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A COMPARATIVE BIOAVAILABILITY STUDY OF FOLIC ACID PRESENT IN SPINACIA OLERACEA USING SOXHLET EXTRACTION, CHEMICAL METHODS, CHROMATOGRAPHIC AND SPECTROSCOPIC STUDIES.

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ABSTRACT

Leafy veggies are taken into consideration as a essential a part of human diet. As they contain low calories and fat and are rich in nutrient fibres. Intake of leafy vegetables in a regular diet pays numerous health benefits and prevents certain types of diseases. There is an enormous demand for leafy vegetables as people nowadays prefer organic and natural ways of dieting. Spinach, a dark green leafy vegetable which is rich in vitamins, potassium, calcium, manganese and also iron and folate has a lot of therapeutic benefits. And therefore, tt is taken as the sample. Folic acid, the synthetic form of folate (Vitamin B9) that's clearly located in a few cereals, nuts, fruits, veggies especially green leafy vegetables such as lettuce , spinach etc is either added in diet as foods or in synthetic supplements in form of tablets. Folic acid is the product to be extracted. This study aspects the extraction and bioavailability of folic acid from spinach and intake of it in the diet. Three samples extracted with the aid of using specific techniques are taken. Extraction is performed the use of Soxhlet and chemical techniques. Bioavailability of folic acid present in spinach is calculated using chromatographic and absorbance using spectroscopic methods. HPLC is used in chromatography technique and UV- VIS spectroscopy for spectroscopy method. Both raw and cooked samples are kept for study. By comparing the recovery rate obtained from both samples after extraction and chromatography, the amount of folic consumption to be taken by a person in a diet in the form of spinach either raw or cooked is concluded.

Keywords*: Green leafy vegetables, Spinach, Folic acid, bioavailability, extraction, chromatography, spectroscopy, Soxhlet method.*

1. INTRODUCTION

Spinach has a high nutritional value and also contains secondary metabolites as health benefits. It is suitable eating vegetable that is eaten both raw or cooked. Spinach is likewise saved via upkeep strategies like canning, freezing and blanching etc. for longer usage. Spinach is a wealthy supply of several vitamins like vitamin A, vitamin B, vitamin C and vitamin K. It also contains minerals, dietary fibers, and omega 3 fatty acids. It offers a very good quantity of folate, iron and consists of many phytonutrients, flavonoids and polyphenolic active ingredients. Spinach helps to prevent cancer cell development. It also helps in age related eye problems, gastrointestinal disorder, anemia, appetite stimulation and so on. Raw spinach carries 91 % of water, 4% of carbohydrates, 3% of protein, and negligible fat. Spinach is more healthy to consume in food plan as it carries low energy that allows to prevent overweight and diabetics. Raw spinach leaves are used in salads. But there arises a difficulty that they contain too much of oxalates and consuming them in large amounts may result in kidney stones. Therefore, one should be aware of the amount to be consumed in raw form. Boiling or cooking of spinach can also additionally lessen the danger of kidney stones because it favors the reduction of oxalate ranges present withinside the spinach leaves.

Folic acid [(2S)-2-[[4-[(2-amino-4-oxo-1H-pteridine-6-yl) methyl amino] benzoyl] amino] pentanedioic acid] is a micronutrient, and a form of B complex vitamin which is soluble in dilute solutions of alkali hydroxides and carbonates. It is an odorless and orange – yellow crystalline powder. Its molecular weight is about 441.404 g/mol and has a melting point of 250°C. The molecular formula of folic acid is C19H19N7O6. Folate, Folacin, Vitamin B9 and Pteroylglutamic acid are different terms of Folic acid. Folic acid naturally occurs in the form of folate by nature. Folate is found in darkish green leafy veggies which include spinach, lettuce, asparagus, broccoli and in beans, meat, mushrooms, yeasts. They are also present in fruits like melons, lemons, and bananas. Whereas the synthetic form i.e., folic acid can be discovered in fortified foods, supplements, etc.

In this study, the comparison is done for finding out the bioavailability of the folic acid present in spinach i.e., Spinacia Oleracea. The samples are taken in two different forms such as raw and boiled. Extraction is done using different methods like centrifugation, chemical extraction and Soxhlet extraction. Then samples are allowed to run in chromatography to perceive the lively content material present withinside the sample. HPLC is used as the chromatography technique. The samples are also allowed to detect through UV-VIS spectroscopy. The wavelength and maximum absorbance are recorded as spectra. Both chromatography and spectroscopy are achieved by comparing the samples with standard and running sample and standard along with blank. After the data collection the calculation is done using the results. The comparison is done among the three samples with the recovery

rate percentage. The efficient way of extraction with a high recovery rate will be found out. With the assist of bioavailability of folic acid found in spinach the sample with excessive recovery rate and quantity of absorption is decided.

2. MATERIALS AND METHODS

Collection and sterilization of the samples:

Samples were collected from the plant species *Spinacia Oleracea*. Healthy plants were chosen and carefully collected from the Chennai region. They were taken in a clean sterile bag and processed within a few hours after sampling. The isolation work was done from the fresh plant material to avoid contamination. To eliminate the dirt debris and debris, the plant samples had been accumulated in easy and sterile baggage and rinsed very well withinside the running water. The leaves were cut carefully and separated from the shoots. Highly aseptic condition was required for the isolation of the sample leaves. Aseptic glass wares were used during the process. The parted leaves were then rinsed in sterile water for three times. Then they were blotted on the sterile blotting paper aseptically. Once the leaves were free of moisture they were packed into sterile bags.

Extraction of the compound:

10g of raw spinach leaves was taken for the preparation of the sample. The leaves had been then positioned in mortar and had been crushed finely the usage of the pestle. The grinded leaves were transferred in the beaker containing a mixture of 5ml of NaOH solution of 0.1N and 5ml of water and sonicated for 10 minutes. The sonicated sample mixture was transferred to centrifuge tubes and then centrifuged for about 5 minutes. The topmost supernatant liquid was transferred in a beaker without disturbing the pellet leaves. The extracted liquid was labelled as sample 1.

The preparation of sample 2 was done using soxhlet extraction method. 20.34g of sample raw leaves were crushed partially using mortar and pestle. As for the soxhlet extraction method, NaOH was used as solvent for extraction because folic acid is soluble in NaOH solution. 100ml of 0.1N NaOH and 100ml water were added together in 50:50 ratio to the round bottom flask of the soxhlet extractor filling 75% of its volume. Glass beads were added to the round bottom flask along with the solvent to stabilize the heat. The crushed leaves sample was transferred in a filter paper and was loaded into the thimble positioned in the Soxhlet extractor. The crushed leaves sample was transferred in a filter paper and was loaded into the thimble placed inside the soxhlet extractor. The soxhlet extraction process heats the solvent to temperature above 60℃. The first cycle was measured after the level of solvent reaches the syphon and pours back into flask. The soxhlet extraction was done for 4 hours till one cycle. Then the process was made to stop and the solution was let to cool down. Then the solvent extraction was concentrated using a heating mantle leaving a small yield of extracted folic acid. Sample 2 was extracted and labelled. The concentrated folic acid was brought with 10ml of diluent or solvent aggregate before running in chromatography.

Raw spinach leaves of 6.99g were taken for preparation of sample 3. The spinach leaves were transferred into the beaker containing solution of 50ml of 0.1N NaOH and 50ml of water. Heating mantle was used to boil the sample leaves in the solution and it was adjusted to a temperature above 80℃ for 1½ hours. Once the heated sample was cooled, glass rod is used for smashing the boiled leaves finely. The sample mixture was then transferred to centrifuge tubes and centrifuged for 5 minutes. The whole supernatant i.e the folic acid extract was transferred in a beaker without disturbing the boiled pellet leaves and labelled as sample 3. After the preparation of all the three samples, the samples were allowed to process through the chromatography for active content estimation. For that the samples were filtered using a syringe filter and transferred to three vials.

Estimation of active content of folic acid present in spinach:

The buffer solution was prepared by dissolving 0.30116g of 1-Hexane Sulphonic Acid in 200ml of water in a beaker. 2ml of acetic acid was added further to the solution and was sonicated in ultrasonicator for 5 minutes. 200ml of mobile phase solution was prepared by dissolving 170ml buffer solution and 30ml of acetonitrile in 85:15 ratio respectively and was transferred into the mobile phase container. 80ml of a solvent mixture or diluent was prepared by dissolving 40ml of 0.1N of NaOH and 40ml water in a beaker in 50:50 ratio. The diluent was also used as the blank solution for chromatography. The standard solution of folic acid was prepared by adding 2.55mg of standard folic acid in the form of powder in a solvent mixture and was made upto 25ml. The standard mixture was diluted absolutely to attain a solution having a regarded concentration of approximately 0.1mg per ml. The prepared standard solution was then added in vial to run in chromatography for active content estimation. The chromatographic condition used to run the samples used in this study are given below in the table 1:

1	Liquid chromatography system	Liquid chromatography equipped with an autosampler, a variable UV wavelength detector and a suitable data collection system.
2	Column	C_{18}, 250 X 4mm, 5□□ or equivalent
3	Detection	UV 282 nm
4	Flow rate	1.0 ml/min

5	Injection volume	20□□
6	Run time	10 minutes

Table 1 : Chromatographic condition for folic acid in HPLC

The chromatographic system was equilibrated until a stable baseline was observed.

S.No	*Solution*	*No. of Injection*	*System suitability parameter*	*Acceptance criteria*
1.	**Specificity** Blank	1	Baseline should be stable. Disregard any peak appearing in the chromatogram less than 0.05% of the size of the peak of interest in all subsequent chromatograms.	Should be complies
2	Standard solution	1	The tailing factor of Folic acid	NMT 2.0
			Theoretical Plates	NLT 2000
			Tolerance for RT of peaks due to Folic acid	NMT± 2.0 minutes

Table 2 : System suitability test

The chromatography was made to run in the following order: Blank, standard folic acid, sample 1 , sample 2, sample 3. The chromatograms were recorded as stated. The percentage of the assay was calculated using the formula mentioned below.

$$\% \text{ Assay} = \frac{U_r}{S_r} \times \frac{S_{wt}}{U_{wt}} \times \text{Potency of the standard}$$

where

U_r = Area of peak due to folic acid obtained with the sample solution.

S_r = Mean peak area of folic acid peak obtained with standard.

S_{wt} = Weight of working standard (standard solution) in mg/ml.

U_{wt} = Weight of test sample in mg/ml.

UV-VIS SPECTROSCOPY:

Cuvettes were cleaned properly with methanol in order to avoid any type of error in the readings. Two cuvettes were first filled with blank solution and were made to run in spectroscopy. Then only one of the cuvettes was removed and refilled with standard folic acid solution. This process was repeated by refilling one of the cuvettes with sample 1,2 &3 one after another. The peaks were observed and recorded.

3. RESULTS AND DISCUSSION

The results obtained are as discussed below

ACTIVE CONTENT ESTIMATION BY HPLC:

uring the process of chromatography, the peak was observed and the chromatogram was recorded for each of the samples. The active content of folic acid in the sample was estimated by comparing the peak observed in the standard folic acid sample along with the retention time

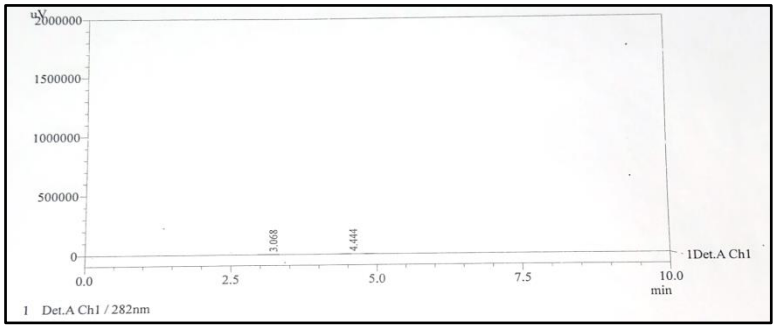


Figure 1 : Chromatogram of the Blank solution

Name	Retention time	Area	Height	Height %	Area %
RT 3.068	3.068	33943	1115	41.236	56.615
RT 4.444	4.444	26012	1589	58.764	43.385
		59955	2705	100.00	100.00

Table 3 : Readings of the Blank solution

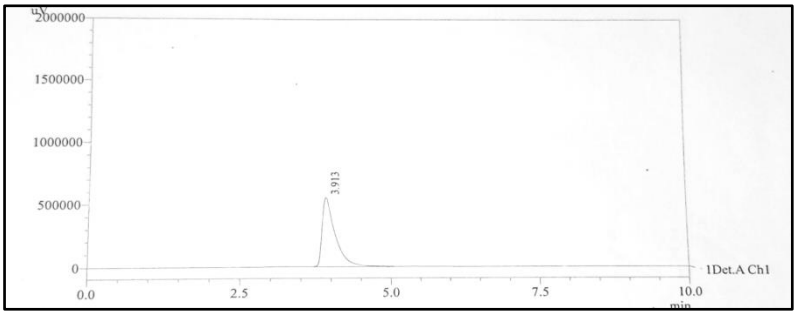


Figure 2 : Chromatogram of the standard folic acid solution

Name	Retention time	Area	Height	Height %	Area %
Folic acid	3.913	8233804	552247	100.00	100.00
		8233804	552247	100.00	100.00

Table 4 : Readings of the Standard folic acid solution

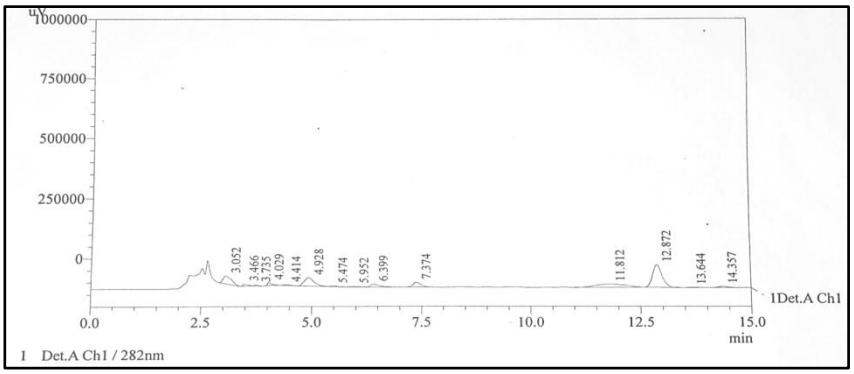


Figure 3 : Chromatogram of the sample 1

Name	Retention time	Area	Height	Height %	Area %
RT 3.052	3.052	439094	33539	13.456	11.799
RT 3.466	3.466	68569	9387	3.766	1.843
RT 3.735	3.735	19065	4419	1.773	0.512
RT 4.029	4.029	83397	12829	5.147	2.241
RT4.414	4.414	36240	3639	1.460	0.974
Folic acid	4.928	535277	35539	14.259	14.384
RT 5.474	5.474	26642	2519	1.011	0.716
RT 5.952	5.952	33097	2452	0.984	0.889
RT 6.399	6.399	149762	11978	4.806	4.024
RT 7.374	7.374	226195	19282	7.736	6.078
RT 11.812	11.812	621273	13086	5.250	16.694
RT 12.872	12.872	1380952	93796	37.632	37.108
RT 13.644	13.644	21290	1309	0.525	0.572
RT 14.357	14.357	80596	5470	2.195	2.166
		3721449	249245	100.00	100.00

Table 5: Readings of the Sample 1

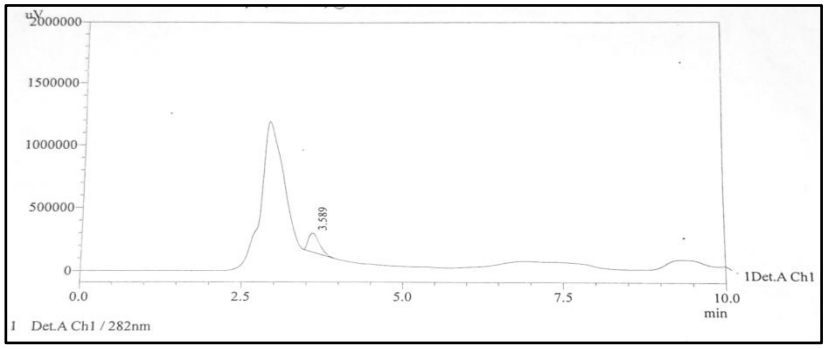
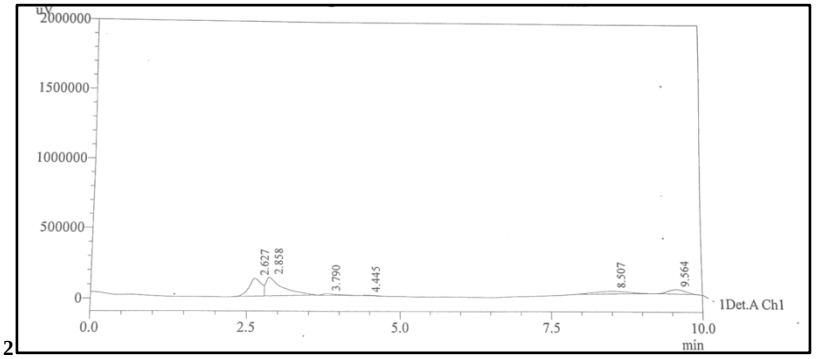


Figure 4: Chromatogram of the sample 2

Name	Retention time	Area	Height	Height %	Area %
Folic acid	3.589	1768376	148984	100.00	100.00
		1768376	148984	100.00	100.00

Table 6: Readings of the Sample



2

Figure 5: Chromatogram of the sample 3

Name	Retention time	Area	Height	Height %	Area %
RT 2.627	2.627	1752128	126563	39.216	29.854
RT 2.858	2.858	2477409	132697	41.116	42.212
Folic Acid	3.790	177094	9564	2.963	3.017
RT 4.445	4.445	43292	2876	0.891	0.738
RT 8.507	8.507	800556	19405	6.013	13.641

RT 9.564	9.594	618458	31630	9.801	10.538
		5868937	322734	100.00	100.00

Table 7: Readings of the Sample 3

SPECTROSCOPIC ANALYSIS OF FOLIC ACID:

The maximum absorbance of the samples was checked using the UV VIS spectroscopy. The range used was 200nm-800nm. The peaks of all the sample solutions were observed and recorded. Referring to the peak formed in standard solution, other peaks of samples were compared and the maximum absorbance was concluded.

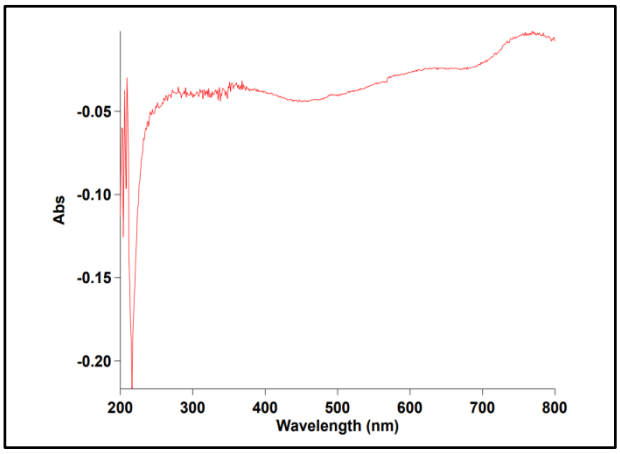


Figure 6: Spectrum of the blank solution

WAVELENGTH (nm)	ABSORBANCE
368.00	-0.032
209.00	-0.030
206.00	-0.037
202.00	-0.060

Table 8: Peak table of the Blank solution

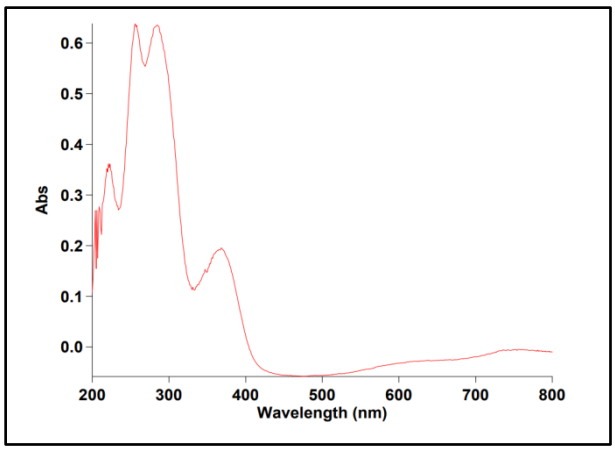


Figure 7: Spectrum of the standard folic acid solution

WAVELENGTH (nm)	ABSORBANCE
368.00	0.196
285.00	0.635
256.00	0.638
221.00	0.362
209.00	0.277
206.00	0.270
204.00	0.270

Table 9: Peak table of the standard solution

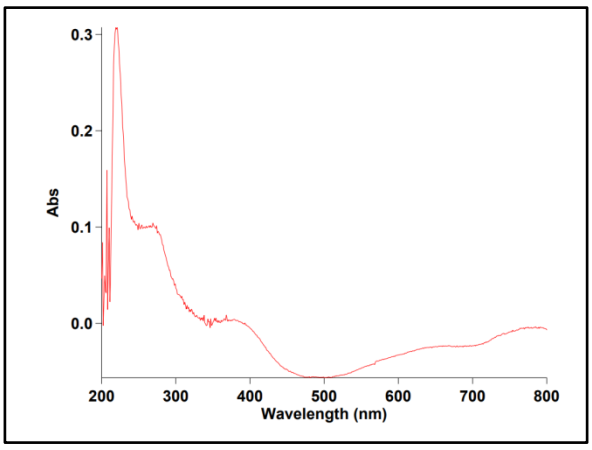


Figure 8: Spectrum of the sample solution 1

WAVELENGTH (nm)	ABSORBANCE
368.00	0.009
221.00	0.307
210.00	0.099
207.00	0.159
204.00	0.049
201.00	0.084

Table 10 : Peak table of the sample solution 1

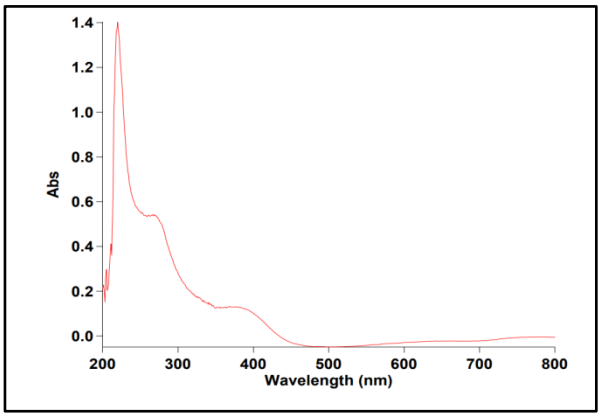


Figure 9: Spectrum of the sample solution 2

WAVELENGTH (nm)	ABSORBANCE
368.00	-0.032
209.00	-0.030
206.00	-0.037
202.00	-0.060

Table 11: Peak table of the sample solution 2

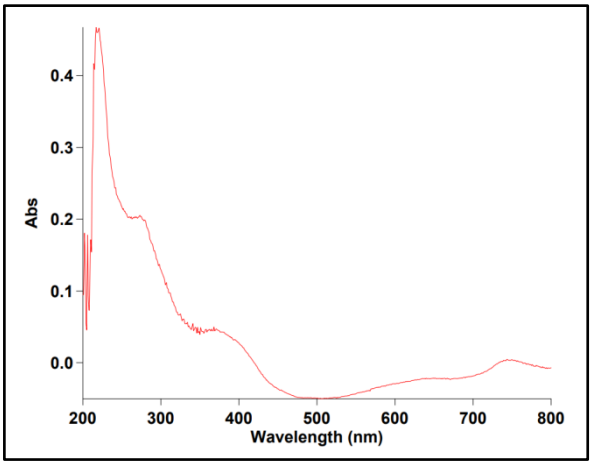


Figure 10: Spectrum of the sample solution 3

WAVELENGTH (nm)	ABSORBANCE
744.00	0.005
368.00	0.050
217.00	0.467
210.00	0.171
206.00	0.178
202.00	0.181

Table 12 : Peak table of the sample solution 3

CALCULATIONS:

The collected data obtained from chromatography was then used for calculation purposes in order to find out the percentage recovery of folic acid that was extracted in the sample leaves of spinach.

Formula:

$$\% \text{ Assay} = \frac{U_r}{S_r} \times \frac{S_{wt}}{U_{wt}} \times \text{Potency of the standard}$$

- Recovery percentage of folic acid in sample 1 = 0.03 %
- Recovery percentage of folic acid in sample 2 = 0.003 %
- Recovery percentage of folic acid in sample 3 = 0.001%

4. CONCLUSION

In this study, we collected the sample leaves and extracted three types of sample solutions using different methods. Then the active content in the leaves extraction were estimated using HPLC method as per the guidelines in the Indian Pharmacopoeia. After doing the calculations it was found that

sample 1 had a high recovery rate of 0.03%. When comparing all the three samples, the extraction done using raw leaves has higher content of folic acid than in boiled leaves. The maximum absorbance of the solutions were also checked using UV VIS spectroscopy. As for the intake of folic acid in form spinach, as per the study to attain the required amount of folic i.e 400 mcg we can approximately take 3g - 7g of spinach in daily diet. This is enough to get required amount of folic acid. In case of folic acid deficiency or anaemia we can take a larger amount than the normal one. As for the pregnant ladies or who tend to get pregnant can consume like 4g - 10g and if baby having NTDs can consume 27g-30g of spinach to get enough folic acid.

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