Research Article
Buckwheat Treatment Ameliorates Transforming Growth Factor Beta-1, its Receptor Gene Expression and Biochemical Parameters in Experimental Steatohepatitis

[1]Sahar Y. Al-Okbi, [1]Doha A. Mohamed, [1]Thanaa E. Hamed, [2]Ahmed M.M. Gabr, [1]Hoda B. Mabrok, [1]Shaimaa E. Mohammed and [3]Oksana Sytar

[1]Department of Nutrition and Food Sciences, National Research Centre, El-Buhouth Street, 12622, Dokki, Cairo, Egypt
[2]Department of Plant Biotechnology, National Research Centre, Cairo, 12622, Egypt
[3]Department of Plant Physiology and Ecology, Taras Shevchenko National University of Kyiv, Institute of Biology, Ukraine

Abstract

Background and Objective: Non-alcoholic fatty liver is recognized as the hepatic component of metabolic syndrome that accused for induction of cardiovascular and chronic liver diseases. This research evaluated the protective effect of two varieties of buckwheat seeds; Rubra and Karadag and their calli prepared by biotechnology towards non-alcoholic fatty liver in rat. **Materials and Methods:** Total phenolics, flavonoids, flavonols and DPPH free radical scavenging activity of buckwheat seeds and calli were assessed. Metabolic syndrome together with non-alcoholic fatty liver (MF) was induced by maintaining rats on high fructose diet. Daily oral dose of buckwheat seeds and calli (40 mg/rat) were given to 4 different groups during MF induction and compared to MF control rats and control group fed on balanced diet (NC). The experiment continued for 5 weeks. **Results:** *In vitro* free radical scavenging activities, total phenolic and flavonols of the calli were shown to be higher than the seeds. *In vivo* study showed significant dyslipidemia, significant increase in malondialdehyde, tumor necrosis factor-alpha, transaminase activity and liver fats in MF control group compared to NC. The relative gene expression of transforming growth factor beta-1 (TGF-β1) and TGF-β1 receptors I in liver as fibrogenic markers was significantly up-regulated in MF control group compared to NC. Treatment with different buckwheat forms produced improvement of the various determined plasma parameters and liver fats with variable degrees. Also, mRNA expression of TGF-β1 and TGF-β1 receptors I genes were significantly down-regulated in rats' liver on treatment with buckwheat seeds and calli. **Conclusion:** Buckwheat seeds and calli showed improvement of fatty liver and fibrogenic biomarkers.

Key words: Buckwheat seeds, callus, steatohepatitis, TGF-β1, plasma and liver lipids, inflammation, oxidative stress

INTRODUCTION

Metabolic syndrome is an increasing health problem Worldwide. It includes a group of tangled maladies composed of type 2 diabetes, obesity, non-alcoholic fatty liver, hyper-triglyceridemia and hypertension. Non-alcoholic fatty liver disease (NAFLD) is highly prevalent in both obese and type 2 diabetic patients[1,2]. The incidence of NAFLD was surveyed to be up to 30% in general population[3]. Progression of simple fatty liver (steatosis) to fatty liver associated with inflammation and fibrosis (steatohepatitis) is a serious condition that may be advanced to liver cirrhosis, hepatocellular carcinoma and liver failure[4]. Cardiovascular events remain to be the main cause of mortality and morbidity in NAFLD, even in the non-obese population[5]. Dyslipidemia and liver dysfunction together with increased inflammatory biomarkers and oxidative stress were demonstrated during steatohepatitis in both animal and human studies[6,7]. TGF-β1 is considered as a biomarker of liver fibrosis[8] which could be highly expressed in steatohepatitis. Amelioration of such changes could alleviate progression of fatty liver disease. So far; there is no exact remedy for fatty liver however some insulin sensitizers and lipid lowering drugs might be helpful. Hence the need for concentrated effort to search new sources of hepatoprotective agents is necessary. Functional foods and nutraceuticals might have a potential role in management of fatty liver; being natural products from food sources could represent a promising safe approach.

Buckwheat is a unique crop that attracted more and more attention due to both its nutritional and medicinal values in recent years[9]. Buckwheat contains many important bioactive constituents such as starch with a low glycemic index, polyphenol compounds including rutin, quercetin, orientin, vitexin, isovitexin and isoorientin[10,11], in addition to many essential minerals[12]. Therefore, buckwheat is rich in antioxidants and anti-inflammatory agents and it is a popular functional food component which is incorporated in different healthy food products[13]. Studies have revealed that buckwheat can help in curing chronic human diseases, decrease blood cholesterol, inhibit mammary cancer and prevent gallstones[14]. It is listed as health protection food in many countries and many nutritional meals related to buckwheat have been developed[15]. So, it was hypothesized that buckwheat could have an impact in prevention of fatty liver. On the other hand, biotechnological techniques that could enhance the production of bioactive constituents within buckwheat using tissue culture techniques might also represent a new source of natural product of promising remedial effect for non-alcoholic fatty liver.

The objective of the present research was to study the impact of treatment with two varieties of buckwheat seeds and their corresponding calli, prepared by applying biotechnological techniques, in rat with metabolic syndrome and fatty liver induced by dietary means. The aim included studying the bioactive constituents and *in vitro* anti-oxidant activity of different forms of buckwheat that may be responsible for any therapeutic effect.

MATERIALS AND METHODS

Plant materials and culture conditions: Seeds of *Fagopyrum esculentum*, Family, Polygonaceae of Karadag (K) and Rubra (R) cultivars were supplied by the Institute of Agriculture, The Ukrainian Academy of Agrarian Science (UAAS). The seeds were sterilized and germinated separately as described previously[16]. Germinated seedlings of *F. esculentum* Karadag and Rubra cultivars were grown on hormone-free Murashige-Skoog (MS) medium[17] supplemented with 30 g L^{-1} sucrose and solidified with 8 g L^{-1} agar. Cultures were incubated at 25±1°C under cool white fluorescent light at 40 µmol/m^2 sec under 16 h photoperiod for 3 weeks.

Establishment of callus culture: Leaf explants were obtained from three weeks old *in vitro* derived plantlets of both *F. esculentum* cultivars and inoculated on MS media supplemented with 2 mg L^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D) and 0.5 mg L^{-1} benzylaminopurine (BAP). These cultures were maintained at 25±2°C for 16/8h (light/dark) photoperiod in growth room. Data of callus induction frequencies was recorded weekly and respective calli were sub-cultured after 3 weeks on MS medium with the same supplements.

Callus preparation and extraction: Callus from both cultivars was harvested, weighed and immersed in liquid nitrogen to prevent any enzymatic metabolite and then followed by freeze-drying. The lyophilized samples were grounded to a fine powder. Ground samples (100 mg) were extracted with methanol (1 mL) overnight in a shaker at room temperature, followed by sonication in ultrasonic with ice for 15 min, then centrifuged at 10000×g for 10 min. The supernatant was collected and the same procedure was repeated for 2 more times.

The same extraction procedure was carried out on the original seeds of both varieties.

Determination of total phenolic: Total phenolic content of both cultivars and calli was determined by the Folin-Ciocalteu

method[18]. Gallic acid (GA) was used as standard for the calibration curve. Total phenolic content was expressed as gallic acid (mg GA/ g dry weight).

Determination of total flavonoids: Total flavonoids content was estimated by the method of Ordonez *et al.*[19]. Total flavonoids content was expressed as µg quercetin equivalent (QE)/g dry weight.

Determination of total flavonols: Total flavonol content was assessed according to the method of Kumaran and Karunakaran[20]. Total flavonol content was expressed as µg quercetin equivalent (QE)/g.

Assessment of free radical scavenging activity using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) method: Free radical scavenging activity of the two varieties of buckwheat seeds and their corresponding calli methanol extract was determined by adopting DPPH test according to Lee *et al.*[21]:

$$DPPH \text{ radical scavenging activity } (\%) = \frac{\text{Abs blank-Abs sample}}{\text{Abs blank}} \times 100$$

Where:
Abs blank = Absorbance of DPPH radical of the blank
Abs sample = Absorbance of DPPH radical of the sample

Design of animal experiment for studying the anti-fatty liver activity of buckwheat seeds and the produced calli.

Animals: Male albino rats of 140-160 g b.wt., were used in the present study. The animals were obtained from Animal House of National Research Centre, Cairo, Egypt. Animals were kept individually in stainless steel cages; water and food were given *ad libtium*. The animal experiment was carried out according to the Ethics Committee of the National Research Centre, Cairo, Egypt and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

Preparation of dosage form of buckwheat seeds and calli: Original R and K seeds were reduced into fine powders after being dried. Both seeds powder and freeze dried calli powder were prepared separately into emulsions using Tween 20 and distilled water. A vehicle containing the same quantity of Tween 20 and distilled water was prepared to be used as placebo (vehicle) for control groups.

Diets: Two experimental diets were prepared as in Table 1, a balanced and a high fructose diet. High fructose diet was

Table 1: Composition of different experimental diets (g/100 g)

Ingredients	Balanced diet	High fructose diet
Casein	12.0*	17.00
Corn oil	10.0	-
Coconut oil	-	7.25
Fructose	-	66.00
Starch	70.0	4.00
Salt mixture	3.5	3.50
Vitamin mix.	1.0	1.00
Fiber	3.5	-
Cholesterol	-	1.00
Cholic acid	-	0.25

*12 g casein used in the present study contains 10 g protein

prepared similar to the previous report of Kawasaki *et al.*[22] with some modifications by addition of cholesterol powder, cholic acid and coconut oil to induce metabolic syndrome with non-alcoholic fatty liver.

Experimental procedure: About 36 rats were divided into six groups, each of six rats. The first was normal group where rats received a balanced diet. The second group was metabolic syndrome and fatty liver (MF) control where rats were fed on high fructose diet (HFD). Group three, four, five and six were fed on HFD and given daily oral dose of 40 mg/rat/day from whole buckwheat seeds R and K and calus K and R, respectively. The first and second control groups were given daily oral dose of the vehicle. The experimental period continued for 5 weeks. During the experiment, body weight and food intake were recorded weekly. At the end of the study total food intake, body weight gain and feed efficiency ratio (Body weight gain/total food intake) were calculated. Blood samples were collected from rats after an overnight fast in heparinized tubes and plasma was separated by centrifugation at 3000 rpm for 15 min. Plasma total cholesterol (T-Ch), high density lipoprotein cholesterol (HDL-Ch), low density lipoprotein cholesterol (LDL-Ch) and triglycerides (TGs) were determined colorimetry according to Watson[23], Burstein *et al.*[24], Schriewer *et al.*[25] and Megraw *et al.*[26]; respectively. T-Ch/HDL-Ch ratio was calculated. Malondialdehyde (MDA) was assessed as indicator of lipid peroxidation adopting the method of Satoh[27]. Plasma tumor necrosis factor-α (TNF-α) the inflammatory biomarker was determined according to Stepaniak *et al.*[28] by applying enzyme-linked immunosorbent assay. The activity of aspartate transaminase (AST) and alanine transaminase (ALT) were estimated as indicator of liver function by the method of Reitman and Frankel[29]. Rats were dissected and liver was immediately received, weighed and a known weight was used for extraction of total hepatic lipids according to the procedure of Folch *et al.*[30] and Cequier-Sanchez *et al.*[31]. Triglycerides and cholesterol were assessed in the extracted hepatic lipids according to the methods of Megraw *et al.*[26] and

Watson[23], respectively. Another part of liver was subjected directly to RNA extraction and cDNA synthesis according to the subsequent procedure.

The mRNA expression of TGF-β1 and TGF-β1 receptor as biomarkers of liver fibrosis

RNA extraction and cDNA synthesis: Total RNA was isolated from <50 mg of liver tissue with PureLink® RNA Mini Kit (ambion® Life technologies™) according to the manufacturer's instructions. RNA concentrations were measured with a Nanodrop spectrophotometer and purity of the extracted RNA was assessed by the A_{260nm}/A_{280nm} ratio. The cDNA was synthesized from 1.5 µg of total RNA in 20 µL reaction with the High capacity cDNA Reverse Transcription kit (ambion® Life technologies™) according to the manufacturer's instructions. RNA template was incubated for 10 min at 25°C followed by 120 min at 37°C. The reaction was stopped by heating at 85°C for 5 min and finally at 4°C.

Real time PCR: Real-time PCR was performed as described previously[32] with a Rotor-Gene®MDx instrument. The RT-PCR reaction mix (25 µL) contained 1 µL template cDNA, 1×the SYBR®Green PCR master mix (ambion® Life technologiestm™) and 0.2 µM of the primer pairs. Primers pair sequence

used for RT-PCR gene expression analysis were adapted from the literature[33,34], primers sequence were presented in Table 2. PCR reactions were performed using the following protocol: 50°C for 2 min, 95°C for 10 min, 45 cycles of 15 sec at 95°C, 60/55 sec at 60°C, 15 sec at 72°C, melting curve program (60-95°C) and finally a cooling step to 4°C. PCR water was used instead of cDNA templates as a negative. Then; $2^{-\Delta\Delta CT}$ was used to calculate the relative expression ratio of the target gene[35]; the target gene expression was normalized to the expression of the house-keeping gene GAPDH.

Statistical analysis: The results of animal experiments were expressed as the Mean±SE and they were analyzed statistically using the one-way analysis of variance ANOVA followed by Tukey multiple comparison test using the SPSS statistical program. In all cases $p \leq 0.05$ was used as the criterion of statistical significance. The *in vitro* scavenging activity and the total phenolic, flavonoids and flavonols were expressed as Mean±SD.

RESULTS

Callus cultures: Callus cultures of both *Fagopyrum esculentum* cultivars (Rubra and Karadag) are shown in Fig. 1(a, b). The calli were obtained from three weeks old

Table 2: Primers used for real-time PCR amplifications

Target genes	Sequences	Annealing temp. (°C)	Size (bp)
TGF-β1	FW(GCCCTGGACAC CAACTATTGCT), RW (AGGCTCCA AATGTAGGGGCAGG)	60	293
TGF-β1 receptor I	FW(GCTCTAGATTTC TGCCACCTCTGTAC), RW (GCGAATTCG ACAGTGCGGTTATGGCA)	55	332
GAPDH	FW(GTATTGGGCGCCTGGTCACC), RW(CGCTCCTGGAAGATGGTGATGG)	60	324

(a)   (b)

Fig. 1(a-b): Callus cultures of both *Fagopyrum esculentum* cultivars, (a) Rubra and (b) Karadag

Table 3: Total phenolics, flavonoids, flavonols and free radical scavenging activity of buckwheat seed and buckwheat calli (Mean±SD)

	Total phenolic (mg GA g^{-1})	Total flavonoids (µg QE g^{-1})	Total flavonols (µg QE g^{-1})	DPPH free radical scavenging activity (%)
Buckwheat seed K	2.85±0.11	511.47±5.52	774.91±4.45	79.02±1.26
Buckwheat seed R	8.83±0.08	502.37±6.13	856.13±3.91	77.62±1.40
Buckwheat callus K	18.36±0.08	499.48±3.50	912.77±11.04	91.25±0.89
Buckwheat callus R	12.17±0.05	605.12±6.74	1378.38±9.19	91.74±0.85

Table 4: Biochemical parameters of different experimental groups (Mean±SE)

	Normal control	Fatty liver control	Buckwheat seeds K	Buckwheat seeds R	Callus K	Callus R
Plasma parameters						
T-Ch (mg dL^{-1})	66.6±1.300[a]	146.3±2.556[d]	107.8±3.695[b]	97.7±3.742[b]	133.2±5.087[c]	111.5±5.151[b]
HDL-Ch (mg dL^{-1})	41.3±1.022[d]	28.0±1.064[a]	33.1±1.713[b]	38.0±1.211[c]	32.1±1.352[b]	29.7±0.912[a]
LDL-Ch(mg dL^{-1})	16.0±0.577[a]	76.8±4.093[d]	54.5±1.477[c]	37.0±2.449[b]	74.7±3.738[d]	54.0±2.619[c]
TCh/HDL-Ch	1.6±0.050[a]	5.3±0.216[e]	3.3±0.177[c]	2.6±0.126[b]	4.2±0.278[d]	3.9±0.291[d]
TGs (mg dL^{-1})	62.7±2.245[a]	116.7±2.089[c]	113.4±2.521[c]	102.0±2.309[b]	107.7±5.149[b]	118.2±5.502[c]
MDA (nmol mL^{-1})	9.9±0.578[a]	18.5±0.889[d]	13.8±0.715[c]	11.5±0.772[b]	14.5±0.903[c]	15.1±0.652[c]
TNF-α (pg mL^{-1})	18.0±0.931[a]	41.7±1.115[e]	34.0±1.460[c]	30.0±0.931[b]	36.5±1.477[c]	39.1±1.166[d]
AST (IU L^{-1})	15.8±0.872[a]	58.5±0.957[d]	47.7±3.657[c]	44.3±2.139[b]	52.2±3.841[c]	48.0±5.691[c]
ALT (IU L^{-1})	10.5±0.764[a]	41.2±1.701[c]	36.8±3.155[b]	32.5±1.910[b]	39.2±1.956[b]	35.2±3.102[b]
Liver parameters						
Total fat (mg g^{-1} tissue)	42.3±1.145[a]	94.3±2.403[e]	57.8±2.675[c]	49.0±1.125[b]	68.7±3.592[d]	81.8±4.237[f]
T-Ch (mg g^{-1} tissue)	2.6±0.204[a]	9.6±0.317[c]	5.0±0.414[b]	5.0±0.413[b]	9.2±0.379[c]	9.4±0.188[c]
TGs (mg g^{-1} tissue)	5.9±0.276[a]	19.1±0.950[d]	11.1±0.373[b]	8.9±0.539[b]	15.3±0.442[c]	15.9±0.583[c]

In each row same superscript letters mean non-significant difference while different letters mean significance difference at p≤0.05

in vitro leaf explant on MS media supplemented with 2 mg L^{-1} of 2, 4-D and 0.5 mg L^{-1} of BAP. Callus cultures from both cultivars were sub-cultured after 3 weeks on MS medium with the same supplements. Callus were harvested from the culture media after the third subculture, immersed in liquid nitrogen, freeze dried and stored at -20°C for further studies.

Total phenolics, flavonoids, flavonols and free radical scavenging activity of buckwheat seeds and buckwheat calli: The data of total phenolics, flavonoids, flavonol and DPPH radical scavenging activity of buckwheat seeds and buckwheat calli were complied in Table 3. It could be observed that total phenolic of buckwheat callus K showed the highest content followed by buckwheat callus R and then buckwheat seed R while buckwheat seed K was of the least value. Total flavonoids of buckwheat callus R was higher than that of the corresponding seed while the value of buckwheat callus K was slightly lower than that of the seed itself. Total flavonol of buckwheat callus K and R were higher than that of their seeds. Both buckwheat seed varieties and their respective calli showed high DPPH (%) free radical scavenging activity; calli of both varieties demonstrated higher activity than their original seeds.

Results of animal experiment: Table 4 illustrated the biochemical changes in different experimental groups. Control group, that fed on high fructose diet showed significant decrease in plasma HDL-Ch with significant elevation in triglycerides, total cholesterols, LDL-Ch and T-Ch/HDL-Ch ratio when compared to the normal control group. Also a significant increase was noticed in liver total lipids, T-Ch and triglycerides in the same group. Significant elevation in plasma transaminase activities was observed in the control group that fed on HFD compared to normal control rats pointing to the induction of liver dysfunction. Plasma level of MDA as lipid peroxidation marker and TNF-α as inflammatory biomarker were significantly high in HFD fed control rats compared to normal control group. Oral administration of different buckwheat forms showed significant improvement in plasma AST and ALT activity; buckwheat seed R was the most promising in improving AST activity. Treatment by different buckwheat forms showed significant improvement in dyslipidemia and reduction in liver fat of HFD fed rats; buckwheat seeds R was the best treatment in this respect. An exception is that buckwheat seed K and callus R did not show any improvement in plasma triglycerides. Different buckwheat treatments showed significant reduction in plasma MDA and TNF-α level compared to fatty liver control rats but still higher than normal rats. Oral administration of buckwheat seed R was the most efficient in reducing lipid peroxidation and inflammation as observed by reduction in MDA and TNF-α plasma levels, respectively.

Figure 2 showed the relative expression of TGF-β1 and TGF-β1 receptor I genes in liver of different experimental groups. The mRNA expression of TGF-β1 and TGF-β1 receptor I in the liver of rats of control fatty liver was significantly up-regulated (p<0.001) compared to NC group. Treatment

Table 5: Nutritional parameters of different experimental groups (Mean±SE)

	Normal control	Fatty liver control	Buckwheat seeds K	Buckwheat seeds R	Callus K	Callus R
Initial body weight (g)	150.2±2.650[a]	150.3±1.26[a]	150.5±2.291[a]	150.2±2.119[a]	150.5±1.384[a]	150.5±2.432[a]
Final body weight (g)	210.8±2.688[a]	212.0±2.097[a]	214.5±2.432[a]	211.5±2.093[a]	212.3±1.646[a]	212.8±2.638[a]
Body weight gain (g)	60.7±0.882[a]	61.7±1.686[a]	64.0±0.966[a]	61.3±0.759[a]	61.8±1.887[a]	62.3±1.333[a]
Total food intake (g)	517.2±2.227[a]	522.5±2.348[a]	522.3±3.241[a]	518.0±2.768[a]	527.0±3.915[a]	528.2±3.581[a]
Feed efficiency ratio	0.117±0.002[a]	0.118±0.003[a]	0.123±0.002[a]	0.118±0.002[a]	0.117±0.003[a]	0.118±0.002[a]
Liver weight/body weight (%)	2.8±0.018[a]	4.0±0.298[b]	4.1±0.179[b]	4.0±0.079[b]	4.1±0.119[b]	4.1±0.244[b]

In each row same superscript letters mean non-significant difference while different letters mean significance difference at 0.05 probability
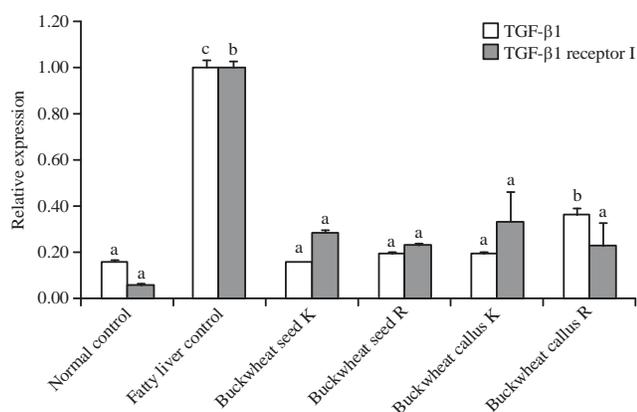

Fig. 2: Relative expression of TGF-β1 and TGF-β1 receptor I genes in liver of different experimental groups (Mean±SE). The mRNA expressions of TGF-β1 and TGF-β1 receptor I genes in the liver were normalized with housekeeping gene (GAPDH), TGF-β1: Transforming growth factor beta-1
Same superscript letters within the same parameter mean non-significant difference while different letters mean significant difference at p≤0.05

with buckwheat seeds (K and R) and buckwheat calli (K and R) significantly (p<0·001) down-regulated the expression of TGF-β1 by 6.4, 5.3, 5.3 and 2.9 fold change compared to fatty liver control, respectively. TGF-β1 receptor I gene expression was significantly (p<0·01) down-regulated with 3.7, 4.5, 3.0 and 4.5 fold change in the liver of rats treated with buckwheat seeds (K and R) and buckwheat calli (K and R), respectively, compared with fatty liver control.

Table 5 showed the nutritional parameters and liver weight (%) of different experimental groups. Non-significant changes were observed when different nutritional parameters of high fructose fed groups with or without treatment were compared with the group fed on balanced diet. Liver weight to body weight (%) of all rats fed on HFD fed rats with or without treatment was significantly higher than that of normal control rats which might be due to deposition of fat in the liver.

DISCUSSION

In the present study, non-alcoholic fatty liver model as a hepatic manifestation of metabolic syndrome was induced in rats by feeding diet high in fructose and supplemented by cholesterol, cholic acid and coconut oil (saturated fat). Animal models simulating non-alcoholic liver disease helps a lot to study the pathophysiology of the disease and to discover new remedies[3]. Diet rich in calories due to the high level of saturated fats and cholesterol along with the inclusion of high fructose level was reported to enhance the induction of steatosis and promote the progression to steatohepatitis, fibrosis and even the hepatocellular carcinoma[36,37]. It was demonstrated that fructose is a key dietary component that triggers the development of steatohepatitis[38] due to enhancing effect on *de novo* lipogenesis together with reduction of fatty acid oxidation[39]. Fructose intake also induced insulin resistance which reported as one of the main mechanism of induction of steatohepatitis[40]. The present rat model showed promising induction of fatty liver and metabolic syndrome represented by elevated total liver fat, TGs and cholesterol together with dyslipidemia, inflammation, high oxidative stress and liver dysfunction which agreed with a previous study[41]. TGF-β was documented as a major profibrogenic cytokine. So, the significant expression of TGF-β1 and TGF-β1 receptor I genes, in the fatty liver model in the present study is pointing to start of liver fibrosis and therefore, become a common target in the treatment of such liver disease[42]. In animal study, enhanced expression of TGF-β1 was noticed during steatohepatitis[43] while in clinical study, no change was noticed concerning genetic expression of TGF-β1[8].

Rats fed on high fructose diet together with cholesterol and cholic acid with simultaneous intake of the two varieties of buckwheat and their respective calli produced improvement in all the biochemical changes related to fatty liver and metabolic syndrome towards normal levels. Original seeds of K and R varieties showed promising improvements compared to their respective calli; with superiority to R variety.

This result was unexpected due to high levels of the bioactive components in the calli compared to that in the original seeds. The result may indicate that the activity of the different tested forms of buckwheat is related to the relative concentration of the bioactive constituents to each other rather than to their high contents. Buckwheat flavonoid fraction was shown previously to improve insulin sensitivity and to possess antioxidant activity in mice given fructose in drinking water[44] which may explain the improvement in fatty liver in the present study. Buckwheat bran extract was reported to reduce TGs accumulation in liver and blood triglycerides and to reduce lipogenic enzyme expression[45] which supports the present results. Buckwheat flour produced hypolipidemic, antiatherogenic and antioxidant effect in high fat fed rats[46].

The significant reduction in the expression of TGF-β1 and TGF-β1 receptor-I genes (as biomarkers of liver fibrosis) on treatment with the different buckwheat varieties and their calli pointed to prevention of liver fibrosis. Again it could be noticed that the original seeds were more efficient than the calli. The TGF-β1 induced hepatic stellate cell activation and extracellular matrix production and leading to liver fibrosis[47,48]. Therefore, the down-regulation of TGF-β1 and its receptor expression may explain the role of buckwheat in preventing the progression of hepatic steatosis to steatohepatitis with fibrosis.

The presence of phenolic compounds, flavonoids and flavonols in the different forms of buckwheat might render them the DPPH radical scavenging activity as could be seen from the *in vitro* result. Previous studies, showed that buckwheat grains and hulls contain flavonoids, flavon, phenolic acid, condensed tannins, phytosterols and fagopyrins of known biological activity. Flavonoids possess chelating properties, acting as antioxidants inhibiting lipid peroxidation and reduce the tissue damage induced by reactive oxygen species[49]. Flavonoids in buckwheat reduced blood cholesterol and prevent an increase in blood pressure. In a previous study; it was reported that there is a statistical relationship between buckwheat antioxidant activity with both total phenolics and rutin content[50]. The flavonoid rutin possess anti-inflammatory and anti-hypertensive effect and it composed of flavonol quercetin and the disaccharide rutinose. Both quercetin and rutin have the ability to reduce the oxidation of the lipoproteins that leads to the reduction of the risk of atherosclerosis[51].

Although, the *in vitro* DPPH radical scavenging activity showed that the calli were more efficient than buckwheat seeds however, this effect is not consistent with the *in vivo*

MDA reduction activity that pointed to the superiority of buckwheat R seed. This might be due to the gastrointestinal effect in the *in vivo* study.

CONCLUSION

Buckwheat seeds and calli have the ability to prevent the progression of fatty liver to steatohepatitis through reduction of fibrogenic, inflammatory and oxidative stress biomarkers, together with lipid and transaminases lowering effect. Thereby buckwheat might be beneficial in the management of fatty liver and metabolic syndrome. Buckwheat R was superior to all treatments; concerning the *in vivo* study while both buckwheat calli were more efficient as scavenger of DPPH radicals than the original seeds. The bioactivity of buckwheat might be attributed to the presence of phenolic compounds, flavonoids and Flavonols.

SIGNIFICANCE STATEMENTS

Buckwheat seeds and calli could be used for prevention or as complementary therapy for metabolic syndrome and fatty liver; variety Rubra proved to be the most promising.

ACKNOWLEDGMENT

REFERENCES

1. Dai, W., L. Ye, A. Liu, S.W. Wen, J. Deng, X. Wu and Z. Lai, 2017. Prevalence of nonalcoholic fatty liver disease in patients with type 2 diabetes mellitus: A meta-analysis. Medicine, Vol. 96. 10.1097/MD.0000000000008179.

2. Kleiner, D.E., 2018. Histopathology, grading and staging of nonalcoholic fatty liver disease. Minerva Gastroenterol. Dietol., 64: 28-38.

3. Van Herck, M., L. Vonghia and S. Francque, 2017. Animal models of nonalcoholic fatty liver disease-a starter's guide. Nutrients, Vol. 9. 10.3390/nu9101072.

4. Lonardo, A., S. Sookoian, M. Chonchol, P. Loria and G. Targher, 2013. Cardiovascular and systemic risk in nonalcoholic fatty liver disease-atherosclerosis as a major player in the natural course of NAFLD. Curr. Pharm. Des., 19: 5177-5192.

5. Yousef, M.H., A. Al Juboori, A.A. Albarrak, J.A. Ibdah and V. Tahan, 2017. Fatty liver without a large "belly": Magnified review of non-alcoholic fatty liver disease in non-obese patients. World J. Gastrointest. Pathophysiol., 8: 100-107.

6. Al-Okbi, S.Y., D.A. Mohamed, T.E. Hamed, A.A. Kassem, S.H. Abd El-Alim and D.M. Mostafa, 2017. Enhanced prevention of progression of non alcoholic fatty liver to steatohepatitis by incorporating pumpkin seed oil in nanoemulsions. J. Mol. Liq., 225: 822-832.

7. Al-Okbi, S.Y., S.I. Shalaby, D.A. Mohamed, T.E. Hamed and A.M.S. Hussein, 2016. Nutrition intervention by functional foods and dietary regimen for management of nonalcoholic fatty liver disease. Der Pharma Chem., 8: 591-599.

8. Yilmaz, Y. and F. Eren, 2019. Serum biomarkers of fibrosis and extracellular matrix remodeling in patients with nonalcoholic fatty liver disease: Association with liver histology. Eur. J. Gastroenterol. Hepatol., 31: 43-46.

9. Guo, X.D., Y.J. Ma, J. Parry, J.M. Gao, L.L. Yu and M. Wang, 2011. Phenolics content and antioxidant activity of tartary buckwheat from different locations. Molecules, 16: 9850-9867.

10. Danihelova, M. and E. Sturdik, 2012. Nutritional and health benefits of buckwheat. Potravinarstvo, 6: 1-9.

11. Sharma, P., A.K. Ghimeray, A. Gurung, C.W. Jin, H.S. Rho and D.H. Cho, 2012. Phenolic contents, antioxidant and α-glucosidase inhibition properties of Nepalese strain buckwheat vegetables. Afr. J. Biotechnol., 11: 184-190.

12. Steadman, K.J., M.S. Burgoon, B.A. Lewis, S.E. Edwardson and R.L. Obendorf, 2001. Minerals, phytic acid, tannin and rutin in buckwheat seed milling fractions. J. Sci. Food Agric., 81: 1094-1100.

13. Starowicz, M., G. Koutsidis and H. Zielinski, 2018. Sensory analysis and aroma compounds of buckwheat containing products-a review. Crit. Rev. Food Sci. Nutr., 58: 1767-1779.

14. Tomotake, H., I. Shimaoka, J. Kayashita, F. Yokoyama, M. Nakajoh and N. Kato, 2000. A buckwheat protein product suppresses gallstone formation and plasma cholesterol more strongly than soy protein isolate in hamsters. J. Nutr., 130: 1670-1674.

15. Xiao Z.P., 2003. Prospect of development and exploitation of buckwheat resources. Modern Agric., 8: 35-35.

16. Gabr, M.M.A., O. Sytar, A.R. Ahmed and I. Smetanska, 2012. Production of phenolic acid and antioxidant activity in transformed hairy root cultures of common buckwheat (Fagopyrum esculentum M.). Aust. J. Basic Applied Sci., 6: 577-586.

17. Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Planta., 15: 473-497.

18. Arabshahi-Delouee, S. and A. Urooj, 2007. Antioxidant properties of various solvent extracts of mulberry (Morus indica L.) leaves. Food Chem., 102: 1233-1240.

19. Ordonez, A.A.L., J.D. Gomez, M.A. Vattuone and M.I. Lsla, 2006. Antioxidant activities of Sechium edule (Jacq.) Swartz extracts. Food Chem., 97: 452-458.

20. Kumaran, A. and R.J. Karunakaran, 2007. In vitro antioxidant activities of methanol extracts of five Phyllanthus species from India. LWT-Food Sci. Technol., 40: 344-352.

21. Lee, S.C., J.H. Kim, S.M. Jeong, D.R. Kim, J.U. Ha, K.C. Nam and D.U. Ahn, 2003. Effect of far-infrared radiation on the antioxidant activity of rice hulls. J. Agric. Food Chem., 51: 4400-4403.

22. Kawasaki, T., K. Igarashi, T. Koeda, K. Sugimoto and K. Nakagawa et al., 2009. Rats fed fructose-enriched diets have characteristics of nonalcoholic hepatic steatosis. J. Nutr., 139: 2067-2071.

23. Watson, D., 1960. A simple method for the determination of serum cholesterol. Clin. Chem. Acta, 5: 637-642.

24. Burstein, M., H.R. Scholnick and R. Morfin, 1970. Rapid method for the isolation of lipoproteins from human serum by precipitation with polyanions. J. Lipid Res., 11: 583-595.

25. Schriewer, H., U. Kohnert and G. Assmann, 1984. Determination of LDL cholesterol and LDL apolipoprotein B following precipitation of VLDL in blood serum with phosphotungstic acid/$MgCl_2$. J. Clin. Chem. Clin. Biochem., 22: 35-40.

26. Megraw, R.E., D.E. Dunn and H.G. Biggs, 1979. Manual and continuous-flow colorimetry of triacylglycerols by a fully enzymic method. Clin. Chem., 25: 273-278.

27. Satoh, K., 1978. Serum lipid peroxide in cerebrovascular disorders determined by a new colorimetric method. Clin. Chim. Acta, 90: 37-43.

28. Stepaniak, J.A., K.E. Gould, D. Sun and R.H. Swanborg, 1995. A comparative study of experimental autoimmune encephalomyelitis in Lewis and DA rats. J. Immunol., 155: 2762-2769.

29. Reitman, S. and S. Frankel, 1957. Colorimetric methods for aspartate and alanine aminotransferase. Am. J. Clin. Pathol., 28: 55-60.

30. Folch, J., M. Lees and G.H.S. Stanley, 1957. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem., 226: 497-509.

31. Cequier-Sanchez, E., C. Rodriguez, G. Ravelo and R. Zarate, 2008. Dichloromethane as a solvent for lipid extraction and assessment of lipid classes and fatty acids from samples of different natures. J. Agric. Food Chem., 56: 4297-4303.

32. Mabrok, H.B., R. Klopfleisch, K.Z. Ghanem, T. Clavel, M. Blaut and G. Loh, 2012. Lignan transformation by gut bacteria lowers tumor burden in a gnotobiotic rat model of breast cancer. Carcinogenesis, 33: 203-208.

33. Ryu, S.H., Y.H. Chung, J.K. Lee, J.A. Kim and J.W. Shin et al., 2009. Antifibrogenic effects of tamoxifen in a rat model of periportal hepatic fibrosis. Liver Int., 29: 308-314.

34. Khan, H.A., M.A. Abdelhalim, A.S. Alhomida and M.S. Al Ayed, 2013. Transient increase in IL-1β, IL-6 and TNF-α gene expression in rat liver exposed to gold nanoparticles. Genet. Mol. Res., 12: 5851-5857.

35. Livak, K.J. and T.D. Schmittgen, 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods, 25: 402-408.

36. Parry, S.A. and L. Hodson, 2017. Influence of dietary macronutrients on liver fat accumulation and metabolism. J. Investig. Med., 65: 1102-1115.

37. Kong, L., Y. Lu, S. Zhang, Y. Nan and L. Qiao, 2017. Role of nutrition, gene polymorphism and gut microbiota in non-alcoholic fatty liver disease. Discov. Med., 24: 95-106.

38. Lambertz, J., S. Weiskirchen, S. Landert and R. Weiskirchen, 2017. Fructose: A dietary sugar in crosstalk with microbiota contributing to the development and progression of non-alcoholic liver disease. Front. Immunol., Vol. 8. 10.3389/fimmu.2017.01159.

39. Ackerman, Z., M. Oron-Herman, M. Grozovski, T. Rosenthal, O. Poppo, G. Link and B.A. Sela, 2005. Fructose-induced fatty liver disease: Hepatic effects of blood pressure and plasma triglyceride reduction. Hypertension, 45: 1012-1018.

40. Chidambaram, J. and A.C. Venkatraman, 2010. *Cissus quadrangularis* stem alleviates insulin resistance, oxidative injury and fatty liver disease in rats fed high fat plus fructose diet. Food Chem. Toxicol., 48: 2021-2029.

41. Al-Okbi, S.Y., D.A. Mohamed, T.E. Hamed and R.S.H. Esmail, 2014. Rice bran oil and pumpkin seed oil alleviate oxidative injury and fatty liver in rats fed high fructose diet. Pol. J. Food Nutr. Sci., 64: 127-133.

42. Dooley, S. and P. ten Dijke, 2012. TGF-β in progression of liver disease. Cell Tissue Res., 347: 245-256.

43. Qin, G., G.Z. Wang, D.D. Guo, R.X. Bai, M. Wang and S.Y. Du, 2018. Deletion of *Smad4* reduces hepatic inflammation and fibrogenesis during nonalcoholic steatohepatitis progression. J. Digest. Dis., 19: 301-313.

44. Hu, Y., Z. Hou, R. Yi, Z. Wang and P. Sun *et al.*, 2017. Tartary buckwheat flavonoids ameliorate high fructose-induced insulin resistance and oxidative stress associated with the insulin signaling and Nrf2/HO-1 pathways in mice. Food Funct., 8: 2803-2816.

45. Hosaka, T., S. Sasaga, Y. Yamasaka, Y. Nii and K. Edazawa et al., 2014. Treatment with buckwheat bran extract prevents the elevation of serum triglyceride levels and fatty liver in KK-A (y) mice. J. Med. Invest., 61: 345-352.

46. Durendic-Brenesel, M., T. Popovic, V. Pilija, A. Arsic and M. Milic *et al.*, 2013. Hypolipidemic and antioxidant effects of buckwheat leaf and flower mixture in hyperlipidemic rats. Bosn J. Basic Med. Sci., 13: 100-108.

47. Biernacka, A., M. Dobaczewski and N.G. Frangogiannis, 2011. TGF-β signaling in fibrosis. Growth Factors, 29: 196-202.

48. Yoshida, K., M. Murata, T. Yamaguchi and K. Matsuzaki, 2014. TGF-β/Smad signaling during hepatic fibro-carcinogenesis (Review). Int. J. Oncol., 45: 1363-1371.

49. Heim, K.E., A.R. Tagliaferro and D.J. Bobilya, 2002. Flavonoid antioxidants: Chemistry, metabolism and structure-activity relationships. J. Nutr. Biochem., 13: 572-584.

50. Holasova, M., V. Fiedlerova, H. Smrcinova, M. Orsak, J. Lachman and S. Vavreinova, 2002. Buckwheat-the source of antioxidant activity in functional food. Food Res. Int., 35: 207-211.

51. Valenzuela, A., J. Sanhueza, P. Alonso, A. Corbari and S. Nieto, 2004. Inhibitory action of conventional food-grade natural antioxidants and of natural antioxidants of new development on the thermal-induced oxidation of cholesterol. Int. J. Food Sci. Nutr., 55: 155-162.