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Cookies fortified with pomegranate seed powder and defatted soybean flour exhibited hypoglycemic and hypo-cholesterolemic effects on type-2 diabetes induced albino rats

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Abstract
Pomegranate seed is a major byproduct from pomegranate fruit juice industries which goes unused most of the time. Beyond its pharmaceuticals uses the potential bioactive phytochemical constitution of pomegranate seed and defatted soybean made to use them as a substitute for functional ingredients in food industries. In the present study, Pomegranate seed powder (PSP) and defatted soybean flour (DSF) based cookies were prepared and its efficacy on glycemic activity at serum level were tested with two concentrations *i.e.*, 5 g and 10 g/rat/day in diabetic and non-diabetic induced rats upto 21 days. Compared to their respective initial observations, serum glucose, cholesterol and haemoglobin levels were influenced positively (-32.43%, -36.99% and +40.94% respectively) in G_6 group of rats *i.e.*, laboratory diet + 10 g cookies/rat/day at the end of experimental period (21 days). Though PSP and DSF incorporated cookies reduced the serum glucose, cholesterol levels and increased haemoglobin content in diabetic and non-diabetic rats, it maintained their levels at recommended range invariable to the dosage of cookies.

Keywords: Pomegranate seed powder, cookies, type 2 diabetes, hypoglycemic activity

Introduction
Pomegranate is one of the most important deciduous shrub fruit crop grown in arid and semi-arid regions of the world. The health benefits of pomegranate are mainly because it is claimed to be an excellent source of vitamins, including A, C, and E, as well as folic acid. It has high anti-oxidant values and hence documented to cure many of the chronic diseases like cardiovascular, cancer, colic and repairs skin damages. Punicalagin and punicic acid, two of their constituents, are maximum responsible for all of their strong advantages towards health benefits. The edible part of a pomegranate is the sweet and juicy arils with seeds. Though the recent varieties of pomegranate have soft seeds, consumer prefer to have cordial and hence seeds are abandon in many of the fruit juice industries with respect to pomegranate. Seeds contribute about 3–5% of the total fruit weight are the major by-product from the pomegranate juice industry, which have a major potential to be converted into value-added products. Similarly, soybean (*Glycine max*) is an annual leguminous crop with pod as a edible product are high in free fats and protein. Soybean flour is mainly used in the bakery industry as a replacement for wheat flour since it is a rich source of protein. It is commercially available in 2 forms *i.e.*, defatted and full-fat form. Defatted soybean flour is a concentrated form of flour wherein oil is removed during the production process.

Cookies are baked or cooked snacks usually fall under the dessert category. Cookies are usually enjoyed by all the group of people, irrespective of their age. It is usually served along with tea or coffee as a refreshment snack. But because of carbohydrate rich wheat as a major ingredient, cookies were not really relished by diabetic people. Diabetes mellitus is a metabolic disorder that causes an increased blood glucose level in the body. This diabetic condition is associated with genetics as well as lifestyle. Maintaining a proper diet and exercise will help in managing type-2 diabetes mellitus. Although there are several hypoglycemic medications on the market right now, including insulin, people are increasingly drawn towards natural remedies with less expense to overcome this disease. Moreover plant based products have no adverse effect compared to synthetic medications.

Hence to bridge this gap, an experiment was formulated to prepare cookies from pomegranate seed powder and defatted soybean flour and its efficacy on glycemic activity were tested at pre-clinical level using different dosage.

Materials and Methods

Biological materials

Fresh pomegranate fruits cv. Bhagwa were harvested from Pomegranate Orchard, Fruit Research Station, Sector 70, University of Horticultural Sciences, Bagalkot (longitude: 75º42' East; latitude: 16º10'). After separation of aril from peel and other fruit parts, the arils are crushed to separated juice using juice extractor. The seeds are subjected to 2% salt solution for 10 minutes in order to remove with mucous layer over the seeds. The seeds are again washed with clean tap water, drained off and air dried to remove excess moisture on the seed surface. These seeds were dehydrated using a tray drier at 65±1 ºC for 24 h to obtain the dried seeds. The dried seeds were pulverised in a mixer grinder. The seed powder obtained was passed through a 0.5 mm size mesh sieve to get uniform size particles. Other ingredients used for cookies preparation *i.e.,* Defatted soybean flour, sugar, baking powder, hydrogenated fat, wheat flour was procured from local market. Thirty six weanling male wistar rats weighing 150-250 g were supplied by the animal house of the Department of Pharmacology, College of Pharmacy, BVVS, Bagalkot, Karnataka after obtaining approval from Animal Ethical Committee (Ref. No: IAEC/HSKCOP/April 2019/UHS 2).

Preparation of cookies

The cookies were prepared as per the procedure suggested by Suresha *et al.* (2015) [18] with little modification by replacing the refined wheat flour partially with 15% of pomegranate seed powder and 25% defatted soybean flour (Fig S1) and used for further pre-clinical studies.

Preparation of Wistar rats and treatment details for pre-clinical studies

Thirty six weaning male Wistar rats weighed 150-250 g were obtained from animal house to laboratory of the Department of Pharmacology, College of Pharmacy, BVVS, Bagalkot, Karnataka. The rats were maintained at the study environment (room temperature: 25± 2 °C with 12 h light/dark cycle) for initial 5 days as acclimatization period. The rats were fed with water *ad libitum* as laboratory diet during acclimatization period (5 days) and experimental period (21 days).

The rats were divided into six major groups consisting of six rats in each group. In first three groups the rats were not treated with Alloxan streptozotocin (non-diabetic rats) in which the first group was given only the laboratory diet (G_1), the second was given 5g cookies per kg body weight along with laboratory diet (G_2) and the third group was given 10g cookies per kg body weight along with laboratory diet (G_3) (Table 1).

The same treatments were replicated in other three groups, where the rats are treated with STZ for induction of diabetic (diabetic rats). Diabetes was induced by a single injection of 140 mg/kg body weight of Alloxan streptozotocin (STZ) @ 0.9% (w/v) saline solution to overnight fasted rats. The treatments in this group will be, rats given only the laboratory diet can also be considered as diabetic control (G_4), rats given 5 g cookies per kg body weight along with laboratory diet (G_5) and rats given 10 g cookies per kg body weight along with laboratory diet (G_6). The cookies were given upto 21 days on daily basis along with regular laboratory diet (Table 1).

Blood sample collection and preparation

The blood samples from all six group of rats were collected on the 0th (after 72 hours of the injection in diabetic rats), 7th, 21st day (end of experimental period) in clean, sterilized and labelled 2 ml capped centrifuge tubes, by retro-orbital plexus puncture method using micro-haematocrit capillary tubes. Immediately after collection, the blood samples were centrifuged at 3000 rpm for 10 min and kept hold in a slanting position for 1-2 h to facilitate serum separation at ambient conditions. The clear non-haemolysed serum was then transferred into a clean, sterilized screw capped vial and used for further analysis.

Estimation of serum glucose, cholesterol and haemoglobin levels

For this analysis, sufficient quantity of test tubes was labeled for standard, control and sample to be tested for all parameters.

Serum glucose estimation in rats was done by Trinders method (Lott and Turner, 1975) [13]. Glucose reagent (500 µl) was pipetted into each test tube and 5 µl of blood samples was added to appropriate test tubes, mixed well and incubated at 37 ºC for 10 min. The absorbance of reaction mixture was measured at 525 nm against water as a blank using UV spectrophotometer. The glucose content in blood is expressed as mg/dL.

PEG-CHOD-PAP method was used for estimation of serum cholesterol (Shah *et al.*, 2011) [20]. Cholesterol reagent (500 µl) was pipetted into each test tube and 5 µl of blood was added to appropriate tube, mixed well and incubated at 37 ºC for 10 min. The absorbance was read at 505 nm on UV spectrophotometer, calculated using following formula and expressed as mg/dL.

$$\text{Total cholesterol } \left(\frac{\text{mg}}{\text{dL}}\right) = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times 100$$

The serum hemoglobin concentration in all blood samples was measured according to the cyanomethemoglobin method using Drabkin's reagent (Alexander and Grifiths, 1993) [3]. Blood samples were analyzed for serum haemoglobin in City Diagnostic Centre, Bagalkot.

The serum glucose, cholesterol and haemoglobin of blood samples collected on 0th, 7th and 21st day were compared with their respective rat samples.

Statistical analysis

In this present study, the six tuplicate data obtained on serum glucose, cholesterol and haemoglobin levels in each experimental group were analyzed by Completely Randomized Design using one way analysis of variance (ANOVA) and Fisher's Least Significant Difference at 1% level (p=0.01). The entire analysis was performed using the online statistical program called Graphpad prism (version 8.0).

Results and Discussion

Pomegranate seed powder and Defatted soybean flour are

inherited with low glycemic index. But the consumption in the raw form is less acceptable except *anar dhana*. Since, cookies are consumed as regular evening snack invariably by all the age groups; both the products were used as ingredients in cookie preparation. The effect of PSP and DSF incorporated cookies on glycemic activity were studied at pre-clinical level and the results of serum glucose, cholesterol and haemoglobin levels are furnished and discussed.

The effect of the different doses of cookies incorporated with PSP and DSF on the serum glucose level of both non-diabetic and diabetic rats are given in Table 2. The initial serum glucose level ranged from 89.80 to 104.23 mg/dL and 159 to 286.05 mg/dL in non-diabetic and diabetic rats groups respectively. The serum glucose level increased at 7th day and decreased at the end of experiment *i.e.*, 21st day in non-diabetic rats irrespective of the feed treatments. Whereas, in diabetic rat groups, the level of serum glucose were gradually reduced throughout the experimental period (Table 2). The serum glucose level of diabetic control rats (G_4) was significantly higher than those of non-diabetic rats (G_1, G_2 and G_3). PSP and DSF incorporated cookies on serum glucose level of diabetic rats over 0th day to 21st day had shown significant reduction in G_6 (-32.43%) followed by G_5 (-26.92%) compared to diabetic control *i.e.*, G_4 (+1.16%) (Table 3). In case of non-diabetic rat groups though the PSP and DSF incorporated cookies increased the glycemic activity at the rate of 6.0% and 4.8% invariable to its dose (G_2 & G_3 respectively), the serum glucose level was maintained in the normal recommended range of ≤140 mg/dL. The results obtained in this study with respect to serum glucose level of diabetic rats are in line with the findings of Das *et al.* (2001) [4] where the PSP showed significant decrease in serum glucose level of diabetic induced rats. Hashemi *et al.* (2021) [6] also got a positive lead in reduced fasting blood glucose level in type 2 diabetic patients by feeding them PSP. Many of the reports say that pomegranate seed or its oil does not have direct effect on serum glucose level of consumers and hence, the pomegranate seed oil present in the PSP of cookies may alter the insulin secretion and thereby regulated the level of serum glucose (Hashemi *et al.*, 2021) [6]. The abundance of phenol derivatives such as Punicic, Gallic, Oleanolic and Uallic acids present in pomegranate seeds involved in insulin regulation by forming new pancreatic tissue and hence acts as a potent anti-diabetic agent. Thus, daily intake of pomegranate juice along little seed residue reduces the blood and serum glucose level in patients with type 2 diabetes (Rosenblat *et al*., 2006) [22].

During the experimental period the non-diabetic control rats (G_1) showed a gradual increase in serum cholesterol level from 29.58 to 33 mg/dL (Table 2). In other rat groups (G_2 to G_6), a steep decrease in serum cholesterol level was observed. On 21st day, the PSP and DSF incorporated cookies *i.e.*, 5 g/rat/day and 10 g/rat/day reduced the serum cholesterol level in non-diabetic rats at the rate of 27% and 12.3% respectively (Table 3). In diabetic rat groups, the PSP and DSF cookies (5 and 10g/rat/day) had the impact of reducing serum cholesterol level at the rate of 35.83% and 36.99% respectively (Table 3). The results obtained in this current study are in confirmation with the findings of Viladomiu *et al.* (2013) [17]. Abdel-rahim *et al.* (2013) [2] also got the similar results of the hyperlipidemic and hypocholestrolemic effects of pomegranate seeds. Hashemi *et al.* (2021) [6] also got the positive results in reducing blood cholesterol levels of type 2 diabetes mellitus patients by treating with 5 g pomegranate seed powder twice a day. Elbandy and Ashoush (2012) [5] stated that the phytochemicals present in the pomegranate seeds effectively regulate the fat metabolism which is responsible for their hypolipidemic and hypocholesterolemic effect. Pomegranate seeds contain oleic acid which enhances the oxidation of low density lipids and cholesterol biosynthesis. Pomegranate seed also has considerable amount of its anti-oxidant activity and makes to increase the High density lipid in blood. The DSF also reported for the cholesterol lowering effect and subsequently reduces the risk of heart diseases because of presence of isoflavones, lecithins, saponins and fiber (Ramdath *et al.*, 2017) [21]. Since, the cookies contains considerable amount of PSP and DSF, the functional properties of biochemical components would have been reduced the serum cholesterol level resulted in hypocholesterolemic effect.

Haemoglobin is an essential biochemical component involved in reducing the risk of glycemic activity especially in women with pregnancy (Huerta-Uribe *et al*., 2022) [1]. The serum haemoglobin increased in different rat groups except in non-diabetic rats given laboratory diet (G_1) (Table 2). At the end of experiment *i.e.*, 21st day, significant increase in serum haemoglobin level was observed in diabetic rats fed with cookies at the rate of 10g/rat/day (G_6) followed by diabetic rats fed with cookies at the rate of 5g/rat/day, the rate of reduction in serum haemoglobin levels in respective treatments were 40.94% and 24.88% (Table 3). The results of this experiment are in concordance with the findings of Viladomiu *et al*. (2013) [18] and Khalil *et al*. (2004) [10] were the folic acid rich pomegranate juice increased the red blood cells of the diabetic induced rats. Though the PSP and DSF cookie had a vital role in increasing the serum haemoglobin level, it was observed that the parameter is increasing in other group of rats also. In both the cases, the serum haemoglobin level was maintained within the range recommended by CDC, 2021. Hence, it can be considered that the haemoglobin increasing component may be included in regular laboratory diet.

Table 1: Details of feed composition for different groups of diabetic and non-diabetic rat

Groups	Treatments
G_1	Laboratory diet in non-diabetic rats
G_2	Laboratory diet with 5g of cookies per rat per day in non-diabetic rats
G_3	Laboratory diet with 10 g of cookies per rat per day in non-diabetic rats
G_4	Laboratory diet in diabetic rats
G_5	Laboratory diet with 5g of cookies per rat per day in diabetic rats
G_6	Laboratory diet with 10 g of cookies per rat per day in diabetic rats

Table 2: Efficacy of pomegranate seed powder and defatted soybean flour cookies on serum glucose, serum cholesterol and haemoglobin levels on non-diabetic and diabetic rats

Groups & Treatments	Serum glucose level (mg/dL)			Serum cholesterol level (mg/dL)			Serum haemoglobin level (mg/dL)		
	0th day	7th day	21st day	0th day	7th day	21st day	0th day	7th day	21st day
G1:Laboratory diet in non-diabetic rats	92.58	95.93	94.78	29.58	30.65	33.00	14.55	13.85	13.70
G2:Laboratory diet with 5g of cookies per rat per day in non-diabetic rats	89.80	110.38	95.20	35.18	29.70	25.68	12.58	13.85	14.18
G3:Laboratory diet with 10 g of cookies per rat per day in non-diabetic rats	104.23	124.78	109.20	33.58	31.58	29.43	13.25	14.30	14.48
G4:Laboratory diet in diabetic rats	286.05	280.30	289.38	60.38	54.78	44.15	10.83	11.08	11.45
G5:Laboratory diet with 5g of cookies per rat per day in diabetic rats	174.83	149.30	127.75	55.95	47.78	35.90	10.65	10.95	13.30
G6:Laboratory diet with 10 g of cookies per rat per day in diabetic rats	159.00	130.40	107.43	40.68	39.80	25.63	10.38	13.15	14.63

Table 3: Effect of pomegranate seed powder and defatted soybean flour cookies in altering the serum glucose, cholesterol and haemoglobin levels on non-diabetic and diabetic rats

Groups & Treatments	Serum glucose level (%)	Serum cholesterol level (%)	Serum haemoglobin level (%)
G_1:Laboratory diet in non-diabetic rats	$+2.37^{e}$	$+11.56^{d}$	-5.8^{f}
G_2:Laboratory diet with 5g of cookies per rat per day in non-diabetic rats	$+6.00^{c}$	-27.00^{b}	$+11.2^{c}$
G_3:Laboratory diet with 10 g of cookies per rat per day in non-diabetic rats	$+4.8^{d}$	-12.3^{c}	$+9.2^{d}$
G_4:Laboratory diet in diabetic rats	$+1.16^{f}$	-26.87^{b}	$+5.72^{e}$
G_5:Laboratory diet with 5g of cookies per rat per day in diabetic rats	-26.92^{b}	-35.83^{ab}	$+24.88^{b}$
G_6:Laboratory diet with 10 g of cookies per rat per day in diabetic rats	-32.43^{a}	-36.99^{a}	$+40.94^{a}$

The symbol + & - prefixed to numerical denotes the increased and decreased activity respectively Values with the same alphabet and no alphabets are non-significant (NS) at 99% confidence level Values with the different alphabet(s) are significant at 99% confidence level

Conclusion

Consumption of pomegranate seed is not relished unlike fresh fruits. Pomegranate seeds are rich in bioactive components but not having commercial value in its right form. Many pharma industries are selling encapsulated PSP for high price which cannot be affordable by middle and lower income groups. Hence, an alternative form such as extrudates, cakes and confectioneries which can be affordable by range of income groups is needed now. The preparation of cookies using wheat flour, PSP and DSF as ingredients is achieved at the ratio of 60:15:25. The output of pre-clinical trail undertaken on diabetic induces male rats showed demotion of serum glucose and cholestrol levels, and elevation of serum haemoglobin level. Therefore hypoglycemic and hypo-cholesterolemic effect of pomegranate seed powder and defatted soybean flour incorporated cookies were proved at pre-clinical level.

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Authors' contributions

Harish T: Biochemical studies, data curation, data analysis and statistical analysis, writing – original draf; G Bhuvaneshwari: Conceptualization, Investigation, Methodology, Data curation; S L Jagadeesh: Supervision; V. M. Chandrashekhar: Methodology and Resources; Deepa Terdal: Supervision; Preethi P and S.V.R. Reddy: writing - original draft, review & editing.

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Data availability

The datasets used and analyzed during the current study are available from the corresponding author.

Code availability: Not applicable

Declarations Conflict of interest: The authors have declared no conflict of interest for this article.

Ethics approval: This work was carried out after the approval taken from Animal Ethical Committee (Ref. No: IAEC/HSKCOP/April 2019/UHS 2).

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