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In-vivo studies featuring antidiabetic studies using millet-fortified functional snack bars as the delivery vehicle

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Abstract
Diabetes mellitus is a world level problem, with India being the diabetes capital of the world. It has many associated complications such as kidney failure, heart attack, limb amputations etc. In such circumstances, turning towards functional foods can prove to be a very intelligent cure since it can help us avoid all the allopathic complications and side effects. In this context, millet-fortified functional snack bars have been developed, both for commercial purpose and *in vivo* study. Present paper focuses on *in vivo* designed snack bars, which contains the goodness of millets, apple pomace powder and camel milk powder.

Keywords: Diabetes mellitus, functional snack bar, millets, apple pomace, camel milk, *in vivo*

Introduction
Diabetes mellitus is a growing global concern as it is affecting around 422 million people every year around the world [1]. Its associated complications are kidney and heart related problems, leg amputations, vision loss [2]. In order to deal with this problem, functional snack bar has been developed in the present study, wherein camel milk powder, apple pomace powder and millet mixture has been added as the major ingredients [3]. In this context, *In-vivo* studies have been conducted in order to validate the studies [4].

Materials and Methods
Apple pomace powder, pasteurized and unpasteurized camel milk powder, millet mixture (Ragi, bajra, kodo, kutki), puffed wheat, puffed rice, jaggery powder and maltodextrin were procured from amazon.
Please look at the figures below in order to refer to the images of the prepared snack bars.

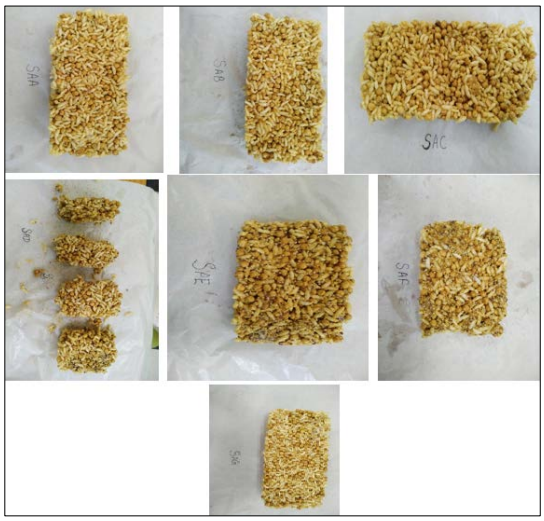

Fig 1: Snack bars (SAA-SAG) prepared especially for conducting *In-vivo* studies

Snack bars were prepared using the equipments available in the nutrition research facility lab. Firstly, the dry ingredients, such as puffed wheat and puffed rice, were roasted. Then, the millet mixture (Ragi, bajra, kodo and kutki in the ratio 1:1:1:1) was roasted using kadhaai on a very sim flame for 3-4 minutes. Then, the bars were bound using jaggery powder and maltodextrin as the binding agents and were given shape using bakery molds. They were wrapped in butter paper having a very thin glace of refined oil and kept in refrigerator for an hour or so.

Procedure

***In vivo* trials**

In phase II, *In-vivo* trials were conducted which consisted of animal trials in which wistar strain rats were used to examine the anti obesetic and antidiabetic reactions of functional snack bars.

Experimental Animals

Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS), Hisar, India had an infection ridden tiny animal house from where adult male wistar strain rats (4 weeks) (n=120) of weight 187.2±1.81 g were obtained. These rats were subjected to rearing according to the CPCSEA guidelines. Considering it as a segment of ethical agreement, the animal study was accepted by IAEC of NIFTEM vide no. 1/N/IAEC/16.

The rats were made accustomed to the new surroundings for a period of 15 days during which they were provided with food and water as and when necessary prior to the beginning of actual trials. Persistent climatic circumstances comprising of 12 hours of light and dark cycle, relative humidity of 60 ± 5% and a temperature of 22 ± 1*C were maintained inside each polycarbonate cage in which rats were accommodated. These polycarbonate cages had a sanitized rice husk bedding wherein microorganism free environment was maintained.

First of all, animals with almost equal weights were randomized into segments in such a way that there were six animals in each segment/ category. Finally, there were two major categories of rats: one was the control group (n=6) and the others were collectively named as the treatment groups (n=114). Obesic (n=54) and diabetic (n=60) were the two categories in which the treatment groups were further divided based on nutritive and medicinal means respectively. The primary diet given to animals was that of standard pellet chow diet obtained from Aashirwaad Industries (Hindustan Lever Ltd.) India. The chow diet constituted of 20% protein, 5% fat, 5% fiber, 60% carbohydrates and 10% mineral as well as vitamin mix. This diet schedule was adhered to for a time frame of 9 weeks. In the complete study duration, animals were checked upon two times a day i.e. once in the morning at 10.00 and once in the evening at 7. A track record of the animals' body weight was maintained on a weekly basis. Based on the amount of food consumed and residue left on a day-to-day basis, the food efficiency ratio (FER) was calculated according to the following formula:

Calculation

Food Efficiency Ratio (FER) = (Increase in Body Weight (g/d) / Food consumed (g/d))

Table 1: Description of dietary groups of diet induced obese rats

S. No.	Dietary Groups	Description
1	Control	Normal diet
2	CD	Cafeteria diet simulated to high fat diet
3	CD + ATV	Cafeteria diet plus Atorvastatin (10 mg/kg body weight)
4	CD + SAA	Cafeteria diet plus SAA bar (46g puffed rice + 35g jaggery + 15g maltodextrin + 8g apple pomace powder + 46g puffed wheat)
5	CD + SAB	Cafeteria diet plus SAB bar (35g puffed rice + 35g jaggery + 15g maltodextrin + 30g camel milk powder (pasteurized) + 35g puffed wheat)
6	CD + SAC	Cafeteria diet plus SAC bar (35g puffed rice + 35g jaggery + 15g maltodextrin + 30g camel milk powder (unpasteurized) + 35g puffed wheat)
7.	CD + SAD	Cafeteria diet plus SAD bar (35g puffed rice + 35g jaggery + 15g maltodextrin + 30g millet mixture + 35g puffed wheat)
8.	CD + SAE	Cafeteria diet plus SAE bar (16g puffed rice + 35g jaggery + 15g maltodextrin + 30g millet mixture + 8g apple pomace powder + 30g camel milk powder (pasteurized) + 16g puffed wheat
9.	CD + SAF	Cafeteria diet plus SAF bar (16g puffed rice + 35g jaggery + 15g maltodextrin + 30g millet mixture + 8g apple pomace powder + 30g camel milk powder (unpasteurized) + 16g puffed wheat
10.	CD + SAG	Cafeteria diet plus SAG bar (50g puffed rice + 35g jaggery + 15g maltodextrin + 50g puffed wheat)

Table 2: Description of dietary groups of diet and STZ induced diabetic rats

S. No.	Dietary Groups	Description
1.	Control	Normal diet (common for DIO and STZ rats)
2.	Diabetic control (DC)	Normal diet plus Nicotinamide – Streptozotocin (NA – STZ) NA – 230 mg/kg body weight STZ – 45 mg/kg body weight
3.	D + CD	Diabetic rats given cafeteria diet
4.	D + CD + GLM	Diabetic rats given cafeteria diet plus glibenclamide (5mg/kg body weight)
5.	D + CD + SAA	Diabetic rats given cafeteria diet plus SAA bar (46g puffed rice + 35g jaggery + 15g maltodextrin + 8g apple pomace powder + 46g puffed wheat)
6.	D + CD + SAB	Diabetic rats given cafeteria diet plus SAB bar (35g puffed rice + 35g jaggery + 15g maltodextrin + 30g camel milk powder (pasteurized) + 35g puffed wheat
7.	D + CD + SAC	Diabetic rats given cafeteria diet plus SAC bar (35g puffed rice + 35g jaggery + 15g maltodextrin + 30g camel milk powder (unpasteurized) + 35g puffed wheat)
8.	D + CD + SAD	Diabetic rats given cafeteria diet plus SAD bar (35g puffed rice + 35g jaggery + 15g maltodextrin + 30g millet mixture + 35g puffed wheat)

9.	D + CD + SAE	Diabetic rats given cafeteria diet plus SAE bar (16g puffed rice + 35g jaggery + 15g maltodextrin + 30g millet mixture + 8g apple pomace powder + 30g camel milk powder (pasteurized) + 16g puffed wheat
10.	D + CD + SAF	Diabetic rats given cafeteria diet plus SAF bar (16g puffed rice + 35g jaggery + 15g maltodextrin + 30g millet mixture + 8g apple pomace powder + 30g camel milk powder (unpasteurized) + 16g puffed wheat
11.	D + CD + SAG	Diabetic rats given cafeteria diet plus SAG bar (50g puffed rice + 35g jaggery + 15g maltodextrin + 50g puffed wheat)

Experimental Design: Two groups of animals were involved in the framework of experimental design. The first one was treated with diet induced obesity (DIO) while the second group was treated with diabetes. Moreover, the first group was further subdivided into nine categories whereas the second group was subdivided into ten categories. The following figure (Figure 3.2) and the above tables 3.1 and 3.2 respectively highlight this information.

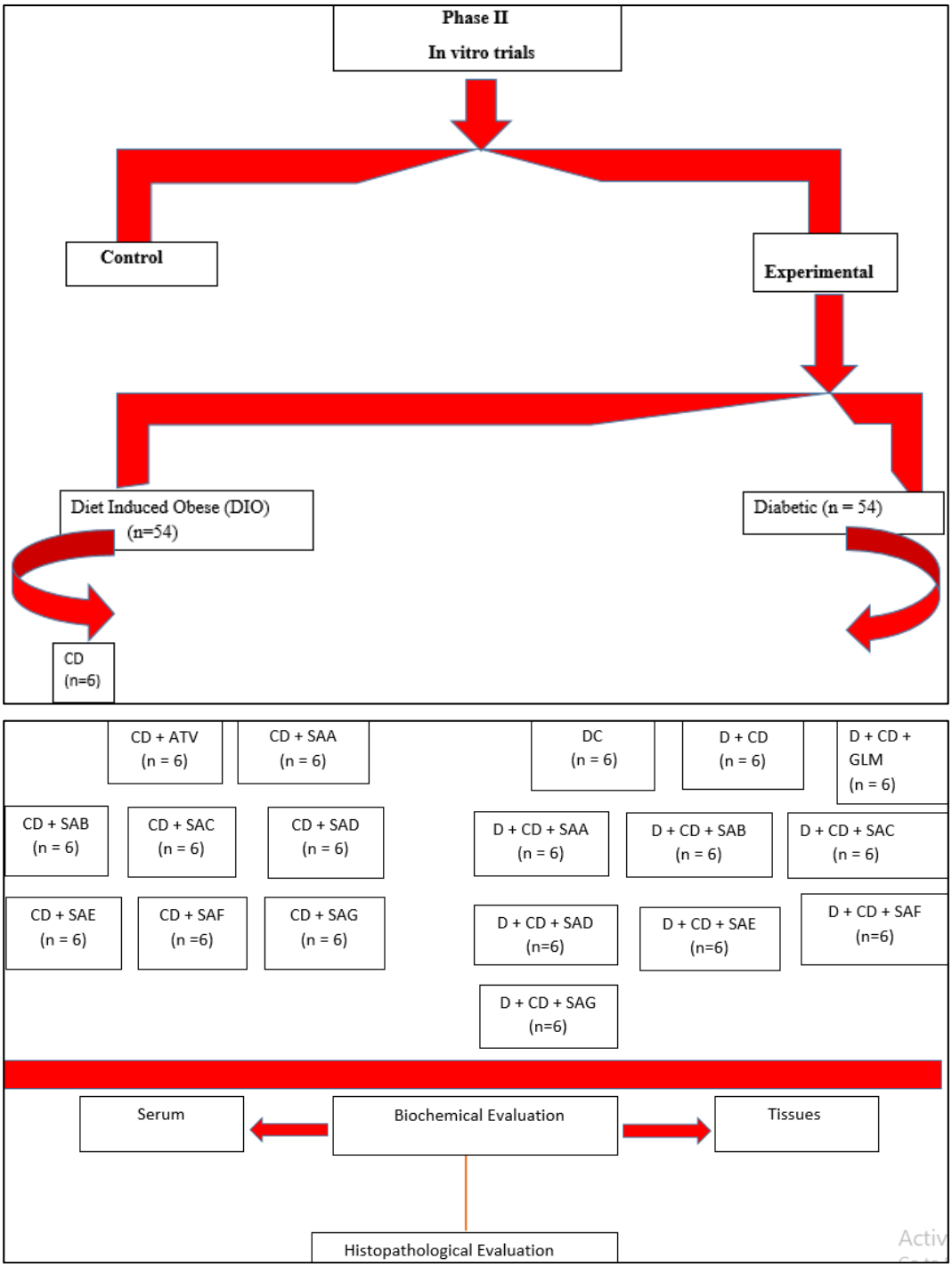

Fig 2: Schematic representation of Experimental design

Exposure to obesity

One set of animals (n=54) were treated for diet induced obesity (DIO) which resembled a lot to cafeteria diets. The cafeteria diets employed for introduction of obesity into the animals was prepared two times in a week and comprised of saturated fat (15%) as well as cholesterol (0.5%).

Exposure to diabetes

Diabetes was introduced into the body of the rats (n =60) by injecting nicotinamide (NA) at a dosage value of 230 mg/kg body weight intravenously and then, streptozotocin (STZ) was injected at a dosage value of 45 mg/kg body weight intravenously with a gap of 15 minutes between the two injections.

Chemical agents required

- **Nicotinamide (NA):** 230 mg/kg body weight; formed through solution preparation with the help of mineral water
- **Streptozotocin (STZ):** 45 mg/kg body weight; newly harvested by making a solution of STZ with the help of 0.1 M glacial citrate buffer having a pH of 4.5
- **Citrate Buffer (0.1 M, pH 4.5):** solution is prepared by mixing 2.94g of sodium citrate in mineral water and the pH was brought to 4.5 by employing 0.1M citric acid. The total volume was brought to 100ml with the help of mineral water.

Method

- The two chemical agents i.e. Nicotinamide (NA) and Streptozotocin (STZ) solutions were newly formed and treated using 20µm syringe filter in order to properly sterilize it.
- In order to eliminate the risk of any trauma or confusion due to sudden inception of insulin or onset of condition such as STZ triggered low glucose level, the animals were given customary chow pellets combined with 5% glucose solution as and when necessary for a time period of three uninterrupted days after the STZ administration.
- The rats were minutely observed for any irregularities in their behavior or any indications or gestures which point towards discomfit, anguishness or death.
- Glucometer based on one press select easy blood glucose detecting technique was employed for ascertaining the fasting blood glucose level of animals.
- The ones which were having their blood glucose level above 200 mg/dl were classified as diabetic and then they were arbitrarily categorized into different sets depending upon almost identical blood glucose values and body weights.

Experimental diets

The food which was provided to animals under experimentation at the time of following the whole dietary schedule (as outlined in the table below) was made ready two times in a week and preserved in refrigerated condition i.e. at 4*C.

Table 3: Constitution of diet provided under experimentation

S. No.	Ingredients (g/100g)	Control	CD	CD + SAA	CD + SAB	CD + SAC	CD + SAD	CD + SAE	CD + SAF	CD + SAG
1.	Crude Protein	20	20	20	20	20	20	20	20	20
2.	Fat	5	15	15	15	15	15	15	15	15
3.	Crude Fiber	5	18.5	13.5	13.5	13.5	13.5	13.5	13.5	13.5
4.	Carbohydrates	60	30	30	30	30	30	30	30	30
5.	Starch	-	5	5	5	5	5	5	5	5
6.	Sucrose	-	2	2	2	2	2	2	2	2
7.	Ash	10	9	9	9	9	9	9	9	9
8.	Cholestrol	-	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
9.	Bile Acids	-	0.125	0.125	0.125	0.125	0.125	0.125	0.125	0.125
10.	*T. aesticum*	-	-	5	-	-	2.5	2.5	-	1.6
11.	*H. vulgare*	-	-	-	5	-	2.5	-	2.5	1.6
12.	*S. bicolor*	-	-	-	-	5	-	2.5	2.5	1.6

Below mentioned minerals constituted g/kg of mineral mixture

Anhydrous calcium carbonate (357.00g), potassium phosphate monobasic (196.00g), potassium citrate (70.78g), sodium chloride (74.00g), potassium sulphate (46.60g), magnesium oxide (24.00g), ferric citrate (6.06g), zinc carbonate (1.65g), magnesium carbonate (0.63g), cupric carbonate (0.30g), potassium iodate (0.01g), anhydrous sodium selenate (0.01g), ammonium paramolybdate (0.007g), sodium metasilicate (1.45g), chromium potassium sulphate (0.275g), lithium chloride (0.01g), boric acid (0.08g), sodium fluoride (0.06g), nickel carbonate (0.03g), ammonium vandate (0.006g) and powdered sucrose (221.02g).

Below mentioned minerals constituted g/kg of vitamin mixture:

Nicotinic acid (3.00 g), calcium pantothenate (1.60 g), pyridoxin – HCl (0.70 g), thiamin-HCl (0.60 g), riboflavin (0.60g), folic acid (0.20 g), D-Biotin (0.020 g), vitamin B_{12} (Cyanocobalamin) (0.1% in mannitol) (2.50g), vitamin E (All-rac-α-tocopheryl acetate) (500 IU/ g) (15.00 g), vitamin A (All-trans-retinyl palmitate) (500,000 IU/ g) (0.80g), vitamin D_3 (Cholecalciferol) (400,000 IU/g) (0.25g), vitamin K (Phylloquinone) (0.075g) and powdered sucrose (974.65g).

Both the vitamins i.e. vitamin E and vitamin A to be employed as a dried-up, jelly-like dispersion (Reeves *et al*., 1993).

The customary drugs used for the study i.e. atorvastatin (ATV) and glibenclamide (GLM) were fed to the animals through an oral gauge meter and intravenously respectively (section 3.3.1 b and c). This was followed by euthanization of rats by employing the method of cervical decapitation according to the CPCSEA and IAEC, NIFTEM rules and regulations. Thereafter, the tissues were separated from the animal's body and subjected to treatment as per the procedure referred to in segment 3.3.7.

Procurement of blood samples

The blood samples were procured at regular time intervals (after every 7 days) as highlighted in the procedural steps through the lateral tail vein with the help of a cannula. Once the blood samples were procured, blood glucose level was evaluated. In addition to this, blood sample was also taken through retro-orbital sampling (from retro-orbital plexus) and through coronary failure by employing gentle anaesthesia with the help of diethyl ether. This was followed

by ultimate renunciation of the rats in order to assess the activity of other biological molecules. The procured blood samples were preserved using pre-sterilized ampules with and without ethylenediaminetetraacetic acid (EDTA) (Plate 3.5).

Conclusion

Timely *In-vivo* studies should be conducted in order to meet the growing challenges of diabetes mellitus. Several hypoglycemic drugs are consumed by the human population so that the disease can be cured but intent is to shift towards functional foods. This way, side effects of drugs can be avoided and diseases can be treated using natural ingredients. Pathways should be opened to validate the efficacy of developed fortified products through animal studies, thus helping the society to move towards a better life.

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