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ORIGINAL ARTICLE

Relative bioavailability of folate from the traditional food plant *Moringa oleifera* L. as evaluated in a rat model

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Abstract *Moringa oleifera* is an affordable and rich source of dietary folate. Quantification of folate by HPLC showed that 5-formyl-5,6,7,8-tetrahydrofolic acid (502.1 μg/100 g DW) and 5,6,7,8-tetrahydrofolic acid (223.9 μg/100 g DW) as the most dominant forms of folate in *M. oleifera* leaves. The bioavailability of folate and the effects of folate depletion and repletion on biochemical and molecular markers of folate status were investigated in Wistar rats. Folate deficiency was induced by keeping the animals on a folate deficient diet with 1 % succinyl sulfathiazole (*w*/*w*). After the depletion period, animals were repleted with different levels of folic acid and *M. oleifera* leaves as a source of folate. Feeding the animals on a folate deficient diet for 7 weeks caused a significant (3.4-fold) decrease in serum folate content, compared to non-depleted control animals. Relative bioavailability of folate from dehydrated leaves of *M. oleifera* was 81.9 %. During folate depletion and repletion, no significant changes in liver glycine N-methyl transferase and 5-methyltetrahydrofolate-homocysteine methyltransferase expression were recorded. In RDA calculations, only 50 % of natural folate is assumed to be bioavailable. Therefore, the bioavailability of folate from *Moringa* is much higher, suggesting that *M. oleifera* based food can be used as a significant source of folate.

Keywords *Moringa oleifera* · Folate bioavailability · Folate repletion · Gene expression · Liver GNMT

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Introduction

Folate is the general term for both naturally occurring food folate and folic acid. Folic acid (not found in food) is the fully oxidized monoglutamate form (pteroylmonoglutamate) of the folate vitamin that is used in fortified foods and dietary supplements. Folate plays an essential role in asssociation with B_{12} and B_6 vitamins in nucleotide synthesis, methionine regeneration in DNA methylation, oxidation and reduction of one-carbon units required for normal metabolism and regulation (Scotti et al. 2013). Folate defeciency causes severe abnormalities in one-carbon metabolism, resulting in DNA hypomethylation, which cause certain types of chronic diseases and developmental disorders, including neural tube defects (Williams et al. 2015). Neural tube defects (NTDs) are a group of abnormalities of the brain, cardiac and spinal cord which normally originate during gestation period causing the failure of the neural tube to close during embryogenesis (Williams et al. 2015). Thus, folate sufficient diet is strongly recommended during pregnancy to prevent the NTDs, and other chronic dysfuctions.

A sustainable food based approach using dietary source of folate, in adequate amount, can be effective in controlling folate deficiency and other usual associated nutritional deficiencies (Neeha and Kinth 2012; Kushwaha et al. 2014). *Moringa oleifera* leaves are one such promising food due to their easy availability (Saini et al. 2012), high nutritional (Saini et al. 2014a, b, c, d) and nutraceutical value (Saini et al. 2014e).

Use of fresh leaves, flowers and tender pods (fruits) of this plant as vegetable is confined to African and Asian countries including India (Anwar et al. 2007; Kushwaha et al. 2014; Nadeem et al. 2014; Pawar et al. 2014). Tender pods and seed and seed oil of *M. oleifera* is well known for medicinal properties (Coppin et al. 2013; Govardhan Singh et al. 2013). However, information on the bioavailability of folate from fresh or dehydrated *Moringa* leaves is not available.

Folate bioavailability refers to the proportion of consumed folate that is absorbed and becomes available for metabolic processes. Poor bioavailability of natural food folates is the major cause of folate deficiency in developing countries. In general, bioavailability of folate is measured compared to folic acid (relative bioavailability), which shows great variation in human studies, ranging from 10 to 98 % (Aiso and Tamura 1998). The bioavailability of folates from various foods depends on the content of monoglutamyl (fortified food; highly bioavailable) and polyglutamyl folates (vegetables; low bioavailable) and on the presence of enhancers and inhibitors of folate absorption (Aiso and Tamura 1998). Radioisotope labelling techniques are the most preferred and the best method for the assessment of folate bioavailability, with high sensitivity and reproducibility. However, the use of radiolabeled folate is difficult in human studies due to safety and regulatory issues (Gregory et al. 1991).

The present investigation was therefore conducted to study the bioavailability of folate from the leaves of *M. oleifera*, in folate depleted rats. Over the past decade, major advance has been made in understanding the molecular mechanism of folate one carbon metabolism and this has led to the identification of key genes, including GNMT (glycine N-methyl transferase) and MTR (5-methyltetrahydrofolate-homocysteine methyltransferase). In the present study, we have also evaluated the effects of different level of dietary folate on modulation of the levels of mRNA encoding genes involved in folate one carbon metabolism. Since dietary antioxidants such as ascorbic acid may enhance folate bioavailability by increasing the stability of folates during food processing and during digestion in the gastrointestinal tract (McNulty and Pentieva 2004), effect of ascorbic acid was also evaluated in folate bioavailability.

Methods and materials

Materials

Fresh leaves were collected from 3 year old *M. oleifera* (c.v. Bhagya) plants grown in Institute's orchard in December 2012 (average temperature was 23–25 °C). Recommended cultural practices were followed to raise the plants in field (Saini et al. 2013). Leaves were collected early in the morning, mixed thoroughly and dried using cabinet tray dryer (Armstrong Smith, India), with a capacity of 40 trays of 400×800 mm at 50 °C (Saini et al. 2014d). Dehydrated leaves were powdered and stored at −80 °C in amber color air tight containers until use. Content of folate in dehydrated leaves was determined using microbiological assays and HPLC.

For folate quantification, Folic Acid Casei Medium, Lactobacillus Agar and Lactobacillus Broth were obtained from HiMedia Mumbai, India. Folate binding protein, α-amylase from *Aspergillus oryzae* (30 units/mg) and protease from *Streptomyces griseus* (Type XIV, 3.5 units/mg) were purchased from Sigma-Aldrich (Bangalore, India). Affigel matrix was purchased from BIO-RAD (Gurgaon, India). Folic acid standards were purchased from Schircks Laboratories (Jona, Switzerland).

Refined Groundnut oil, corn starch, and cane sugar were procured from local supermarkets. Minerals, vitamins, cellulose, choline chloride and L-cystine were purchased from Himedia Laboratories (Mumbai, India). Casein was purchased from Nimesh Corporation (Mumbai, India). Clinical enzyme kits namely LDL cholesterol, HDL cholesterol, triacylglycerol, total cholesterol, glucose, albumin and total protein were purchased from Agappe Diagnostics, Kerala (India).

Quantification of folate in dehydrated leaves

Trienzyme extraction of folate

Folate was extracted from the dehydrated leaves *M. oleifera* (under subdued light) according to the method of Aiso and Tamura (Aiso and Tamura 1998), with some modifications. Dehydrated leaves (200 mg) were homogenised with 10 ml 0.1 M sodium phosplate buffer (pH 6.1) containing 1 % ascorbic acid (*w*/*v*) and 0.1 % 2-merceptoethanol (*v*/*v*). Similarly, a blank was prepared without adding the leaf extract. Loosely capped, 50 ml digestion tubes containing the samples and the blank were placed in a boiling water bath for 5 min and then allowed to cool to room temperature. After cooling, 0.5 ml charcoal treated rat serum (De Brouwer et al. 2010), and 300 units of α-amylase from *Aspergillus oryzae* were added. Tubes were gently swirled; their caps were secured and they were incubated at 37 °C for 4 h. Then, the tubes were kept in a boiling water bath for 5 min to deactivate the enzymes and allowed to cool at room temperature. After cooling, 35 units of protease from *Streptomyces griseus* (Type XIV) were added, gently swirled and incubated overnight at 37 °C in a water bath. To deactivate the enzymes after the incubation period, 2 ml extraction buffer was added and the tubes heated for 5 min in a boiling water bath followed by cooled to room temperature. Subsequently, the tubes were centrifuged at 10, 000*g* for 5 min and 2 ml supernatant was mixed in 18 ml water containing 2 % extraction buffer. This sample was used for the microbiological assay. Undiluted sample was used for folate purification followed by HPLC analysis. All samples were

stored at −80 °C in amber colour tubes to avoid the light mediated degradation of folate. Total folate was quantified by the microbiological assay on the same day as extraction.

Preparation of glycerol cryoprotected *Lactobacillus casei*

To minimize the analysis time and error between the analyses, cryoprotected *Lactobacillus casei* (ATCC 7469), were prepared according to standard methods (Grossowicz et al. 1981; Pandrangi and LaBorde 2004; Ortiz-Escobar et al. 2010). Briefly, 20 μl *L. casei* culture from Lactobacillus broth was transferred to 10 ml assay medium (Folic Acid Casei Medium) containing 250 mg/l ascorbic acid and 30 ng/l folic acid, and incubated at 140 rpm at 37 °C. After obtaining a constant optical density (OD: 0.5) at 550 nm at 14 to 20 h after inoculation, the whole culture was inoculated to 20 ml assay medium containing 250 mg/l ascorbic acid, with no added folic acid, and incubated at 140 rpm and 37 °C. After obtaining the constant OD, the culture was centrifuged and pellets were dissolved in assay medium to obtain the OD value 0.2, followed by dilution to equal volumes with sterile glycerol and water (80:20, *v/v*). Then, the cultures were aliquoted to 2 ml sterile cryo-vials and stored at −80 °C.

Microbiological assay of total folate

Folic acid content was assayed according to the method of AOAC (1990). Sample extracts (0.5, 1.0, 2.0, 4.0 and 5.0 ml) were assayed in triplicate. The volume in each tube was adjusted to 5 ml with deionized water. Five ml single-strength assay medium was added to each tube. Prepared assay tubes, standard curve tubes, blank and enzyme blank tubes were sterilised by autoclaving at 121 °C for 5 min. To prepare inoculum, 1.0 ml glycerol cryoprotected *L. casei* culture was diluted with 9.0 ml assay medium to obtain a 10 times dilution. The diluted medium was inoculated (50 μl) to each assay tube incubated at 37 °C for 20 to 24 h, and the growth response was measured at 550 nm.

Folate purification and HPLC analysis

Folate was purified by immunoaffinity chromatography using folate binding protein and quantified by RP-HPLC from dehydrated leaves essentially according to the method of Konings (Konings 1999). The outline of column preparation with affigel matrix and folate binding protein, along with the purification procedure is given in Fig. 1. The binding capacity of the column was analysed by loading an excess amount of 5CH_3-H_4- folate. The HPLC analysis of purified folate derivatives was performed using a Shimadzu chromatograph (LC 20-AS HPLC), equipped with dual pump, fluorescent detector (RF-20A), YMC-Pack ODS-AQ column (250×4.6 mm ID× 5 μ) and YMC ODS-AQ guard column (10×5 mm ID×5 μ). The separation and elution was accomplished by employing a binary gradient mode using solvent A (0.1 %*v/v* trifluoroacetic acid in water) and solvent B (acetonitrile) with an injection volume of 20 μl sample and at a flow rate of 0.8 ml/min for 25 min. The solvent system was ran as follows (% solvent A/solvent B): 0 min (10/90), 20 min (50/50), and 25 min (10/90). To quantify the folate derivatives, the fluorescent detector was set in dual wavelength mode; H_4 folate, 5-CH_3-H_4 folate and 5-HCO-H4-folate were detected at (Ex/Em) 290/360 nm. Similarly, 10-HCO-folic acid was detected (Ex/Em) at 360/460 nm (Shohag et al. 2011). Folate standards were diluted to a concentration of 1 μg/ml (Patring et al. 2005). The actual concentrations of the folate standards solution were checked spectrometrically using the molar extinction coefficients (Jastrebova et al. 2003). Quantification of folate derivatives was based on an external calibration curve with a linear range of 5–100 ng/ml for H_4-folate and 5-CH_3-H_4-folate, and 100–1000 ng/ml for 5-HCO-H_4-folate and 10-HCO-folic acid.

Dehydrated *Moringa* leaves were found to be very rich in total folate content as quantified by the microbiological assay (502.1 μg/100 g DW) and HPLC (223.69 μg/100 g DW). 5-HCO-H4-folate (502.1 μg/100 g F\DW) and H_4-folate (223.9 μg/100 g FW) were found as the prominent forms of folate in dehydrated leaves of *M. oleifera* (Fig. 2, Table 1). In the *Moringa* leaf based diet formulation, dehydrated leaf powder was incorporated at the expense of corn starch to supply the 1.0 and 2.0 mg of total folate per diet, on the basis of folate content data obtained by the microbiological assay and HPLC.

Rats and diets

Male Wistar rats (OUTB—Wistar, IND-cft (2c) weighing 60± 5 g, were used in this study. The experimental protocol adopted was approved by the Institute's Animal Ethical Committee. Animals were grouped according to the outline given in Fig. 3, housed in metabolic cages, in groups of four animals, under a 12 h light/dark cycle, at 25±2 °C and 40–60 % relative humidity. Diet plan, duration of treatment and details of folate depletion and repletion schedule is given in Fig. 3. Diets (AIN-93 M) were formulated (Reeves et al. 1993) according to Table 2. In *Moringa* leaf based diet, 10 and 20 % dehydrated *Moringa* leaves were incorporated at the expense of corn starch to supply the 1.0 and 2.0 mg folate per kg diet. In folate deficient diet, 1 % succinyl sulfathiazole (*w/w*) was also added to prevent the growth of folate biosynthetic micro flora in gut (O'Leary and Sheehy 2001). To study the effect of ascorbic acid (AA) supplementation on folate absorption, ascorbic acid was included at 2:1 molar ratio (AA:FA) (Teucher et al. 2004).

At the end of experimental depletion and repletion periods, rats were fasted overnight and sacrificed under ether anaesthesia. Blood was drawn by cardiac puncture and serum was

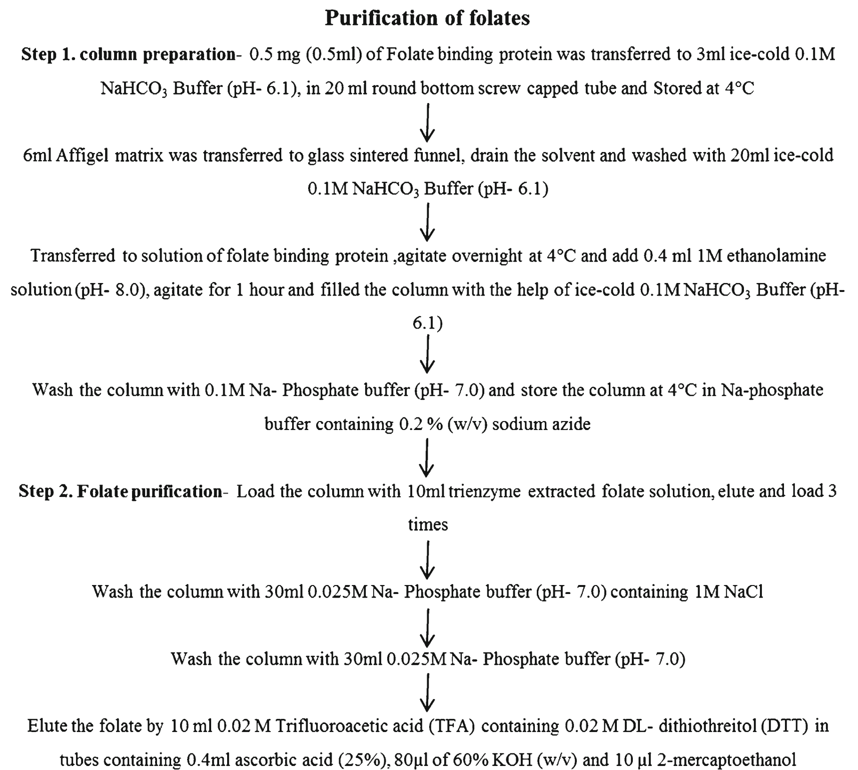

Fig. 1 Outline of column preparation and folate purification procedure

separated by centrifugation at 4 °C. Livers were dissected, rinsed with DEPC treated water, approximately 1 g of liver sample was freezed in liquid nitrogen in 15 ml RNase free falcon tubes and immediately stored at −80 °C for RNA extraction. Serum clinical enzymes and metabolites such as LDL, HDL, total cholesterol, triglycerides, glucose, albumin and total protein were analyzed by using Agape diagnostic kits, as per the manufacturer's guidelines.

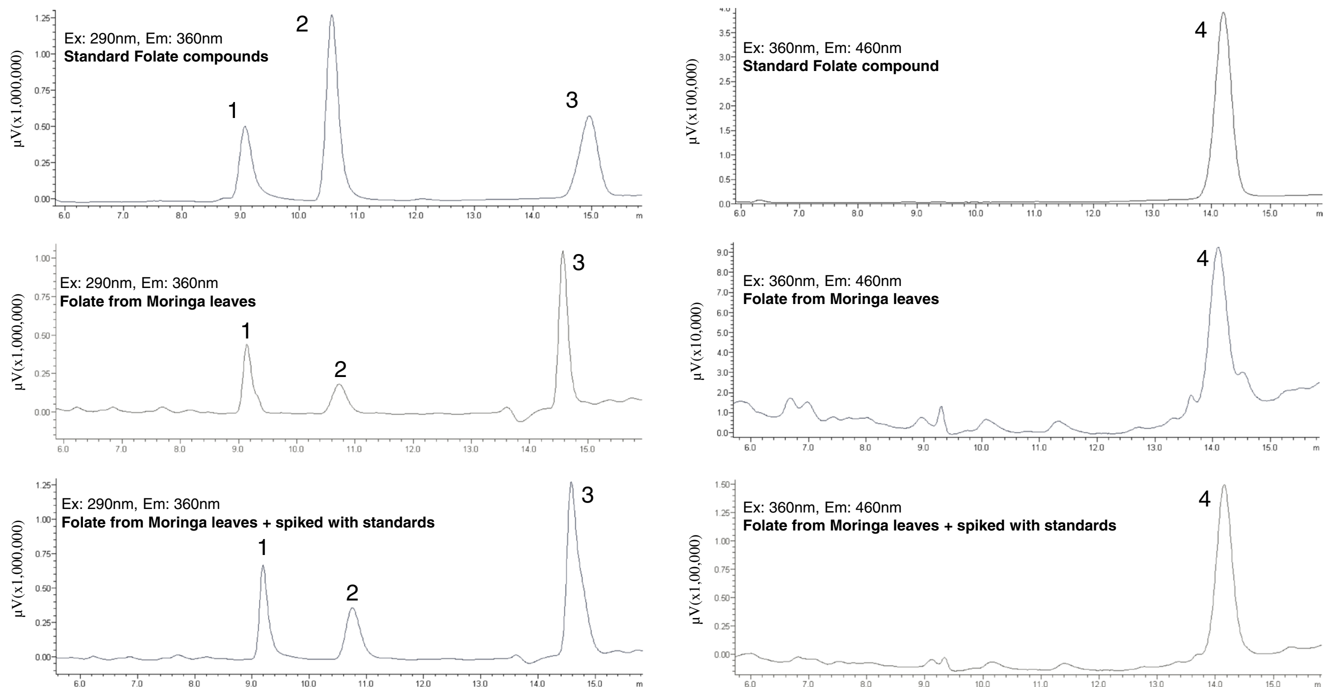

Fig. 2 HPLC chromatograms of standards and purified folates form *M. oleifera* leaves. (*1*) 5,6,7,8-Tetrahydrofolic acid (H_4 folate); (*2*) 5-Methyl-5,6,7,8-tetrahydrofolic acid (5-CH_3-H_4 folate); (*3*) 10-Formylfolic acid (10-HCO folic acid) and (*4*) 5-Formyl-5,6,7,8-tetrahydrofolic acid (5-HCO-H_4 folate)

Table 1 Folate content of fresh leaves of *Moringa oleifera* (c.v. Bhagya), purified by immunoaffinity chromatography and analysed by HPLC

S/No	Folate form	Folate (μg/100 g DW)
1	5,6,7,8-Tetrahydrofolic acid (H_4 folate)	223.9±15.7
2	5-Methyl-5,6,7,8-tetrahydrofolic acid (5-CH_3-H_4 folate)	144.9±16.4
3	10-Formylfolic acid (10-HCO-folic acid)	29.0±8.2
4	5-Formyl-5,6,7,8-tetrahydrofolic acid (5-HCO-H_4 folate)	502.1±43.2
5	Total folate	899.9

Values are mean±S.D. of three replicates

RNA extraction and real-time quantitative PCR (qPCR) analysis

Total RNA extraction was carried out using TRIzol reagent (Invitrogen, CA, USA) from 50 mg frozen liver. RNA concentration and purity were determined by spectrophotometry using Nano Drop 1000 (NanoDrop Technologies, inc., Wilmington, DE, USA). After quantification, RNA quality and integrity was analysed in a 1.5 % (*w*/*v*) agarose formamide gel. cDNA was synthesized in 40 μl reaction, using 2 μg of total RNA, 0.5 mM dNTP mix, 5 μM random hexamer, 5 μM oligo dT primers, 40 units of RNase inhibitor and 400 units of M-MLV Reverse transcriptase (Sigma Aldrich, Bangalore). cDNA was diluted with 160 μl of water to achieve 5-fold dilution, and used as the template for real-time quantitative PCR. Quantitative RT-PCR was performed in a total volume of 10 μl, including 2 μl of diluted cDNA, 100 μM for each primer, and 5 μl of 2× SsoFast EvaGreen Supermix (Bio-Rad Inc., CA, USA) on an CFX96 Touch Real-Time PCR Detection System (Bio-Rad Inc., CA, USA). The qPCR programme included a preliminary step of 95 °C for 1 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 20 s. Melt Curve was performed from 65.0 to 95.0 °C, with increment of 0.5° at every 5 s. *Rattus norvegicus* Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to normalize the gene expression. Relative gene expression was calculated according to a $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). qPCR was performed in triplicates and the fold change in each target gene was compared with non-depleted control (90 days old), which was set to 1. Primer sequences, Tm values, PCR product length and NCBI accession numbers of the genes used in the study is given in Table 3.

Statistical analysis

Statistical analysis was performed using the SPSS statistics 17.0 (SPSS Inc. Chicago, IL, USA). Data were analysed by one-way ANOVA, at 95 % confidence level ($P<0.05$). The values are means with standard deviation for all treatments.

Results

Body weight rats fed with folate-deficient diets grew in the same way during depletion period, as control diet (2 mg folate/kg diet) (Fig. 4a). During the repletion period, animals fed with 20 % *Moringa* leaf alone and with ascorbic acid (Group 5), showed marginally but significant lower body weight (214.8 g), compared to animals fed with folate and 10 % *Moringa* leaf diet. Whereas, rats fed with control diet (Group 2), 10 % *Moringa* leaf (Group 3), 20 % *Moringa* leaf (Group 4), 2.0 mg folic acid (Group 6), and 2.0 mg folic acid with ascorbic acid (Group 7), did not show any significant difference ($p<0.05$) in final body weight (229.0–237.1 g).

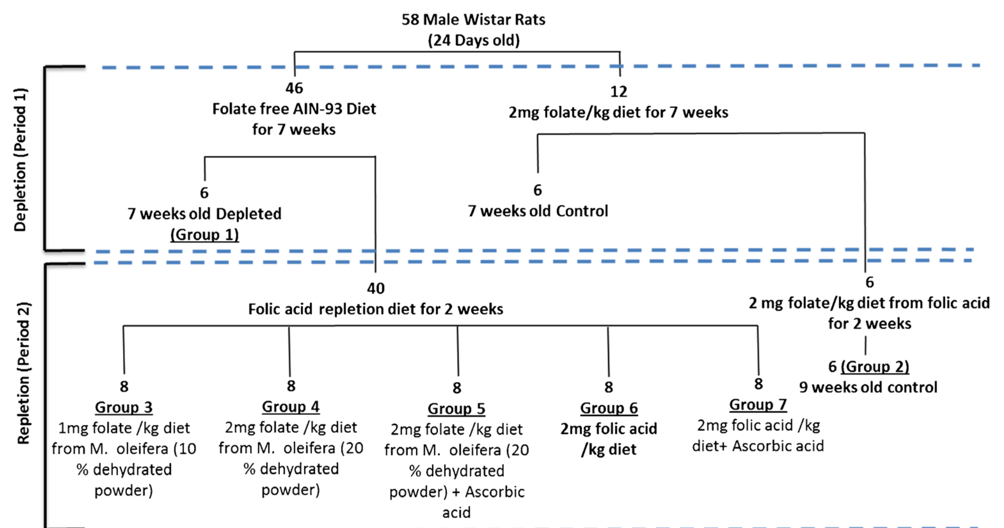

Fig. 3 Experimental outline of the folate depletion and repletion protocol used in the present investigation

Table 2 Ingredient composition of the folate depleted and repleted diets

Ingredients	Folate deficient diet	Control (No depletion) diet	10 % MO diet	20 % MO diet	20 % MO+AA diet	2 mg Folic acid diet	2 mg folic acid+AA diet
Corn starch	500.0	500.0	400.0	300.0	300.0	500.0	500.0
Casein (85 % protein)	200.0	200.0	200.0	200.0	200.0	200.0	200.0
Sucrose	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Groundnut oil	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Fibre (cellulose)	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Mineral mix (AIN-93G-MX)	35.0	35.0	35.0	35.0	35.0	35.0	35.0
Vitamin mix (AIN-93-VX)	10.0	10.0	10.0	10.0	10.0	10.0	10.0
L-cystine	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Choline bitartrate (41.1 % choline)	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Moringa dry powder	0	0	100	200	200	0	0
Ascorbic acid	0	0	0	0	0.217	0	0.217
Folic acid	0	0.002	0	0	0	0.002	0.002

MO Moringa oleifera, *AA* Ascorbic acid, #(AIN-93G-VX) deficient in folate. All the Values are in g/kg diet

Serum folate profile Serum folate concentrations (Fig. 4b) showed a complex pattern in rats fed with different diets. Serum folate level decreased to 9.1 ng/ml in rats fed with folate depleted diet for 7 weeks compared to rats fed with non-depleted diet (31.0 ng/ml), folate content was further increased in rats fed with non-depleted (control) diet for 9 weeks (35.2 ng/ml). During folate repletion period (2 weeks), final serum folate content was absolutely highest (33.0 and 32.3 ng/ml) in rats fed with 2 mg folate and 2 mg folate with ascorbic acid, respectively. Addition of ascorbic acid in 2:1 molar ratio (ascorbic acid:Fe), did not show any significant improvement ($p<0.05$) in serum folate content (Fig. 4b).

Other serum parameters Significantly ($p<0.05$) higher amount of serum triglycerides (134.0–165.6 mg/dL) was recorded in folate repleted rats (group 3–7), compared to non-depleted and depleted rats (Table 4). Similarly, content of serum LDL and total cholesterol was recorded higher in non-depleted rats (9 weeks) compared to depleted rats. Content of serum glucose, albumin and total protein was not significantly influenced ($p<0.05$) with different treatments.

Relative folate bioavailability Relative bioavailability was calculated as response of *Moringa* folate relative to the response of folic acid (Hannon-Fletcher et al. 2004).

$$\text{Relative folate bioavailability } (\%) = \frac{\text{Gain in serum folate in rats fed with } \textit{Moringa} \text{ folate (X)}}{\text{Gain in serum folate level in rats fed with folic acid (Y)}} \times 100$$

X = (Serum folate in rats fed with 2 mg folate/kg diet from *Moringa* for 2 weeks-serum folate in 7 weeks deficient rats), Y = (Serum folate in rats fed with 2 mg folate/kg diet from folic acid for 2 weeks- serum folate in 7 weeks deficient rats)

$$= \frac{(28.1-9.1)}{(32.3-9.1)} \times 100 = \mathbf{81.9\%}$$

Table 3 List of oligos designed for liver folate metabolism in *Rattus norvegicus*

S/No	Primer/Gene Name	Forward Primer (5′-3′)	Reverse primer (5′-3′)	NCBI Accession No.
1	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	AGAACATCATCCCTGCATCC	AGTCACAGGAGACAACCTGG	NM_017008.4
2	5-methyltetrahydrofolate-homocysteine methyltransferase (MTR)	GGAGGTTTCAGTGTGCTTGC	CAGGCGGTGGTACCTGTAAG	NM_001039003.1
3	Glycine N-methyltransferase (GNMT)	CGTGCTCAAGAAGACAGGCT	TTGTCGACTCCCTGTTTGCC	NM_017084.1

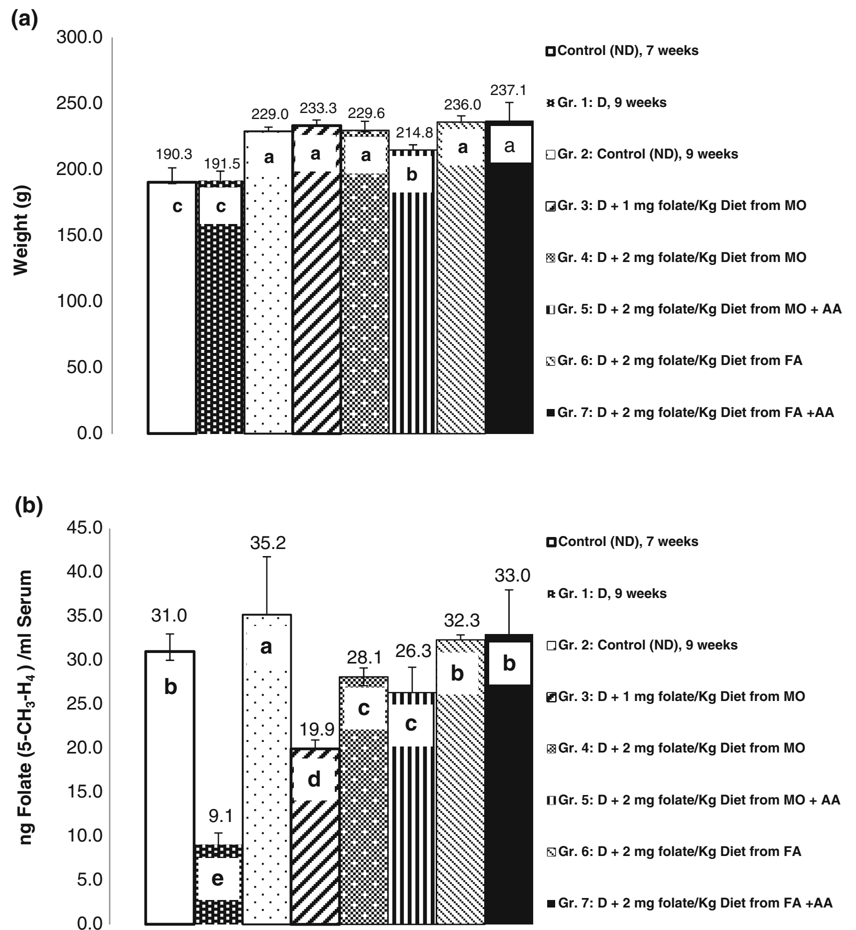

Fig. 4 Final body weight (**a**) and serum folate (**b**) of depleted and repleted rats. Values are mean± S.D. of 6 (control) and 8 (treated) animals. Different letters indicate statistically significant differences between the means ($P<0.05$)

Relative folate bioavailability from Moringa leaves was found to be ≈82 % when compared to equivalent amount of folic acid.

Expression of genes relevant to folate metabolism The relative expression pattern of genes relevant to folate metabolism; glycine N-methyl transferase (GNMT) and 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR) is given in Fig. 5. Both the studied genes did not show any significant changes in expression pattern in folate depleted as well as repleted rats ($p<0.05$).

Table 4 Influence of dietary *Moringa* dehydrated powder on serum lipid, glucose and protein profile

	Triacylglycerol (mg/dl)	Cholesterol (mg/dL)			Glucose (mg/dL)	Total protein (g/dl)	Albumin (g/dl)
		HDL (mg/dl)	LDL (mg/dl)	Total (mg/dl)			
Non-depleted, 52 days	109.2 ± 12.4^{c}	16.8 ± 2.2^{c}	7.0 ± 1.3^{bc}	49.8 ± 8.2	72.5 ± 11.3^{c}	8.81 ± 0.7^{c}	2.89 ± 0.23^{b}
Depleted, 52 days	109.2 ± 18.6^{c}	17.1 ± 3.9^{c}	7.3 ± 2.2^{cd}	34.9 ± 4.0^{d}	87.1 ± 9.2^{b}	9.20 ± 0.45^{c}	3.03 ± 0.071^{ab}
Non-depleted, 66 days	106.3 ± 18.3^{c}	21.6 ± 1.1^{ab}	13.2 ± 1.8^{a}	59.9 ± 2.7^{a}	84.2 ± 10.1^{b}	9.95 ± 0.24^{ab}	2.94 ± 0.09^{ab}
Depleted+10 % MO	158.9 ± 18.4^{a}	19.0 ± 1.5^{bc}	$9.3\pm2.6b^{c}$	44.7 ± 4.2^{bc}	84.9 ± 13.9^{b}	9.84 ± 0.58^{ab}	3.01 ± 0.11^{ab}
Depleted+20 % MO	134.0 ± 18.0^{b}	19.6 ± 1.8^{a}	10.6 ± 1.8^{ab}	51.2 ± 4.5^{bc}	80.5 ± 12.3^{b}	10.33 ± 0.47^{a}	3.04 ± 0.05^{ab}
Depleted+20 % MO+AA	165.6 ± 15.5^{a}	23.8 ± 7.1^{a}	8.7 ± 2.8^{bc}	51.9 ± 5.7^{b}	94.2 ± 13.0^{a}	10.16 ± 0.71^{b}	3.04 ± 0.17^{ab}
Depleted+2 mg Folic acid	160.9 ± 17.9^{a}	19.2 ± 2.6^{bc}	7.9 ± 2.7^{bc}	45.8 ± 10.0^{bc}	89.1 ± 22.9^{a}	9.82 ± 0.94^{ab}	2.91 ± 0.20^{b}
Depleted+2 mg folic acid+AA	161.0 ± 25.3^{a}	19.09 ± 2.3^{bc}	4.8 ± 1.3^{d}	43.4 ± 8.5^{c}	98.3 ± 17.8^{a}	9.85 ± 0.58^{b}	3.09 ± 0.08^{a}

Values are mean±S.D. of 6 (control) and 8 (treated) animals. Different letters indicate statistically significant differences between the means ($P<0.05$)

MO Moringa oleifera, *AA* Ascorbic acid

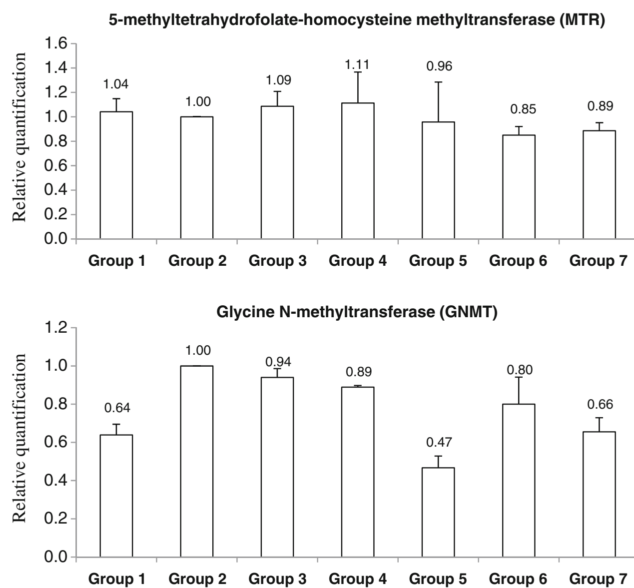

Fig. 5 Effect of folate deficient and repletion diets on the expression of liver folate metabolite genes, determined by qRT-PCR. Relative transcript abundances of each gene were normalised to the housekeeping gene GAPDH. Values are means±S.D. Group 1: Depleted (7 weeks), Group 2: Control (non-depleted, 9 weeks), Group 3: Depleted+1.0 mg folate/kg diet from *M. oleifera* leaves (MO), Group 4: Depleted+2.0 mg folate/kg diet from MO, Group 5: Depleted+2.0 mg folate/kg diet from MO+ ascorbic acid (AA), Group 6: Depleted+2.0 mg folate/kg diet from folic acid (FA), Group 7: Depleted+2.0 mg folate/kg diet from FA+AA. *Significant ($P<0.05$) compared to non-depleted control (Group 2)

Discussion

The present study was designed to investigate the effect of dietary folate from *Moringa* leaves on body folate status and modulation of folate responsive genes in rat model by using well established folate depleted and repleted approach (Clifford et al. 1989; Saini et al. 2014a). In the present study, *M. oleifera* in particular was selected to establish the readily available and folate rich underutilized leafy vegetable as a source of dietary folate to prevent the folate deficiency. Folic acid was used as the source of folate in control diet. In our previous studies, dietary iron supplements from Moringa leaves were more beneficial compared to ferric citrate in overcoming the effects of iron deficiency (Saini et al. 2014a).

The different forms of folate in *M. oleifera* leaves have not been studied earlier. We found, for the first time that 5-CHO-H_4-folate was the major form of folate in *Moringa* leaves, accounting for 55.8 % of the total folate, followed by H_4-folate (24.9 %), 5-CH_3-H_4-folate (16.1 %) and 10-HCO-folic acid. The total folate content, quantified by HPLC (899.9 μg/100 g DW) was 10.2 % lower than shown by the microbiological assay. This could be due to lack of folate standards for all folate forms and loss of folate during purification. These results are in agreement with previous studies, which reported folate contents determined by HPLC to be 20–52 % lower when determined by the microbiological assay (Ruggeri et al. 1999; Konings 1999). Recently, in a comparative study among Fijian vegetables, including *Moringa oleifera* leaves, taro leaves, bele leaves, amaranth leaves, okra and French bean, maximum total folate was observed in *M. oleifera* leaves (Maharaj et al. 2015).

The diets differed in the source of dietary folate viz. folic acid and *Moringa* leaf, with and without ascorbic acid supplements was well accepted by the rats. Feeding of folate deficient diet for 7 weeks caused significant decrease in serum folate content, compared to non-depleted control (7 weeks). At the end of 7 weeks depletion period, significant difference in body weight was not observed among folate depleted and control rats. However, very small, but significant difference was recorded at the end of 2 weeks repletion period. Significant decrease in body weight of folate deficient anaemic rats has been recorded in previous studies (Endoh et al. 2013).

Liver plays a crucial role in folate metabolism and expresses almost all the genes related to folate metabolism and homeostasis. GNMT is the most abundant methyltransferase in mammalian liver, comprising nearly 1 % of the soluble protein in rat liver (Takusagawa et al. 1999). An outline of one carbon metabolism and specific role of glycine N-methyl transferase (GNMT) and 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR) is shown in Fig. 6 (Supplement material). Among the different forms of folates, the 5-methyl tetrahydrofolate (5-CH_3-H_4 folate or 5-methyl-THF) plays the major role in transfer of methyl group to homocysteine by the action of methionine synthase (MTR; 5-methyltetrahydrofolate-homocysteine methyltransferase), and generate methionine (Zetterberg 2004). Glycine N-methyltransferase (GNMT) is a folate-binding protein, express in liver that converts S-adenosylmethionine (AdoMet) to S-adenosylhomocysteine (AdoHcy) while donating the methyl group. Suppression or deletion of GNMT imparts, lower cellular folate, AdoMet to AdoHcy balance, leads to cancer and other metabolic syndromes (Martínez-Chantar et al. 2008). Overexpression of GNMT, reduces the 5-methyl-THFdependent homocysteine remethylation due to higher affinity binding of GNMT with 5-methyl-THF. Overdose of retinoid compounds (Vitamin A) and methionine is reported to markedly elevate the activity and abundance of hepatic GNMT (Rowling et al. 2002). Whereas, folate derivatives inhibit the activity of GNMT (Wagner et al. 1985). In the present study, significant changes in GNMT and MTR expression were not observed during folate deficient and normal conditions. This may be because, we have supplied the sufficient amount of methionine (30 g/kg diet) and B_{12} vitamin, which is also known to regulate the activity of GNMT in rats (Rowling et al. 2002). Similarly, with folate, other metabolites might influence the regulation of MTR expression in liver. Supplementation of ascorbic acid, which is known to prevent the oxidation of folates, was not effective in modulating the expression of folate one carbon metabolism

related genes, and folate absorption. This indicates, oxidation of folate will not influence its absorption in gastrointestinal tract of the rats.

In human and other mammals, majority of dietary folates (polyglutamates) are absorbed in the intestine by proton coupled folate transporter (PCFT) and reduced folate carrier (RFC1) after hydrolysing to monoglutamates by the action of brush-border membrane γ-glutamyl hydrolase (GCPII). Deconjugation of polyglutamates by GCPII strongly depends on pH, with optimum pH of 6.5 (Chandler et al. 1986), thus folate bioavailability is generally influenced by the pH of intestinal lumen. Tamura et al. (1976) recorded the significant decrease in the bioavailability of heptaglutamyl folate ingested with orange juice at a pH of 3.7, compared to monoglutamyl folate. Thus, alterations in the pH of the intestinal lumen during gastrointestinal diseases (atrophic gastritis) have the potential to impair the hydrolysis of polyglutamyl folates, resulting in decrease of their bioavailability.

The bioavailability of folates from various foods is depends on the content of 16 monoglutamyl (fortified food; highly bioavailable) and polyglutamyl folates (vegetables; low bioavailable) and on the presence of enhancer and inhibitors of folate absorption (Aiso and Tamura 1998). Folate intake directly influences the plasma folate concentrations, whereas tissue folate levels saturate at high folate intakes (Clifford et al. 1990). To elevate the tissue folate levels, dose of 5-formyl-THF are more beneficial than folic acid or 5- methyl-THF. Intestinal microbiota, can synthesize the folate and it may yield inaccuracy in the estimation of available folate. So, in the present study, 1 % succinyl sulfathiazole (*w*/*w*) was also added to prevent the growth of folate biosynthetic micro-flora in gut (O'Leary and Sheehy 2001). In general, bioavailability of folate is compared to folic acid (relative bioavailability), which shows great variation in human studies, ranging from 10 to 98 % (Aiso and Tamura 1998). The bioavailability of folate form dried cabbage is reported to be 68 % in rat bioassays (Abad and Gregory 1987). In another study from green leafy vegetables, bioavailability of folate from spinach was 84 % compared with folic acid, based on folate repletion of folate-depleted rats (Babu and Lakshmaiah 1987). In the present study, relative bioavailability of folate from dehydrated leaves of *Moringa* was observed 81.9 %. In RDA calculations, only 50 % of natural folate is assumed to be bioavailable (DRI, Institute of Medicine 1998). Result shows that bioavailability of *M. oleifera* leaf folate is much higher and it can be used as a significant source of folate in diet.

In conclusion, *Moringa oleifera* leaf based food can be used as a significant source of folate due to equal or higher bioavailability compared to other vegetables, which are known as significant source of folate. Easy availability of Moringa leaves in rural areas can also make it a promising food for rural communities with high nutritional and nutraceutical value.

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References

Abad AR, Gregory JF 3rd (1987) Determination of folate bioavailability with a rat bioassay. J Nutr 117:866

Aiso K, Tamura T (1998) Trienzyme treatment for food folate analysis: optimal pH and incubation time for alpha-amylase and protease treatment. J Nutr Sci Vitaminol (Tokyo) 44:361–370

Anwar F, Latif S, Ashraf M, Gilani AH (2007) *Moringa oleifera*: a food plant with multiple medicinal uses. Phytother Res 21:17–25. doi:10.1002/ptr.2023

Association of Official Analytical (1990) Official methods of analysis. Vol 15th Ed AOAC Arlingt. VA

Babu S, Lakshmaiah N (1987) Availability of food folate by liver folate repletion in rats. Nutr Rep Int 35:831–836

Chandler CJ, Wang TT, Halsted CH (1986) Pteroylpolyglutamate hydrolase from human jejunal brush borders- Purification and characterization. J Biol Chem 261:928–933

Clifford AJ, Heid MK, Müller HG, Bills ND (1990) Tissue distribution and prediction of total body folate of rats. J Nutr 120:1633–1639

Clifford AJ, Wilson DS, Bills ND (1989) Repletion of folate-depleted rats with an amino acid-based diet supplemented with folic acid. J Nutr 119:1956–1961

Coppin JP, Xu Y, Chen H et al (2013) Determination of flavonoids by LC/MS and anti-inflammatory activity in *Moringa oleifera*. J Funct Foods 5:1892–1899. doi:10.1016/j.jff.2013.09.010

De Brouwer V, Storozhenko S, Stove CP et al (2010) Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) for the sensitive determination of folates in rice. J Chromatogr B Anal Technol Biomed Life Sci 878:509–513. doi:10.1016/j.jchromb.2009.12.032

DRI, Institute of Medicine (1998) Dietary reference intakes for thiamin, riboflavin, niacin, vitamin b6, folate, vitamin B12, pantothenic acid, biotin, and choline. National Academies Press (US), Washington (DC)

Endoh K, Fenech M, Umegaki K (2013) Green tea is a poor contributor to tissue folate in a Folate Depletion-Repletion Rat Model. Food Nutr (Roma) 4:136–143

Govardhan Singh RS, Negi PS, Radha C (2013) Phenolic composition, antioxidant and antimicrobial activities of free and bound phenolic extracts of *Moringa oleifera* seed flour. J Funct Foods 5:1883–1891. doi:10.1016/j.jff.2013.09.009

Gregory JF, Bhandari SD, Bailey LB et al (1991) Relative bioavailability of deuterium-labeled monoglutamyl and hexaglutamyl folates in human subjects. Am J Clin Nutr 53:736–740

Grossowicz N, Waxman S, Schreiber C (1981) Cryoprotected *Lactobacillus casei*: an approach to standardization of microbiological assay of folic acid in serum. Clin Chem 27:745–747

Hannon-Fletcher MP, Armstrong NC, Scott JM et al (2004) Determining bioavailability of food folates in a controlled intervention study. Am J Clin Nutr 80:911–918

Jastrebova J, Witthöft C, Grahn A et al (2003) HPLC determination of folates in raw and processed beetroots. Food Chem 80:579–588. doi:10.1016/S0308-8146(02)00506-X

Konings EJ (1999) A validated liquid chromatographic method for determining folates in vegetables, milk powder, liver, and flour. J AOAC Int 82:119–127

Kushwaha S, Chawla P, Kochhar A (2014) Effect of supplementation of drumstick (*Moringa oleifera*) and amaranth (*Amaranthus tricolor*) leaves powder on antioxidant profile and oxidative status among postmenopausal women. J Food Sci Technol 51:3464–3469. doi:10.1007/s13197-012-0859-9

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) Method. Methods San Diego Calif 25:402–408. doi:10.1006/meth.2001.1262

Maharaj PPP, Prasad S, Devi R, Gopalan R (2015) Folate content and retention in commonly consumed vegetables in the South Pacific. Food Chem. doi:10.1016/j.foodchem.2015.02.096

Martínez-Chantar ML, Vázquez-Chantada M, Ariz U et al (2008) Loss of the glycine N-methyltransferase gene leads to steatosis and hepatocellular carcinoma in mice. Hepatology 47:1191–1199

McNulty H, Pentieva K (2004) Folate bioavailability. Proc Nutr Soc 63: 529–536

Nadeem M, Azeem MW, Rahman F (2014) Assessment of transesterified palm olein and *Moringa oleifera* oil blends as vanaspati substitutes. J Food Sci Technol 1–7. doi:10.1007/s13197-014-1271-4

Neeha VS, Kinth P (2012) Nutrigenomics research: a review. J Food Sci Technol 50:415–428. doi:10.1007/s13197-012-0775-z

O'Leary K, Sheehy PJA (2001) Influence of folic acid-fortified foods on folate status in a folate depletion-repletion rat model. Br J Nutr 85: 441–446

Ortiz-Escobar TB, Valverde-González ME, Paredes-López O (2010) Determination of the folate content in cladodes of nopal (*Opuntia ficus indica*) by microbiological assay utilizing *Lactobacillus casei* (ATCC 7469) and enzyme-linked immunosorbent assay. J Agric Food Chem 58:6472–6475

Pandrangi S, LaBorde LF (2004) Optimization of microbiological assay of folic acid and determination of folate content in spinach. Int J Food Sci Technol 39:525–532. doi:10.1111/j.1365-2621.2004.00812.x

Patring JDM, Jastrebova JA, Hjortmo SB et al (2005) Development of a simplified method for the determination of folates in baker's yeast by HPLC with ultraviolet and fluorescence detection. J Agric Food Chem 53:2406–2411. doi:10.1021/jf048083g

Pawar N, Gandhi K, Purohit A et al (2014) Effect of added herb extracts on oxidative stability of ghee (butter oil) during accelerated oxidation condition. J Food Sci Technol 51:2727–2733. doi:10.1007/s13197-012-0781-1

Reeves PG, Nielsen FH, Fahey GC Jr (1993) AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr 123:1939–1951

Rowling MJ, McMullen MH, Chipman DC, Schalinske KL (2002) Hepatic glycine n-methyltransferase is up-regulated by excess dietary methionine in rats. J Nutr 132:2545–2550

Ruggeri S, Vahteristo LT, Aguzzi A et al (1999) Determination of folate vitamers in food and in Italian reference diet by high-performance liquid chromatography. J Chromatogr A 855:237–245

Saini RK, Manoj P, Shetty NP et al (2014a) Dietary iron supplements and *Moringa oleifera* leaves influence the liver hepcidin messenger RNA expression and biochemical indices of iron status in rats. Nutr Res 34:630–638

Saini RK, Prashanth KVH, Shetty NP, Giridhar P (2014b) Elicitors, SA and MJ enhance carotenoids and tocopherol biosynthesis and expression of antioxidant related genes in Moringa oleifera Lam. Leaves. Acta Physiol Plant 36:2695–2704

Saini RK, Saad KR, Ravishankar GA et al (2013) Genetic diversity of commercially grown *Moringa oleifera* Lam. cultivars from India by RAPD, ISSR and cytochrome P450-based markers. Plant Syst Evol 299:1205–1213. doi:10.1007/s00606-013-0789-7

Saini RK, Shetty NP, Giridhar P (2014c) Carotenoid content in vegetative and reproductive parts of commercially grown *Moringa oleifera* Lam. cultivars from India by LC–APCI–MS. Eur Food Res Technol 238:971–978

Saini RK, Shetty NP, Giridhar P (2014d) GC-FID/MS analysis of fatty acids in Indian cultivars of *Moringa oleifera*: potential sources of PUFA. J Am Oil Chem Soc 91:1029–1034

Saini RK, Shetty NP, Giridhar P, Ravishankar GA (2012) Rapid *in vitro* regeneration method for *Moringa oleifera* and performance evaluation of field grown nutritionally enriched tissue cultured plants. 3. Biotech 2:187–192. doi:10.1007/s13205-012-0045-9

Saini RK, Shetty NP, Prakash M, Giridhar P (2014e) Effect of dehydration methods on retention of carotenoids, tocopherols, ascorbic acid and antioxidant activity in *Moringa oleifera* leaves and preparation of a RTE product. J Food Sci Technol 51:2176–2182. doi:10.1007/s13197-014-1264-3

Scotti M, Stella L, Shearer EJ, Stover PJ (2013) Modeling cellular compartmentation in one-carbon metabolism. WIREs Syst Biol Med. doi:10.1002/wsbm.1209

Shohag MJI, Wei Y, Yu N et al (2011) Natural variation of folate content and composition in spinach (*Spinacia oleracea*) germplasm. J Agric Food Chem 59:12520–12526

Takusagawa F, Ogawa H, Fujioka M (1999) Glycine N-methyltransferase, a tetrameric enzyme. In: Cheng X, Blumenthal RM (eds). Adenosylmethionine-Depend. Methyltransferases Struct. Funct. World Scientific publishing, Singapore, pp 93–122

Tamura T, Shin YS, Buehring KU, Stokstad ELR (1976) The availability of folates in man: effect of orange juice supplement on intestinal conjugase. Br J Haematol 32:123–134

Teucher B, Olivares M, Cori H (2004) Enhancers of iron absorption: ascorbic acid and other organic acids. Int J Vitam Nutr Res 74: 403–419

Wagner C, Briggs WT, Cook RJ (1985) Inhibition of glycine N-methyltransferase activity by folate derivatives: implications for regulation of methyl group metabolism. Biochem Biophys Res Commun 127:746–752

Williams J, Mai CT, Mulinare J et al (2015) Updated estimates of neural tube defects prevented by mandatory folic acid fortification—United States, 1995–2011. MMWR Morb Mortal Wkly Rep 64:1–5

Zetterberg H (2004) Methylenetetrahydrofolate reductase and transcobalamin genetic polymorphisms in human spontaneous abortion: biological and clinical implications. Reprod Biol Endocrinol RBE 2:7. doi:10.1186/1477-7827-2-7