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In-vivo studies on supplementation of multigrain flour mixes on plasma lipid profile

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Abstract
A healthy food is well balanced with respect to quality and quantity of ingredients from different food groups and not just concentrating on one food group, giving rise to a concept of multigrain foods. The present work on quality evaluation of multigrain flour mix was carried to standardize a simple, nutritional blends by utilizing wheat, foxtail millet, maize, Bengal gram and pumpkin seed with the objectives to develop a multigrain flour mix and study its hypolipidemic effect in Wister rats. Five blends were made with whole wheat, foxtail millet, maize, Bengal gram and pumpkin seed in different proportions. Plasma HDL level was significantly increased and plasma LDL level significantly decreased when compared with the reference group. The supplementation of multigrain mixes decreased the plasma triglyceride level. Therefore, the developed formulations can be utilized for development of such functional foods that are beneficial to improve the lipid profile and general health status of populations.

Keywords: Multigrain, wistar rats, HDL, LDL, cholesterol, triglyceride

Introduction
A healthy food is well balanced with respect to quality and quantity of ingredients from different food groups and not just concentrating on one food group, giving rise to a concept of multigrain foods (Shinde, 2017) [18]. Health effects of diets rich in whole grains and cereal fibres are well known and are associated with decrease risk of diabetes, myocardial infarction and certain cancers (Banu *et al.*, 2010) [1] due to the presence of phytochemicals, phenol compounds, carotenoids, vitamins, lignans, β glucan, inulin, resistant starch, sterols and phytate (Rui, 2007) [17]. In addition to whole grain benefits, multigrain blend concept helps to mix different whole grains to maximize their nutritious, functional and sensory properties (Mandge *et al.*, 2014) [14].

Wheat is considered good source of protein, minerals, B-group vitamins and dietary fibre *i.e*, an excellent health- building food. Wheat is a good source of thiamine and nicotinic acid (Kumar *et al.*, 2011) [10]. The maize kernel is high in fat (33.3%) in addition to enzymes, vitamin B complex, antioxidants such as vitamin E and polyunsaturated fatty acids (54.7%) (Gwirtz and Casal, 2014) [9]. Millets are unique among the areas because of their richness in calcium, dietary fibre, polyphenols and protein (Devi *et al.*, 2011) [6]. Foxtail millet (*Setaria italica*) is tasty, with a mildly sweet, nut-like flavor and contains a myriad of beneficial nutrients. This millet contains 12.3 percent crude protein and 63-64 percent carbohydrate 3.3 percent minerals (Gopalan *et al.*, 1991) [8]. It is considered to be one of the least allergenic and most digestible grain (Prashant *et al.*, 2005; Xue *et al.*, 2008) [16, 24]. Bengal gram also called chickpea or gram (*Cicer arietinum*), is a major pulse crop in India and accounts for nearly 40% of the total pulse production (FAO/WHO, 2008) [7]. Pulse is an important food crop due to the high protein and essential amino acid content. Pulses are 20-25% protein by weight (Slavin, 2004). Oilseeds have long been used extensively as they are excellent source of protein (25.2-37%), vitamins and oil (37.8-45.4%) (Barbara and Murkovic, 2004) [3] especially omega-6 fatty acids which have a number of biological application along with significant antioxidant activity in addition to anti-inflammatory and hypolipidemic activities (Suresh and Das, 2003) [21]. Pumpkin seeds contain about 40% of fat rich in unsaturated fatty acids (FA), lecithin and antioxidants as well as in cellulose and minerals particularly iron (Makni *et al.*, 2010) [13]. Therefore an attempt has been made to formulate multigrain flour mixes by utilizing cereals, pulses, millets and oil seeds with the objective to study the *in-vivo* supplementation of multigrain flour mixes on plasma lipid profile.

Methodology

The ingredients for the development of multigrain flour mix were selected according to the guidelines given by ICMR for multigrain flours. Wheat grains (PBW621) were procured from Punjab Agricultural University, Ludhiana. Foxtail millets were procured from RARS, Gossaigaon. Bengal gram flour was bought from local markets of Jorhat. Pumpkin seeds were collected from local households and college hostels of Assam Agricultural University, Jorhat. All the ingredients were processed into flour according to standard methods and kept separately in airtight containers at refrigerated temperatures.

Four different variations of multigrain flour mixes were developed using flours of whole wheat, foxtail millet, maize, Bengal gram and pumpkin seed at different level of incorporation and whole wheat (100%) as control (Mix 1). Mix 2 was developed using whole wheat and foxtail millet (90:10), Mix 3 with whole wheat, foxtail millet and maize in the ratio 80:10:10, whereas Mix 4 was developed using whole wheat, foxtail millet, maize and Bengal gram (70:10:10:10) and Mix 5 with whole wheat, foxtail millet, maize flour, Bengal gram flour and pumpkin seed flour (60:10:10:10:10) respectively.

***In vivo* studies using animal model on plasma lipid profile**

Considering the limitation of animals for the study to be conducted on all the five treatments, the three formulations were selected for carrying out the *in vivo* study for further quality parameter assessment of the multigrain flour mixes on Wister strain albino rats procured from Kolkata. Animals were housed in polypropylene cages in small groups of 6 rats per cage. Animals had free access to standard balanced ration and drinking water *ad-libitum* and were maintained in standard laboratory conditions (12:12 hour light/dark cycle at ambient temperature ranging between 12-25 °C). The use of experimental animals and the study protocol was duly approved by the Institutional Animal Ethics Committee (IAEC) of the college.

Experimental design

Thirty healthy animals were selected and divided into five groups of 6 animals each. Group I served as normal control while in Groups II, III, IV and V hyperlipidemia was induced by allowing animals to feed high fat and carbohydrate diet for 21 days. Following the induction of hyperlipidemia, Group I received normal diet, Group II received high fat diet, Group III received Mix 3, Group IV received Mix 4 and Group V received Mix 5 (Table 1). For inducing hyperlipidemic action, commercial coconut oil was utilized and lipid profiles of the animals were monitored after introduction of coconut oil containing diet for a period of 21 days.

Table 1: Proportion and composition of diets given to the experimental animals

Groups	No. of animals	Diet
I	6	Normal controls (Negative control): 100% RR
II	6	Positive control (HFD): 80% RR + 20% CO
III	6	40% RR + 40% Mix 3 + 20% CO
IV	6	40% RR + 40% Mix 4 + 20% CO
V	6	40% RR + 40% Mix 5 + 20% CO

RR - Rat ration
HFD - High fat diet
MIX 3 - Whole wheat flour (80%): foxtail millet flour (10%): maize flour (10%)
MIX 4 - Whole wheat flour (70%): foxtail millet flour (10%): maize flour (10%): Bengal gram flour (10%)
MIX 5 - Whole wheat flour (60%): foxtail millet flour: maize flour (10%): Bengal gram flour (10%): Pumpkin seed flour (10%)
CO - Coconut oil

Estimation of lipid profile

The blood of the animals were collected by retro-orbital puncture on 0^{th}, 7^{th}, 14^{th} and 21^{st} day of the experimentation and centrifuged at 3000 rpm for about 15 min. Serum was then separated and high density lipoprotein (HDL), low density lipoprotein (LDL), total cholesterol and total triglyceride levels were estimated with commercially available HDL-D cholesterol kit, LDL-D cholesterol kit, cholesterol kit and triglyceride kit (Tulip Diagnostic Pvt. Ltd).

Statistical analysis

All the data of the experiment were statistically analysed by using mean, standard deviation and CRD in SPSS software.

Results and Discussion

Supplementation of high fat diet (HFD) with 20 percent coconut oil were used to induce hyperlipidemia (Group II) and were compared with incorporation of different multigrain flours fed to the different groups *i.e.,* Group III, Group IV and Group V.

Impact of supplementation of multigrain flour mixes on plasma High Density Lipoprotein (HDL) level (mg/dl) in Wister rats

The impact of supplementation of multigrain flour mixes on plasma HDL level (mg/dl) in rats is presented in Table2 and 3.

Table 2: Impact of supplementation of multigrain flour mixes on plasma HDL level (mg/dl) in Wister rats

Experimental groups	Effects of feeding of multigrain flour mixes on HDL level (mg/dl)			
Days	0 days	7days	14 days	21 days
Group I (Normal diet)	58±0.85^{bc}	60±0.73^{c}	58±0.93^{cd}	59±0.77^{d}
Group II (HFD)	55±0.57^{d}	50±0.57^{e}	47±0.57^{e}	46±0.73^{e}
Group III (Mix 3)	50±0.77^{e}	54±0.73^{d}	57±0.85^{c}	61±0.73^{c}
Group IV (Mix 4)	59±0.57^{a}	64±0.57^{b}	66±0.57^{b}	72±0.57^{b}
Group V (Mix 5)	58±0.57^{b}	66±0.89^{a}	72±0.81^{a}	75±1.03^{a}
		F- value	CD at 5%	
For factor time		126.20**	1.30	
For factor treatment		33.09**	1.82	
For time*treatment		12.19**	3.63	

Values are expressed in Mean±SEM (n=6) **Significant at $p \leq 0.05$
Means followed by the same letter shown in superscript(s) are not significantly different

Table 3: Mean increment in plasma HDL level (mg/dl) in supplementation of multigrain flour mixes in comparison to HFD control in Wister rats

Days	Mean increase or decrease of plasma HDL level after feeding of multigrain flour mixes				
	Group I	Group II	Group III	Group IV	Group V
7 days	+2	-5	+4	+5	+8
14days	+0	-8	+7	+7	+14
21 days	+1	-9	+11	+13	+17

The mean increment in the HDL level from the supplementation of multigrain flour mixes [Table 3] implied that as the days progressed from 7 to 21 days the incorporation of the developed multigrain mixes in the diet, gradually increased the HDL level. However, the highest increase was observed in Group V followed by Group IV Group III. Similar results were also reported by Makni *et al.*, (2010) [13] which showed hypotriglyceremic and hypocholesterolemic effect on rats fed with seed mixture was rich in PUFAs. Park *et al.*, (2008) [15] also reported that the protein present in Korean foxtail millet significantly elevated plasma HDL cholesterol levels in mice. Similarly, Thatola *et al.*, (2011) [22] found that the serum HDL cholesterol level increased significantly because of higher proportion of fibre in the feed.

Impact of supplementation of multigrain flour mixes on plasma Low Density Lipoprotein (LDL) level (mg/dl) in Wister rats

Data pertaining to the plasma LDL level (mg/dl) in experimental rats are presented in Table 4 and Table 5. The experimental groups fed on HFD with Mix 5 (Group V), significant decline in the plasma LDL cholesterol level was observed in the experimental group from baseline to 28 days of intervention in comparison to HFD control group.

Table 4: Impact of supplementation of multigrain flour mixes on plasma LDL level (mg/dl) in rats

Experimental groups	Effects of feeding of multigrain flour mixes on LDL level (mg/dl)			
Days	0 days	7days	14 days	21 days
Group I (Normal diet)	85±0.57^{a}	90±0.68^{ab}	93±0.44^{b}	94±0.68^{b}
Group II (HFD)	84±1.06^{bc}	91±0.57^{a}	95±0.81^{a}	109±0.68^{a}
Group III (Mix 3)	83±0.77^{cd}	87±0.57^{c}	89±0.25^{c}	91±0.57^{c}
Group IV (Mix 4)	84±0.57^{b}	88±0.57^{b}	88±0.36^{d}	90±0.44^{cd}
Group V (Mix 5)	82±0.51^{c}	83±0.36^{d}	85±0.57^{c}	87±0.36^{c}
		F- value	CD at 5%	
For factor time		112.04**	1.84	
For factor treatment		1099.80**	2.06	
For time*treatment		13.80**	4.10	

Values are expressed in Mean±SEM (n=6) **Significant at $p \leq 0.05$
Means followed by the same letter shown in superscript(s) are not significantly different

Table 5: Mean increment in plasma LDL level (mg/dl) in supplementation of multigrain flour mixes in comparison to HFD control in Wister rats

Days	Mean increase or decrease of plasma LDL level after feeding of multigrain flour mixes				
	Group I	Group II	Group III	Group IV	Group V
7 days	+5	+7	+4	+3	+1
14days	+8	+11	+6	+4	+3
21 days	+9	+25	+8	+6	+5

Gradual increase of LDL level in all the experimental groups (Groups III, IV and V), indicated that all the test diet treated groups had lesser extent of increase as compared with the HFD group which could be attributed to the beneficial effects of multigrain mixes. Hypocholesterolemic potential of multigrain mixes observed in the present study might also be due to the presence of bioactive phytochemicals in the mixes. Chandrasekara and Shahidi (2012) [4] reported that the

phenolic content millet was higher that some major cereals and are distributed mostly in the outer layer of the grain and are liable to losses during the separation of seed coat upon dehulling. Therefore, in the present study it was shown that Mix 5 which is concentrated with fibre along with the seed coats of pumpkin seeds possessed more prominent improvement in the plasma LDL-level as compared to other two mixes. Earlier studies has shown that whole grains have antioxidant capacity due to their redox properties which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers and thus remove LDL lipid oxidation (Wolfe and Lui, 2007; Soobratte *et al.*, 2008) [23, 20].

Impact of supplementation of multigrain flour mixes on plasma total cholesterol level (mg/dl) in Wister rats:

It is evident from the study that supplementation of HFD with multigrain mixes significantly ($p \leq 0.05$) reduced the plasma total cholesterol level from baseline to 21 days of intervention in comparison to the group fed with HFD [Table 6 and 7].

Table 6: Impact of supplementation of multigrain flour mixes on plasma cholesterol level (mg/dl) in Wister rats

Experimental groups	Effects of feeding of multigrain flour mixes on total cholesterol level (mg/dl)			
Days	**0 days**	**7days**	**14 days**	**21 days**
Group I (Normal diet)	130±0.57^{c}	133±0.60^{d}	131±1.17^{c}	132±0.95^{c}
Group II (HFD)	134±0.44$^{a\,b}$	141±0.85^{a}	154±0.57^{a}	161±0.57^{a}
Group III (Mix 3)	132±0.57^{c}	135±0.93^{c}	139±1.06^{b}	140±0.51^{c}
Group IV (Mix 4)	134±1.06^{a}	136±0.89^{b}	139±0.36$^{b\,c}$	141±0.81^{b}
Group V (Mix 5)	131±0.57^{d}	132±0.93$^{d\,e}$	134±0.89^{d}	135±0.68^{d}
		F- value	CD at 5%	
For factor time		98.70**	1.98	
For factor treatment		2950.67**	2.22	
For time*treatment		10.59**	4.43	

Values are expressed in Mean±SEM (n=6) **Significant at $p \leq 0.05$ Means followed by the same letter shown in superscript (s) are not significantly different

Table 7: Mean increment in plasma total cholesterol level (mg/dl) in supplementation of multigrain flour mixes in comparison to HFD control in Wister rats

Days	Mean increase or decrease of plasma cholesterol level after feeding of multigrain flour mixes				
	Group I	**Group II**	**Group III**	**Group IV**	**Group V**
7 days	+3	+7	+3	+2	+1
14 days	+1	+20	+7	+5	+3
21 days	+2	+27	+8	+7	+4

The decrease in plasma total cholesterol could be due to the presence of beneficial fibres and phytochemicals with potent antioxidant activity (Chethan *et al.*, 2008) [5]. Barakat and Mahmoud (2011) [2] in their study showed that the decline in hepatic cholesterol levels in flaxseed and pumpkin seed fed hypercholesterolemic group indicated the possible influence of relatively higher fibre content of seed mixture. The unsaturated fatty acids present in seed mixture which could have played a crucial role in reducing blood cholesterol in human and rats. In addition, fibres are known to interfere with cholesterol absorption and enterohepatic bile circulation and resulted in depletion of hepatic cholesterol pools. The low total cholesterol content in the livers of rats fed with high fibre diet could be attributed to reduce synthesis of cholesterol (Ylitalo *et al.*, 2002) [25].

Impact of supplementation of multigrain flour mixes plasma triglyceride level (mg/dl) in Wister rats

The present study [Table8 and 9] showed that the supplementation of HFD in Group III, Group IV and Group V significantly $p \leq 0.05$ decrease the plasma triglyceride level from baseline to 21 days of intervention in comparison to reference group (Group II). The positive alteration in the triglyceride level could be attributed due to the presence of high fibre and protein in the developed mixes.

Table 8: Impact of supplementation of multigrain flour mixes on plasma triglyceride level (mg/dl) in Wister rats

Experimental groups	Effects of feeding of multigrain flour mixes on Triglyceride level (mg/dl)			
Days	**0 days**	**7days**	**14 days**	**21 days**
Group I (Normal diet)	75±1.23^{ab}	73±0.77^{c}	70±1.02^{d}	74±0.68^{b}
Group II (HFD)	72±1.06^{d}	76±0.68^{a}	78±0.51^{a}	81±0.57^{a}
Group III (Mix 3)	68±1.48^{e}	75±0.93^{b}	73±0.81^{b}	70±0.81^{c}
Group IV (Mix 4)	75±1.06$^{b\,c}$	73±0.68^{cd}	71±0.81^{c}	67±0.57^{d}
Group V (Mix 5)	77±0.93^{a}	72±0.57^{e}	69±1.39^{e}	68±1.06^{e}
		F- value	CD at 5%	
For factor time		531.59**	2.06	
For factor treatment		18.30**	2.32	
For time*treatment		4.72**	4.64	

Values are expressed in Mean±SEM (n=6) **Significant at $p \leq 0.05$ Means followed by the same letter shown in superscript (s) are not significantly different

Table 9: Mean increment in plasma triglyceride level (mg/dl) in supplementation of multigrain flour mixes in comparison to HFD control in Wister rats

Days	Mean increase or decrease of plasma triglyceride level after feeding of multigrain flour mixes				
	Group I	Group II	Group III	Group IV	Group V
7 days	-2	+4	-3	-2	-5
14 days	-5	+6	-5	-4	-8
21 days	-4	+9	-8	-7	-9

Thatola *et al.*, (2011) [22] found that the supplementation of foxtail millet based product (biscuits) exhibited slight but non-significant decrease in the triglyceride levels. Another study by Lee *et al.*, (2010) [11] suggested that foxtail millet grain feeding decreased the triglycerides level and reduced C-reactive proteins in hyperlipidemic rats which contributed in lowering the risk for cardio-vascular diseases. Luo *et al.*, (2017) [12] revealed that rats when supplemented with high fibre diet exhibited decreased level of total cholesterol (31.53%), triglyceride (21.35%) and low density lipoprotein (31.53%), respectively while the high density lipoprotein increased by 37.60 percent when compared with the normal rats.

Conclusion

The study focussed on use of underutilized nutritious ingredients such as maize, foxtail millet and pumpkin seeds showed hypolipidemic effects in Mix 3, Mix 4 and highest in Mix 5. These mixes can be utilised to supplement vulnerable populations at a low cost. Considering the increase prevalence of non-communicable diseases, these mixes having potential health benefits for which human studies could also be done.

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