# Effects of bread fortification with pomegranate peel powder on health status: Study design and protocol

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# Abstract

Introduction: The oxidative stress caused by the creation and breakdown of reactive oxygen species affects glucose tolerance, B-cell function, insulin resistance, and metabolites containing free fatty acids. Therefore, owing to their health benefits and ability to prevent oxidative stress, functional foods have received particular attention. This research aims to assess strategies used to ameliorate oxidative stress and inflammation in patients with type 2 diabetes (T2DM).

Methods and Analysis: A randomized controlled trial will be conducted on 90 patients with T2DM. Run-in courses will last for two weeks. The intervention and control groups will receive wheat bread with and without pomegranate peel powder, respectively. Several variables, including anthropometric data, fasting plasma glucose, hemoglobin A1C, lipid profile, insulin level, high-sensitivity C-reactive protein (hs-CRP), malondialdehyde (MDA), and mood state, will be measured at baseline and after three months of the intervention. Beta-cell function (HOMA-B) and insulin resistance (HOMA-IR) will also be assessed. Research findings will increase the scientific knowledge about the use of bread in health intervention targeting.

Ethics and dissemination: The protocol has been approved by the Ethics committee of the faculty of Medicine of the University of Isfahan (IR.MUI.RESEARCH.REC.1399.087). The study results will be disseminated through peer-reviewed publications and presentations at scientific meetings.

Trial Registration: Registered in the Iranian Registry of Clinical Trials (ID: IRCT2019209045672N1) on September 21, 2020.


# Introduction

Type 2 diabetes mellitus (T2DM) is a growing global health issue (1). The disease imposes high healthcare costs and deteriorates the patients' quality of life (2). The International Diabetes Federation estimates that there are currently 425 million diabetics worldwide and the number will increase to 629 million by 2045 (3). Diabetes and insulin resistance are associated with oxidative stress, a pathogenic mechanism (4), which elevates the risk of T2DM complications including nephropathy, neuropathy, retinopathy, and accelerated coronary artery disease (5).

T2DM is currently managed by lifestyle modification and increased physical inactivity. Drug therapy becomes necessary overtime to ensure good glycemic control (6). Medical nutrition therapy has been recommended as an effective method of controlling blood glucose levels in patients with T2DM without the side effects of medication (7). Considering the confirmed benefits of functional food components, these compounds should be a part of public health diet behaviors (8). Based on previous systematic reviews, pomegranate peel powder (PPP) consumption has a positive association with disease prevention (9). PPP possesses anti-inflammatory, hypoglycemic, anti-apoptotic, and prebiotic properties (10).

PPP consists of an internal network of membranes that makes up almost 30% of the total weight of the fruit. It contains not only fiber, but also various polyphenols such as ellagic acid, flavonoids, and hydrolysable tannins. Since bread is an essential part of the Iranian diet (11, 12), its fortification can play a significant role in reversing the nutritional deficiencies at the national level. Unlike previous studies that mainly assessed the effects of PPP on rheological indices (13), we will design a clinical trial in which PPP is added to wheat bread to determine the optimal percentage that would enhance the rheological, organoleptic, and antioxidants properties of this food staple. Moreover, a parallel-group randomized clinical trial will be designed to evaluate the effects of bread fortification on metabolic indices of patients with T2DM.

## Study aims

### General aim:

The current study aims to evaluate the quality and antioxidant properties of bread fortified with PPP and to compare the metabolic effects of the 12-week consumption of fortified and plain wheat breads in patients with T2DM.

### The specific objectives and hypotheses

#### Aim 1

To determine the glycemic effect of bread form (flat or bulk) by measuring circulating levels of pre- and post-prandial blood sugar.

#### Aim2

To determine the properties of PPP-fortified bread by sensory method and selecting the appropriate PPP percentage.

#### Aim 3

To determine whether PPP improves HbA1c and insulin resistance (IR). We hypothesize that PPP will improve fasting blood sugar (FBS), IR, and beta-cell function.

#### Aim4

To determine the number of polyphenols in bread fortified with PPP and wheat bread.

#### Aim 5

To analyze the antioxidant capacity of bread fortified with PPP and wheat bread using the 2, 2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) assay.

#### Aim 6

To determine the anthropometric indices in the intervention and control groups before and after the intervention. We hypothesize that PPP should reduce body mass index (BMI) and waist circumference.

### Aim 7

To determine if wheat bread with PPP enhances the lipid profile, i.e., triglyceride (TG), high-density lipoprotein-C (HDL-C), and direct low-density lipoprotein LDL-D, of patients with T2DM.

### Aim 8

To determine mean levels of malondialdehyde (MDA) in the intervention and control groups before and after the intervention. We hypothesize that PPP-fortified wheat bread will reduce serum MDA in these individuals.

### Aim 9

To determine the total antioxidant capacity (TAC) in the intervention and control groups before and after the intervention. According to our hypothesis, PPP-fortified wheat bread will improve TAC in individuals with T2DM.

### Aim 10

To determine high-sensitivity C-reactive protein (hs-CRP) levels in the intervention and control groups before and after the intervention.

### Aim 11

To determine whether PPP-fortified wheat bread improves mood indices (stress, anxiety, and depression). We hypothesize that PPP can improve mood indices.

# Materials And Methods

### Experimental protocol

The current research will be conducted in three phases:

Phase one: To explore the postprandial effects of five wheat bran bread products on individuals with T2DM.

Phase two: To evaluate the organoleptic characteristics of bread prepared with four doses of PPP.

Phase three: To determine the effects of consuming PPP-fortified bread on risk factors including obesity, oxidative stress, insulin resistance, inflammation factors, and lipid profile in type 2 diabetes patients.

### Sample preparation

Pomegranates of a specific variety will be collected and purchased from cultivation areas in Neyriz, Fars Province, southwest of Iran. They will be washed with cold water and drained. They will be then cut open and the leathery skin on the outside will be removed. After that, the pomegranate and white parts of the interior pomegranate will be separated. The skin will be ground using an electric mill and sieved through a #12 mesh sieve. The powder will be stored in closed containers in a -20 °C freezer until use.

### Organoleptic (sensory) tests

Different amounts of PPP will be added to wheat flour to obtain final percentages of 1.5%, 2.5%, 3.5%, and 5%. PPP-free samples will also be used as controls. The prepared traditional and bulk breads will be cut into equal-sized slices and served in unmarked dishes for 25 untrained panelists (university employees and students) to score their organoleptic properties on a five-point hedonic scale from one (extremely poor or lowest quality) to five (remarkably good or excellent quality). They will be ask to drink some water after testing each sample to cleanse their palate. We will also record and rate the internal and external characteristics of texture, color, aroma, taste, and mouth feel of the bread samples on a score sheet.

### Determination of total phenol

Folin-Ciocalteu (FC) colorimetric method will be employed to determine the total phenolic content (TPC) of methanolic extracts of bread samples. Results will be expressed in milligrams of Gallic acid equivalents per gram of bread (mg GAE/g) (10, 14).

### DPPH radical scavenging activity

DPPH will be used to assess the antioxidant activity of the extract. Using a spectrophotometer, the absorbance will be measured at 515 nm, and the percentage of DPPH scavenging activity will be calculated using the following equation (15):

Radical scavenging activity (%) = (Blank OD-sample OD/Blank OD) *100

The experiment will be performed three times and the average value will be reported. The amount of radical scavenging activity will then be calculated. Half-maximal inhibitory concentration (IC50) will be used to determine the substance's ability to neutralize 50% of the initial free radicals in the environment. Phenolic and DPPH tests will be conducted to determine the phenol content of bread after adding PPP.

### Setting

In the beginning, a list of outpatients with T2DM will be obtained from the Endocrine and Metabolism Research Center and various health centers in Isfahan, Iran. Study inclusion and exclusion criteria are in table 1.

Table 1: Eligibility criteria participation for the study

| **Inclusion criteria** |
| --- |
| Age between 40 and 60 years |
| Informed consent |
| HbA1c < 8 |
| Diagnosis of diabetes during the past five years |
| BMI < 30 kg/m² |
| Absence of complications |
| **Exclusion criteria** |
| Changes in diet or physical activity within the past six months |
| Current smoking and/or alcohol or drug abuse history |
| Pregnancy and lactation |
| Renal failure (stage 3-5) |
| Liver disease (cirrhosis and hepatitis) |
| Insulin treatment or sulfonylureas |
| Current involvement in a clinical trial |
| Recent weight change |
| Use of non-steroidal anti-inflammatory drugs |
| History of malignancy |
| Being on a special diet (e.g., vegetarian and ketogenic) |
| Weight loss over 10% in the past six months |

Volunteer patients will be invited to contact the research team by phone, attend an examination, and complete a brief questionnaire about their health history. The study protocol will be explained to the participants and they will be asked not to make any changes to their usual diet during the study. Groups 1 and 2 will receive wheat bread (100 g) named A and B, respectively. Both the researchers and the patients will be blinded to the randomization code of the intervention and placebo breads (A and B).

### Run-in period

The study will last for three months, beginning with a 14-day run-in period. The participants will be asked to maintain their dietary habits, medicines, and exercise. The run-in period will help the researchers to become familiar with the patients' diets and to identify their concerns. During the run-in period, the participants will be consuming regular wheat bread. The run-in phase is necessary to test the volunteers'

motivation to comply with the intervention. Since there is no biomarker to measure bread intake, we will use diaries to assess their compliance.

### Study design

The study will be conducted on 90 diabetic patients referred to Isfahan Endocrine and Metabolism Research Center and other health centers in the city. Patients will be given the reasons for the work and how the plan is implemented. A questionnaire containing demographic data, age, gender, telephone number, cell phone, address, and medical records will be used to evaluate the inclusion criteria. Individuals who wish to participate in the study will be asked to sign an informed consent form after the approval of the study. The participants will be assigned to either the intervention or control group (Figure 1) and both groups will continue their routine treatment plans. In addition to their routine diet, the participants in the intervention group will also have to eat 100 g regular bread every day. Variables will be assessed at baseline and at the end of the trial.

### Informed consent form process

Informed consent will be obtained from all participants. An approval from the Medical Ethics Committee of Isfahan University of Medical Sciences will also be needed. Before their enrollment, the patients will be assured that their unwillingness to participate will not affect their usual health care at health centers. The participants will have enough time to review the consent form and ask any questions they should have. They will also receive a copy of the consent document after signing it.

### Anthropometric Assessment

### Anthropometric parameters

We will measure height, weight, and waist and hip circumferences at baseline and three months post-intervention. Height and weight (with a precision of 0.01 kg) using a scale-mounted stadiometer while the patients wear light clothing and no shoes. Each participant’s BMI will be calculated as the result of dividing their weight (in kg) by their height (in m) squared. A flexible tape will be used to measure waist and hip circumference to the nearest 0.1 cm. Waist circumference will be measured at the midpoint between the lowest rib and the iliac crest while the patient’s body is level and they are wearing minimal clothing. Hip circumference will be measured around the widest portion of the buttocks. Only one person will do measurements to reduce error rates.

### Physical activity and dietary intake evaluation

At the beginning of the intervention and 12 weeks later, food and activity records will be assessed. For the purpose of dietary intake evaluation, the participants will be asked to record their daily food and beverage intake for three days. We will assess dietary intake using the food records of three nonconsecutive days (two weekdays and a weekend). A food scale and a model will also be used to improve accuracy. The three-day food record will be reviewed by the investigator in the presence of the patient. We will convert

the portion sizes to grams. Using Nutritionist 4 (First data bank Inc., Hearst Corp., San Bruno, CA), each food and beverage item will be coded and analyzed for energy content and macro- and micronutrients. Individuals consuming less than 1200 kcal/day or more than 4000 kcal/day will be excluded
The Participants will complete the Metabolic Equivalent Questionnaire (MET) for physical activity before and after the intervention and will also be asked to report their physical activity on a daily basis. They will receive bread packages twice a week during the 12-week intervention period. As there is no biomarker for assessing the consumption of PPP-fortified bread, compliance with the research protocol (i.e., daily intake of bread) will be evaluated using a self-reported questionnaire.

## Sample size calculation

The sample size was calculated by considering the first type error (α=0.05), the second type error (β=0.2), SD= 32 and 42, and the minimum significant difference for cholesterol (23mg/dl) as the main outcome variable.

The sample size for each group was 40 participants. Including a 10% chance of dropping out, 45 people were determined. Sampling is expected to last for 14 months. The participants' data will be recorded even if they withdraw from the program (16).

## Blood pressure

Blood pressure will be measured after 10 minutes of resting in a seated position using a mercury barometer calibrated in that position. The patients will be asked not to drink tea/coffee or engage in heavy physical activity for approximately 30 minutes before the measurement. At the beginning and end of the study, blood pressure measurements will be performed twice and the mean of two consecutive readings will be recorded.

## Biochemical assays

In order for laboratory tests, patients who meet the inclusion criteria (Table 1) will be asked to attend Isfahan Endocrine and Metabolism Research Center between 8:00 am and 10:00 am after fasting for 12 hours. During their appointment, 10 cc blood samples will be obtained. The collected samples will be centrifuged at 3500 rpm, 25 ℃ for 15 minutes. The serum will then be transferred and stored in a -80 ℃ freezer until further analysis. The participants will be tested for their biochemical parameters at baseline and after the intervention. Homeostatic model assessment of insulin resistance (HOMA-IR) and homeostatic model assessment of beta-cell function (HOMA-B) index will be used to determine insulin resistance (primary outcome) and beta-cell function, respectively. To determine insulin sensitivity, check index (Quick) will be measured based on the following equation:

HOMA-IR= (glucose-insulin)/405; Where glucose is fasting glucose (mg/dl) and insulin is fasting insulin (mu/ml) (17-19).

## Statistical analyses

SPSS software will be used for the statistical analysis of data. Data will be expressed as mean ± SD for continuous variables and frequency report for qualitative variables. Kolmogorov-Smirnov test will be used to determine the compliance of the data with the normal distribution. Paired samples t-test will be used to compare baseline data between and within the two study groups for normal data. Paired samples t-test will be used to compare baseline data between and within the two study groups for normal data and Manwitny test for non-normal data. The baseline demographics as well as clinical and diabetes-related Characteristics of the intervention and the control groups will be presented and compared. The average changes between baseline and 3 months in primary and secondary outcomes will be calculated for each of the groups. Intention-to-treat (ITT) analysis will be performed as the primary analysis on all primary and secondary outcomes after the last participant has ended participation. Missing values will be handled with the last observation carried forward approach for ITT analysis with the use of the multiple imputation approach in a sensitivity analysis.

Two-sided tests will be used. Also, in all analyzes, the value of P-value <0.05 will be considered statistically significant.

### ETHICS AND dissemination

Individuals provided written informed consent the study will be conducted in compliance with the Declaration of Helsinki. The trial has received ethical approval from the Ethics committee of Isfahan University of Medical Sciences (number:IR.MUI.RESEARCH.REC.1399.087). The study results will be disseminated through peer-reviewed publications and presentations at scientific meetings.

### Study outcomes

The primary outcome of this study is total Cholesterol. Secondary outcomes are insulin resistance and beta-cell function measured by the homeostasis model of assessments (HOMA) and quantitative insulin sensitivity check index (QUICKI). Further secondary outcomes include weight, waist circumference, blood pressure, lipid profile, hs-CRP, and oxidative stress biomarkers such as MDA and TAC.

## Discussion

Through our research, we will seek to determine the efficacy and safety of PPP for T2DM. The Iranian meal is incomplete without bread. In many countries, bread is economically accessible to everyone. Increasing consumer demand for functional foods opens up a massive market for this category.

### Strengths and limitations

▪ While previous studies have assessed the health effects and organoleptic characteristics of PPP, we will use this additive in a clinical trial.

▪ Appropriate measures will be adopted to determine the best percentage of PPP to improve the rheological, organoleptic, and antioxidant characteristics of wheat bread.

▪ Effects of bread intervention on diabetes profile, antioxidant capacity, inflammatory markers, and mood disorders in patients with T2DM will also be assessed.

▪ PPP-fortified bread will be applied in a trial to show whether it can reduce signs associated with diabetes, hyperlipidemia, and oxidative stress as a functional food.5) All participants will be obliged to maintain a stable pattern of physical activity and diet.

▪ Compliance could decline over the 12-week follow-up. We hypothesize that PPP consumption can improve markers of blood glucose and inflammation, reduce oxidative stress, and increase the quality of life.

# Declarations

### Ethics approval and consent to participate

All patients will agreed to participate in the study and signed informed consent forms. All experiments will performed in accordance with the compliance with the Declaration of Helsinkiand were approved by The Ethics Committee of Iran University of Medical Science confirmed the Study protocol (ID: IRCT2019209045672N1).

### Consent for publication

all authors read and approved this manuscript for publication.

### Competing interests

The authors declare that they have no competing interests.


### Funding resource

This study was financially supported by the nutrition faculty of Isfahan University of Medical


### Availability of data and materials

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.


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## Patient and Public Involvement

No patient or public involvement

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Contributors: Maryam Zare designed the initial idea of this study, statistical analysis plan, carrying out the trial, protocol manuscript writing and final revision.

Amir Hossein Goli was study design, statistical analysis plan, and final revision.

Mozhgan Karimifar was partially responsible for study design, carrying out the trial, manuscript writing and final revision.

Mohammad Javad Tarrahi was study design, protocol manuscript writing and Final revision.

Atefe Rezaeiwas partially responsible for study design, statistical analysis plan, carrying out the trial, protocol manuscript writing and final revision.

## Conflict of interests

Sciences (grant number 398978).

## Data sharing statement

The data that supports the findings of this study will be available from the corresponding author upon reasonable request.

## Trial status

The estimated registration is 90 subjects. Recruitment began in July 2020. Currently, recruitment completed in March 2022, and intervention and the last follow-up are expected to be completed in June 2022. Due to the prevalence of Covid-19, many subjects were canceled and sometimes the work was stopped due to quarantine therefore, took longer than expected.

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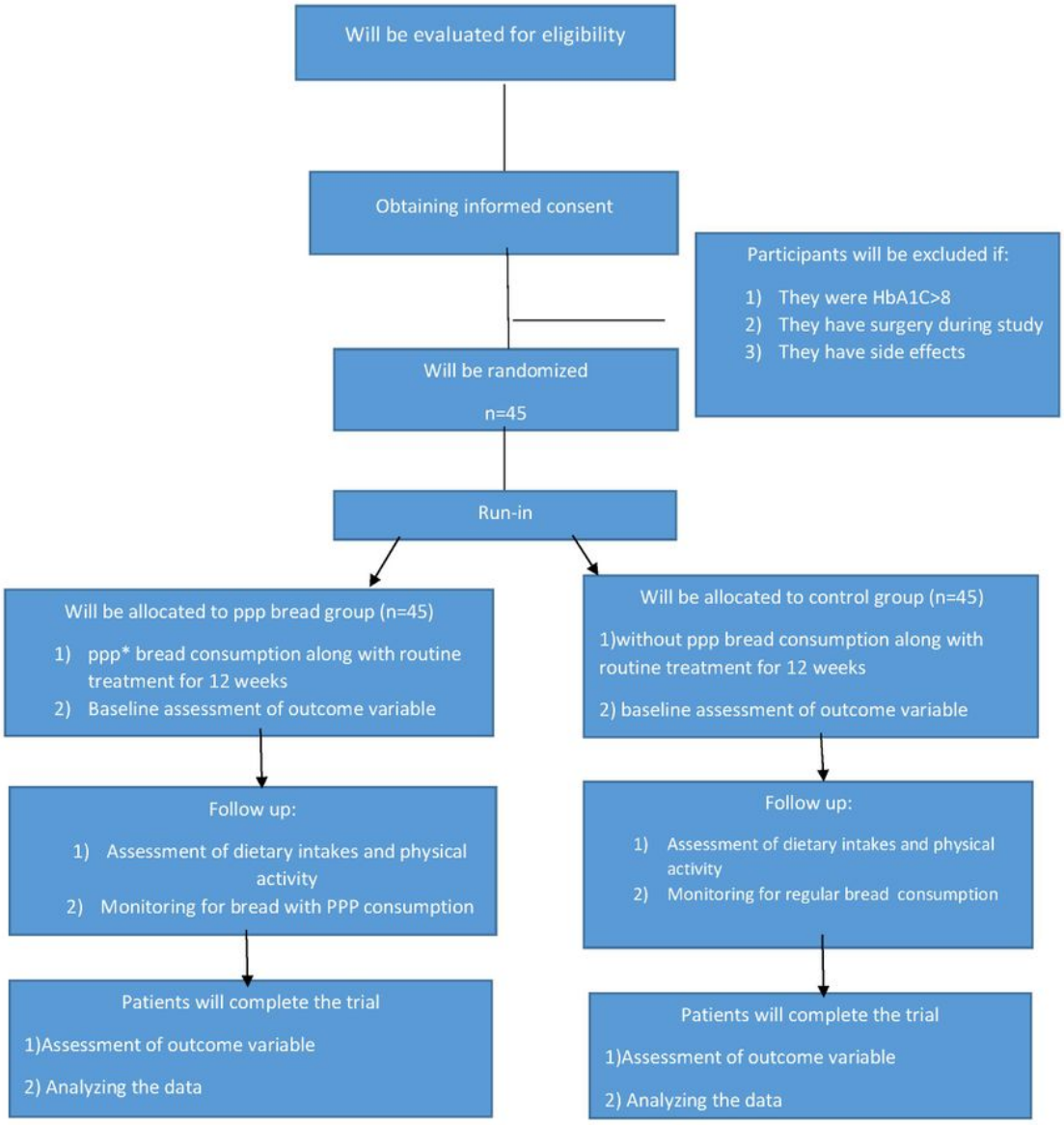
# Figures




## Figure 1

Trial flowchart

ppp*: pomegranate peel powder