native to central and western Asia is called spinach. It belongs to the subfamily Chenopodioideae of the family Amaranthaceous in the order Caryophyllales. Its leaves are a popular edible vegetable that can be eaten either raw or cooked after being preserved through canning, freezing, or dehydration. The taste varies greatly depending on whether it is consumed cooked or raw, and steaming can help to lower the high oxalate level

larger leaves are found at the plant's base and smaller leaves are found higher up on the flowering stem. The leaves are alternate, simple, ovate to triangular, and very variable in size, measuring 2–30 cm (1–12 in) long and 1–15 cm (0.–4.–9.9 in) wide. The flowers are unassuming, yellow-green, and 3–4 mm (0.10.2 in) in diameter. They develop into a small, lumpy, hard, dry fruit cluster that is 5–10 mm (0.2–0.4 in) across and contains a number of seeds. A half-cup of cooked spinach contains about 130 micrograms of folic acid, which is more than a quarter or your daily recommended dosage. And yes, cooking it matters, as there's more folic acid available if it's cooked versus raw, Palin ski-Wade says. Try sautéing the leaves in olive oil and season to taste.[11]

Fig. 3.1 Spinach

**3.2 Asparagus:**

Biological Source: *Asparagus Racemases*

Folate is among the several vitamins and minerals that are abundant in asparagus. In fact, 134 mcg, or 34% of the DV, of folate may be found in a half-cup (90 gramme) meal of cooked asparagus. Asparagus is also a good source of antioxidants and has been demonstrated to be antibacterial and anti-inflammatory. With just one serving, you can meet up to 6% of your daily fiber requirements, making it a great source of fiber that's good for your heart. This nutrient is crucial for the growth of cells. A vital nutrient, folate is particularly crucial during periods of fast growth, such as during pregnancy, infancy, and adolescence. During pregnancy, taking folic acid supplements may help reduce miscarriage and shield the developing embryo from neural tube defects. Additionally, anemia brought on by a folate shortage in persons who do not consume enough folate in their diets might make them feel weak and exhausted. Around 17% of an adult's daily need of folate can be found in one cup of asparagus, which weighs 134 grammes (g). Taking folic acid supplements during pregnancy may help lower the risk of miscarriage and protect the developing fetus from neural tube abnormalities. In addition, individuals with a folate deficiency brought on by inadequate folate intake may experience fatigue and weakness. One cup of asparagus provides 134 grams which counts around 17% of an adult's daily requirement for folate (g).[12]

Fig. 3.2 Asparagus

**3.3 Beet:**

Biological source: *Beta Vulgaris*

A single cup (136 grammes) of raw beets contains 148 mcg of folate, or roughly 37% of the DV, making them a fantastic source of folate as well. Beets are rich in nitrates, a type of plant molecule that has been linked to a number of health advantages, in addition to their vitamin content. One small study found that consuming beetroot juice temporarily reduced healthy adults' systolic blood pressure by 4-5 mmHg.[13]

Fig. 3.3 Beet

**3.4 Broccoli:**

Biological Source: *Brassica oleracea*

Known for its many health benefits, including broccoli in your diet can give you access to a variety of important vitamins and minerals. Approximately 57 mcg of folate, or 14% of the DV, may be found in one cup (91 grammes) of raw broccoli. Additionally rich in manganese and the vitamins C, K, and A is broccoli. It also contains a wide range of advantageous plant substances, such as sulforaphane, which has been extensively researched for its potent anti-cancer capabilities. [14,15]

Fig. 3.4 Broccoli

**3.5 Sunflower seeds:**

Biological Source: *Helianthus annuus*

Sunflower seeds are popular for snacking directly from the bag as well as being used in trail mix, multigrain bread, and nutrition bars. They include a variety of vitamins, minerals, and healthy fats as well as advantageous plant chemicals. These vitamins and minerals may help lower your chance of developing common health issues including heart disease and type 2 diabetes. In particular, sunflower seeds are rich in selenium and vitamin E. These serve as antioxidants to shield the cells in your body from the harm caused by free radicals, which is a factor in a number of chronic diseases. Sunflower seeds contain vitamin E, magnesium, protein, linoleic fatty acids, and numerous plant chemicals, which may help decrease blood pressure, cholesterol, and blood sugar. This B vitamin is essential for women who are of childbearing age. It aids in preventing birth abnormalities of the neural tube such spinal bifida and anencephaly. Additionally, folate may enhance memory function, enhance heart health, and prevent cancer. But further analysis is required to demonstrate its efficacy.[16]

Fig. 3.5 Sunflower seeds

**3.6 Brussels Sprouts:**

Biological Source: *Brassica oleracea*

Brussels sprouts contain a lot of the crucial vitamin folate. B9, commonly

known as folate, aids in the body's maintenance of new cells. The synthetic version of folate, folic acid, may be more familiar to you. Folate is necessary for fertility because it lines a woman's womb with nutrients that feed the womb and improve sperm survival rates. The lack of folate during pregnancy has been related to birth abnormalities because it prevents the proper division of the cells that make up the nervous system in the developing child. Natural sources of folate in whole foods can help lower the risk of miscarriage and birth abnormalities. Folate can help everyone, not just those who are pregnant or attempting to get pregnant, because it is also DNA-protective. Back to the sprouts, though. Eating Brussels sprouts provides your body with some potent phytonutrients that can promote the body's detoxification process and optimize estrogen metabolism. Additionally, you are consuming some anti-inflammatory substances, which are advantageous to your health because inflammation can impair variety of physiological processes, including conception.[17,18]

Fig. 4.6 Brussels Sprouts

Potential source of vitamin B9 used in this project:

**3.7 Peanut:**

Biological Source: *Arachis hypogea*

A legume crop produced primarily for its edible seeds is the peanut (Arachis hypogea), also known as the groundnut, 2 goober (US), Pindar (US), or monkey nut (UK). It is widely cultivated throughout the tropics and subtropics, and both small and significant commercial producers depend on it. Due to its high oil content, it is categorized as both a grain legume and an oil crop. World yearly production of shelled peanuts was 44 million tons in 2016, topped by China with38%of the world total. Atypically among legume crop plants, peanut pods form underground (geocarpy) rather than above ground. The specific name hypogea, which translates to "beneath the soil," was given to peanuts by naturalist Carl Linnaeus in honor of this trait. The peanut is a member of the Leguminosae family, also known as the legume, bean, or pea family. Like the majority of other legumes, the root nodules of peanuts contain symbiotic bacteria that fix nitrogen. The capacity to fix nitrogen means peanuts require less nitrogen-containing fertilizer and improve soil fertility, making them valuable in crop rotations. As a

culinary nut, peanuts are frequently prepared similarly in Western cuisines to tree nuts like walnuts and almonds because they have a comparable flavor and nutritional profile. According to botany, a nut is "a fruit whose ovary wall hardens at maturity." According to this standard, the peanut is not a nut. However, for culinary purposes and in ordinary English, peanuts are typically classified as nuts. Nutrient-rich peanuts are available (right table, USDA nutrient data). As an excellent source (defined as more than 20% of the Daily Value, DV) of several B vitamins, vitamin E, several dietary minerals, including manganese (95% DV), magnesium (52% DV), and phosphorus (48% DV), as well as dietary fiber, peanuts provide 2,385 kilojoules (570 kilocalories) of food energy per 100 grammes (31/2 ounce) of reference serving (right table). They also have a larger proportion of protein than many tree nuts, with roughly 25 g per 100 g serving. According to several research, eating peanuts frequently is linked to a lower specific risk of death from particular diseases. However, it is impossible to conclude cause and effect from the study designs. The risk of heart disease may be decreased by eating 1.5 ounces of most nuts (such as peanuts) per day as part of a diet low in saturated fat and cholesterol, according to the US Food and Drug Administration. The second-largest source of vegetable oil in the world, behind soy beans, is peanuts. They are manufactured commercially as salad and cooking oils and are the main component of margarine [19,20].

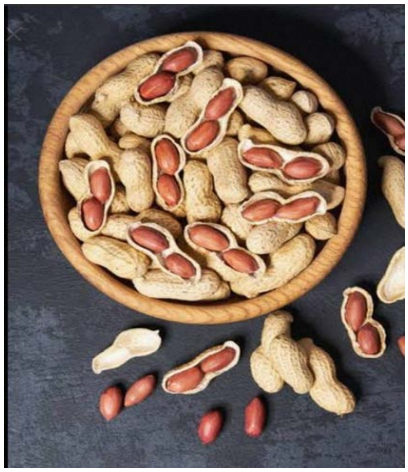


Fig. 4.7 Peanuts

## 4. Available medicines in market

### 4.1 Herbal Formulations[21]

| Drug Name | Generic Name |
|---|---|
| 5-HTP | 5-Hydroxytryptophan |
| Azo-Cranberry | Cranberry |
| Evening Primrose Oil | Evening Primerose |
| Valerian Root | Valerian |
| Ginkgo Biloba | Ginkgo |
| Sambucol | Elderberry |
| Ginger Root | Ginger |
| Venastat | Horse Chestnut |
| Ellura | Cranberry |
| Thera Cran HP | Cranberry |
| Oral Magic | Aloe vera |

### 4.2 Allopathic Formulations[22]

| Drug Name | Brand Name |
|---|---|
| Calcium, folic acid, Ginger, Pyridoxine systemic | Zingiber, focalgin B, B-Nexa |
| Cholecalciferol/folic acid systemic | Ciferex, zolate, ortho D, Genicin Vita-D, DermacinRx, foliplus D |
| Cyanocobalamin/folic acid systemic /pyridoxine//strontium gluconate systemic | Bonisara |
| Ethinyl estradiol/folic acid /levonorgestrel systemic | Falessa kit |
| Ferrous fumarate/folic acid systemic | Hematinic with folic acid ferrocite F, Hemocyte -F, equi -cyte, f Nephro -fer RX |
| Ferrous fumarate /folic acid /iron polysaccharide systemic | Tandem f |

## 5.Methods of determination of Folic acid:

### 5.1 Materials used in extraction of folic acid[23]

1) **Chemicals:**
   - Sodium phosphate dibasic-anhydrous-(Na2HPO4-CRS: 531900)
   - L-Ascorbic acid (1%, w/v)
   - Folic acid USP-Cat No: 1286005, 500mg
   - Acetone
   - Ethanol
   - 0.05 HCl
   - 20%NaOH
   - Hexane
   - Toluene
2) **Conjugate**
   - Chicken pancreas
3) **Enzymes**
   - Pronate R
   - α-Amylase
   - Folic Acid Casein Media
   - Lactobacillus broth AOAC
   - Lactobacillus agar AOAC
   - Depletion media (Lactobacillus broth diluted with folic acid casein media 1:1)
4) **Standard stock solution**
5) **Apparatus**
   - Incubator
   - Water bath
   - Autoclave
   - pH meter
   - 96 Well microplate reader (The Bio-Rad Benchmark Microplate Reader (Bio-Rad, Serial No.11562, USA)
   - Bunsen burner
   - Disposable culture tube
   - Disposable sterile filter system
   - 250 ml receiver, 12/case.
   - Pipette tips
   - Disposable multi-channel pipette basins
   - Disposable syringe- non-sterile, 1 to 5ml.
   - Syringe filter
   - 1-channel pipette
   - 12-channel pipette

- 96 well microplates

**5.2 Analysis of Total Folate:**

**1. Preparation of Standards:**

In a 200-ml conical flask with 20 ml of (95% v/v) ethanol and 50 ml of deionized water, 20 mg of folic acid (US Pharmacopoeia) was added. To aid dissolve the folic acid, the initial pH was raised to 10.0 with 0.1N NaOH, and the final pH was reduced to 7.0 using 0.05N HCl. With distilled water, the final amount was increased to 100 ml. The solution was then transferred to 10-ml Pyrex tubes and kept at 4°C. After six months, a new standard should be created. The stock standard solution (0.2 mg/ml) was diluted with phosphate buffer (0.1 M, pH 7.0) to a final concentration of 0.01 mg/ml (1:20 dilution), and the absorbance of the diluted standard at 282 nm in a 1 cm quartz cell was measured while using phosphate buffer (0.1 M, pH 7.0) as a blank.[24]

The purity was calculated using the following equation:

**Purity of standard (%) = 100 (analyzed concentration C2 / known concentration C1)**

Calculated concentration C2 = $(A_{std} - A_{blank}).M/(\varepsilon.b)$,

Where C1=0.01 mg/ml, C2=analyzed concentration of diluted stock standard (mg/ml),

A=absorbance, $\varepsilon=27.00* 10^{3}M^{-1}$ $cm^{-1}$, b=1cm, M=molar mass of folic acid (441.40).

**2. Extraction of Food by Trien zyme Digestion**

Extraction of Total Folate

First Day of Assay:

1. Turn on water bath to reach 100 ºC
2. Put 1 g of the ground sample into a glass tube with a 3.5 cm O.D. and 15 cm length.
3. Add 20 ml of hexane and vortex for around 45 seconds to extract fat. Phase separation occurs after 10 minutes of letting the mixture stand.
4. Utilizing a Pasteur pipette, remove the hexane layer.
5. Remove any leftover hexane from the nitrogen stream.
6. Add 20 ml of anhydrous Na2HPO4 buffer with L-ascorbic acid at 1% (w/v), pH 7.8, to the defatted sample and vortex. Fill a 150 ml Erlenmeyer flask with the sample. To fill the glass tube to the 50 ml mark, add two 15 ml aliquots of deionized water
7. Create the recovery working standard and apply the appropriate amount of standard to the recovery sample.
8. Add 30 ml of water and 20 ml of pH 7.8 phosphate buffer to each sample.
9. Aluminum foil is used to cover the flasks.
10. Heat for 15 minutes at 100 °C.
11. After cooling, mix in 10 ml of pH 7.8 buffer.

12. Fill each flask with 1 ml of the Pronate R solution (2 mg/ml in water), then stir.
13. Place aluminum foil over the flasks and incubate them at 37 °C for three hours.
14. After 5 minutes of heating at 100 °C, cool to room temperature (turn on water bath 45 minutes before heating).
15. Combine 0.5 ml of toluene and 1 ml of the -amylase solution (20 mg/ml in water).
16. Place aluminum foil over the flasks and incubate them at 37 °C for two hours.
17. Create a conjugated solution in assay buffer (5 mg/ml). Conjugate is weighed, pH 7.8 assay buffer is added, stirred for 10 minutes, and then filtered through glass wool.
18. Mix in 4 ml of the conjugase solution.
19. Place aluminum foil over the flasks and incubate them at 37 °C for 16 hours.[25]

**The second day of assay**

1. Heat samples for 5 minutes at 100°C, then let them cool.
2. Use HCl to bring sample pH to 4.5 and add up to 100 ml of distilled water.
3. Use filter paper to separate each sample (Whatman, 185 mm, Cat No: 1001-185). Stack a 150 ml beaker on top of a funnel, then add a folded piece of filter paper to the funnel.
4. Use pH 6.8 phosphate buffer and microplate assay after any additional dilutions, if necessary.
5. Transfer the original sample extracts into disposable tubes, then carefully cover them before storing them in a -50 C freezer until test.

Caution: 1

Preparations for the enzymes should be made immediately prior to each use of steps 12, 15, and 17. Don't leave enzyme solutions sitting for longer than 10 minutes. Temperature of the extraction solution must be below 37°C before adding the enzyme solution to maintain enzyme activity.

Caution: 2

Prepare extra 1 ml more than the needed amount of Pronate R and α-amylase solutions needed. Since glass wool holds some conjugated solution, prepare 4-5 ml more than the amount of conjugated solution needed for the assay.

Caution: 3

After placing sample flasks in a boiling water bath, temperature goes down. Count heating time after temperature goes back to 100 ºC (Step 8, 12, and 18).

Caution: 4

A large dilution factor (> 1:20) should be used for samples that are high in folic acid or total folate (> 1000 g/100 g). The standard deviation of the data could rise with a large dilution factor. Reduce the sample weight that will be analyzed. It is advised to choose a sample weight of 0.500 or 0.250 g to make data calculations easier. Make a note of the sample weight in the event that it changes. When calculating data, double check that the dilution factor to be entered into "Entering Dilutions of unknown" is accurate.[26]

**3. Preparation of Working Standard Solution for Recovery (0.2 µg/ml)**

In a volumetric flask, the stock solution of 0.1 ml (200 g/ml) was diluted with water to produce a final concentration of 0.2 g/ml. The day of use sees fresh preparation. It is recommended that 100% of the folic acid and total folate be considered. The level of total folate present in foods of the same kind as the sample to be analyzed determined the amount of working standard that was to be added. For comparable samples, estimated values were obtained from the USDA Nutrient Data Bank.

**4. Preparation of the 0.1M, pH 7.8 Phosphate Buffer (Assay Buffer)**

1.42 g of anhydrous sodium phosphate dibasic was dissolved in 100 ml of water. The phosphate buffer was supplemented with ascorbic acid (1%, w/v) to stop the oxidation of folate during the extraction procedure. pH 7.8 was achieved by adding a few drops of 20% (20 mg/100 ml) NaOH to the mixture.

A Detailed Description of the Microplate Assay

Set up for the Microplate Assay

- Preparation of the Working Standard for the Microplate Assay

First, the intermediate standard (0.2 g/ml) was made by diluting 0.1 ml of the standard stock solution (200 g/ml) to 100 ml with distilled water in a volumetric flask. The working standard (2 ng/ml) was created by dilution of the intermediary solution (0.2 g/ml) to 100 ml in a volumetric flask using 1 ml of distilled water. This standard was freshly made the day it was used.[27]

- Preparation of Lactobacilli Broth AOAC

Weighted Lactobacillus broth powder was added to water, where it was dissolved (3.8 g/100ml). The mixture was heated while being stirred until it began to boil. It was then allowed to boil for two to three minutes, cooled, and dispensed (10ml) into screw-cap tubes. This broth was prepared by autoclaving it at 121 °C for 15 minutes, allowing it to cool to room temperature, and then storing it at 4 °C until use.

- Preparation of Lactobacilli Agar AOAC

Weighed (4.8g), lactobacilli agar was diluted with 100ml of distilled water. It was

warmed while being stirred until it began to boil. After boiling for two to three minutes, until it had entirely dissolved, it was allowed to cool. Agar was produced, placed in screw-cap 43 tubes, and autoclaved for 15 minutes at 121 °C. The tubes were positioned at an angle to create a slant and stored in the refrigerator at 4°C until usage while cooling to room temperature.

- Preparation of the Depletion Media (Lactobacilli broth: media=1:1)

In a 1:1 ratio, Lactobacillus broth (3.8g/100ml) and Folic acid casein medium (9.4g/100ml) were weighed, dispensed, and heated with stirring until it began to boil. It was entirely dissolved and chilled to about 37°C after a 2- to 3-minute boil. The resulting mixture was put into screw-type tubes, autoclaved for 15 minutes at 121 °C, allowed to cool to room temperature, and then kept in the refrigerator at 4 °C until it was needed. This media can last for up to six months before needing to be changed.

- Preservation and Transmission of the Culture

Since maintaining the activated state of the culture during a culture transfer is crucial for the consistency of the assay. L. casein ssp. rhamnoses was the culture that was employed in the experiment (ATCC 7469). The American Type Culture Collection's L. casein ssp. rhamnoses pellet was dissolved in 10ml of the Lactobacillus broth solution. A 10ml portion of another Lactobacillus broth solution was added to this solution (0.5–1.0ml), and it was then incubated at 37°C for 18 hours. The culture from the solution was then transferred to the Lactobacillus agar slant and incubated there for 24 hours at 37°C. The slant was then kept chilled, at 4°C, in the refrigerator. Every week on the same day, the culture was moved and incubated at 37°C for 24 hours on the new slant to maintain the activated culture. The new slant was kept at 4°C in the refrigerator.

- Preparation of the Inocula

The depletion media (Lactobacillus broth: media, 1:1) was removed from the refrigerator and warmed to room temperature on the day of the microplate assay. The culture from the 3- to 4-day-old slant was then added to the depletion media, where it was incubated for 6 hours at 37°C. After the 6 h of incubation, this depletion medium was used for the microplate test. Only very slight growth is visible right now in the depleting media. This timetable must be closely followed to produce consistent standard curves with comparable growth times.

- Sample Dilutions

If necessary, distilled water was used to dilute the sample extractions (1g/100ml) that were filtered following the 16-hour digesting period. Dilutions were prepared to obtain growth spanning the concentration range of the standard curve based on the expected level of folic acid and the total amount of folate present in the sample. The dilution factor selected for the samples was about equal to:

a. For samples containing a folate concentration of 0 to 30 g/100 g, there is no dilution.
b. 1:3 dilutions for samples with folate levels between 30 and 80 mg per 100 grammes.
c. 1:5 dilutions for samples containing 80–150 mg of folate per 100 grammes
Higher dilutions are needed if higher folate concentrations are anticipated, such as in supplements. The concentration of the folate is 1.5 ng/ml following the dilutions. This diluted extract is used for micro plating, and 150 liters of it are mixed with 150 liters of distilled water (the maximum concentration well has a folate content of 0.225 ng/300 liters) (G3 through G12). Then, until A3 through A12, 7 successive dilutions are made in each row. Since the working standard has a 2 ng/ml concentration, 150 L of this standard are introduced to the highest concentration wells (G1 and G2) that already have 150 L of distilled water in them. The greatest concentration of final folate is 0.3 ng/300 L, which is about the same as the concentration in the sample (0.225 ng/300 L). This calculation demonstrates how samples are diluted to match the concentration range of the standard curve.

- Autoclaving

Following the dilutions, the tubes were autoclaved at 121°C for 5 minutes with a loose cap on them. A flask, a 100ml measuring cylinder, an Erlenmeyer flask filled with distilled water, and a flask covered in aluminum foil were all autoclaved together with the sample tubes.
To reduce the possibility of microbiological contamination, the assay bench was thoroughly cleaned with 70% alcohol before the microplate was set up next a lit Bunsen burner. Close to the flame, the microplates were removed from their respective packaging, and the cover was labelled with an erasable pen. The sterile water was poured into the reservoir, and 150 ml of water were pipetted into the wells A1-G12 (from row A (A1-A12) to row G) using a 12-channel pipette (G1-G12). The wells H1–H12 received 300 microliters of water through pipet (blank row). Using a syringe and a sterilized syringe filter, the working standard was filtered into the reservoir (300µL of standard solution per plate). Using a 12 – channel pipette, 150 µL of the working standard was pipetted into the wells G1-G2. Then, 150µL of the sample dilutions, control, and recovery and QC dilutions were pipetted into wells G3-G12. Through G3-G12, duplicates of each sample or unknown are pipetted into two wells. Using the 12-channel pipette, serial dilutions of the standard and the samples were made were made by transferring 150 µL from the wells G1-G12 to F1-F12, mixing 3 times in each well (by pipetting it up and down the contour of each well with 12-channel pipette 3 times). Then 150µL of the mixture is transferred from F1-F12 to E1-E12 by mixing it 3 times. This process is continued through A1-A12 and the final 150µL from A1-A12 is discarded. After the dilutions were made, the media with the culture was prepared. Using a sterilized measuring cylinder, the necessary volume of the media (15 ml/plate) was placed into the sterilized flask. The required quantity of ascorbic

acid solution (100 mg/100ml) was filtered into the flask holding the media using a syringe and a sterile syringe filter after making an ascorbic acid solution (1g/10ml). With a 1ml pipette, the inoculum culture that had been incubated at 37°C for 6 hours earlier was then transferred to the flask containing the media and ascorbic acid. The medium and its contents were carefully shaken before being added to the reservoir. Using a12-channel pipette, 150µL of the media mixture was added into wells G1-G12 through A1-A12.The plates were then sealed in Ziploc bags to prevent evaporation. These plates were incubated immediately at 37°C for 24-28 h.

- Reading of the Microplate

The microplate was taken out of the incubator after the 24-28-hour incubation period and mixed three times from the lower concentration (row A) to the higher concentration (row G). The microplates were read at the 595nm absorbance filter using the Bio-Rad Benchmark Microplate Reader with Microplate Manager application. The G1–G2 standard's highest reading point was intended to be over 0.9 absorbance, which serves as a check for maximum growth. The additional microplates were readied when this absorbance level was attained.

Each plate was taken out of the incubator and mixed using a 12-channel pipette, ranging from low concentration (A1-A12) to high concentration (G1-G12) close to the flame. The incubation was not tested until the 24-hour mark, even though the growth typically reaches this stage in 24-28 hours has ended. The microplate reader measures each microwell's absorbance. Once the file had been saved, the standard concentrations were computed by using the highest standard concentration, S7, at a concentration of 0.2 and a dilution factor of 2 as the multiplier. Similar information was entered for the sample dilutions. Regression analysis utilizing Logistic 4PL, Linear-Linear Transformation, and Linear-Linear in Axis Transformation was used to create the standard curve. For the purpose of removing the controls from the sample data and calculating the recovery, the unknown concentration file is exported to the Excel application.

The following formula is used to determine the recovery:

**% Recovery = 100(folic acid in spiked recovery sample a – folic acid in unspoked sample b) / folic acid added in spiked sample c**

Were a, b, c Unit: µg/100g of sample

The calculated recovery values were used to adjust the sample data.

Corrected data = (Assay data) × (100 / % recovery)

Recovery values are typically greater than 85%. Then, outliers were eliminated in accordance with the rule that stipulates that the mean values obtained for each observation should not deviate by more than 10% from the mean. After calculating the means and standard deviations for each sample and eliminating outliers, the final concentrations were obtained.[29]

## 5. Preparation of conjugated:

Using a mortar and pestle, fresh chicken was cut into little pieces (1-2 cm) and

grounded into a fine powder. A beaker containing cold acetone was used to transfer the powdered chicken pancreas (75ml). Using an ultrasonic disintegrator, the chicken pancreas-acetone mixture was reduced to a finer particle size. Remaining acetone was allowed to evaporate from the solid residue after the acetone slur was filtered through cheesecloth that was put over a Buchner funnel filled with filter paper. After being placed in a one-ounce glass bottle with a tight cap and kept at -20°C, the dry powder was moved there. Following the hydrolysis of pteroyl tetra-L-glutamic acid, the conjugated activity of the chicken pancreas preparation was tested. 20 mg of chicken pancreatic conjugated preparation had a measured activity of 0.00003 mol/min (0.03 nmol/min).

Chicken Pancreas Conjugate Activity Measurement:
The standard solution (0.001 mg/100 ml) was made using the reference pteroyl tetra-glutamic acid (MW 828.7). One tenth milliliter of this standard solution (1µg/0.1ml) was added to the twelve flasks containing 30ml of deionized water and 30 ml of 0.1 M phosphate buffer (pH 7.8). The experimental setup was similar to the one used for the folate study. Each flask was filled with four milliliters of chicken pancreas acetone powder (conjugated) solution (5mg/ml of 0.1 M, pH 7.8 phosphate buffer). Digests were heated for five minutes at 100° C during the first hour to inactivate the conjugated. Digests were taken out during the second hour at 15-minute intervals and cooked for 5 minutes at 100° C. The microplate test was carried out according to a standard procedure for folate analysis. After the first hour, 1 µg of the measurable folates were recovered. A rate of 0.00003 mol/min (0.03 nmol/min) per 20 mg of chicken pancreatic conjugated was computed since measurably present folates include pteroyl Mon glutamic acid, pteroyl Di glutamic acid, and pteroyl polyglutamic acid, all of which had an average molecular weight of 570.5. Additionally, the activity can be quantified in nano katal SI units of enzyme activity (nkat). The amount of enzyme needed to convert one mole of substrate per second is called a katal.

1 katal (kat)= 1 mol/s, 1 µmol/min=16.65nkat.

## 6. Control:

Throughout the entire extraction process, an enzyme blank control was used to measure the total folate. The contribution of the enzymes to the growth response of the L. casein ssp. rhamnoses was assessed using the control, which included all enzymes and components aside from the food sample. In an assay to measure the quantity of folate provided by the enzymes, the control is crucial. Additionally, a crucial aspect of the total procedure's quality control is the enzyme control.[30]

## Conclusion:

The present review article demonstrates the prevalence of folic acid profile, its biosynthesis and significance in the synthesis of DNA and RNA and metabolism of cell cycle, further the said review emphasized on the marketed allopathic and herbal medications and the methods of its isolation and estimation in the various preparations along which brief overview of potential herbs having significant content of folic acid can be reduced to efficacious formulation and could be potential natural remedy as an alternative in future.

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