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Immunodetection and quantification of insulin-like antigens in sprouts: development of an efficient functional food

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Running title: Sprouts can be a good source of insulin

Abstract:

Background: Hormone Insulin is a drug used for the treatment of type 1 and type 2 Diabetes Mellitus. Insulin used in this experiment is derived from bovine and pork pancreas, as well as through recombinant technology. Patients with diabetes mellitus face an inability to utilize glucose from blood due to either less secretion of insulin, or the inability of the insulin to act; As a result of this glucose levels in the blood rise. The prevention and treatment of type 2 Diabetes Mellitus one is world's major public health issues. Natural alternatives have a big role to play in this field. This study aims at discovering functional foods rich in Insulin like proteins. Here we are reporting Insulin-like proteins synthesizing during the embryo development stage of *Glycine max*: soybean, *Vigna radiata*: moong and *Vigna unguiculata*: cowpea seeds. Hence, germination transforms these seeds containing human insulin like proteins.

Methods: In our investigation we have provided protein extraction with Enzyme-linked immunosorbent assay (ELISA). The plant materials weighing 1g were crushed in mortar and pestle, and the protein from the plant materials was extracted with 20 ml of 0.05 M sodium phosphate buffer (pH 7.6). The suspensions were centrifuged at 6000 rpm for 15 min, and the clear supernatants were subjected to Enzyme linked immunosorbent assay (ELISA) for the

detection of insulin-like proteins. We have used USDA nutritional data sources for the analysis of new products.

Results: Our results demonstrate that Insulin is not expressed in dry mature dormant seeds, but is expressed only during the embryo development stage. Dry mature dormant seeds and the seeds germinated for 24 hours, 48 hours, 72 hours, and 96 hours of *Glycine max*, *Vigna radiata* and *Vigna unguiculata*, were investigated for expression of insulin through immunodetection using anti-insulin antibodies. Dry dormant seeds of all the three seeds showed zero expression at 450 nm for insulin, while significant presence of insulin showing positive immuno-reactivity towards anti-insulin antibodies were observed at 24 hours, 48 hours, 72 hours, and at 96 hours of germination.

Conclusion: The study is suggesting that insulin-like proteins are synthesized only during the process of embryo development, the sprouts of such legumes, particularly soybeans, can be a good source of insulin.

Key words: Germination, insulin, seed embryo development, sprouts.

INTRODUCTION:

Peptides are a major class of signal molecules in animals that regulate a wide variety of physiological and developmental processes. It was once thought that plants have not evolved signaling systems that use a peptide as a signal.

The first peptide hormone, insulin, was discovered in 1922 [1]. The role of insulin and insulin-like growth factors in animals has been well established to be associated with glucose metabolism, cell growth, and survival, respectively. Though there are many arguments that reject the presence of the peptide hormone insulin in plants, there is strong evidence, since the discovery of insulin that suggests the presence of insulin-like proteins in plants with similar characteristics as those of animal insulin [2].

Seed germination is a central process that is the key to plant development and plant survival. This important event involves metabolism reactivation, translation of stored mRNAs, rapid growth, and cell division. Insulin has been shown to promote seed germination of monocot and dicot plants and accelerate the post-germination development of fat-storing seeds [3]. Also, insulin regulates selective translation in the maize embryonic axis through activation of corresponding maize S6 kinase (ZmS6 kinase) [4]A 4 kDa peptide, leginsulin, was found to be present in radicles of the germinated seeds of soybean, moong bean, and different legume species, which can bind to soybean basic 7S globulin (Bg), which showed similarity to animal hormone receptors and compete with insulin for binding to Bg [5]. This 4 kDa peptide stimulates protein kinase activity of Bg and regulates the growth and cell proliferation of plant callus [6]. A protein with amino acid sequence homology to bovine insulin was shown to be present in the developing fruits of cowpeas [7]. This evidence suggests the possible role of insulin-like proteins in growth, cell proliferation, and sugar transport, during seed germination. This study was carried

out to investigate the effect of seed germination on synthesis of insulin in seeds of *Glycine max* (soybean), *Vigna radiata* (moong bean), and *Vigna unguiculata* (cow pea).

It was found that insulin-like proteins were not detected in the raw seeds, where as significant levels of insulin were detected after the germination of the seeds. This report is another source of evidence for the presence of insulin-like proteins in legumes, with emphasis on its role during the phase of seed embryo development. The report also suggests that the expression of insulin is highly regulated. We reported earlier on the high blood sugar regulating efficacy of Glycine max sprouts [8].

METHODS AND MATERIALS:

Plant material: Dry dormant seeds of *Glycine max* (soybean), *Vigna radiata* (mung bean), and *Vigna unguiculata* (cow pea), were washed thoroughly with water and soaked for 8 hours. After 8 hours, the water was drained, and the seeds were kept in wet cotton clothes and allowed to germinate. The clothes were always kept wet through the sprinkling water at frequent intervals. Germinated seeds were collected at 24, 48, 72, and 96 hours, and dried at 50°C in an incubator.

Antibodies: Anti-pig insulin antibodies produced in guinea pigs, with 100% cross reactivity to human and bovine insulin (Sigma Aldrich, USA), and anti-guinea pig IgG produced in goat, conjugated to horse radish peroxidase (HRP) (Sigma Aldrich, USA), were used.

Protein extraction: The plant materials weighing 1g were crushed in mortar and pestle, and the protein from the plant materials was extracted with 20ml of 0.05M sodium phosphate buffer (pH 7.6)[10, 11]. The suspensions were centrifuged at 6000rpm for 15 min, and the clear supernatants were subjected to Enzyme linked immunosorbent assay (ELISA) for the detection of insulin-like proteins.

Enzyme-linked immunosorbent assay (ELISA): The 10µl of protein samples prepared from dry dormant seeds and 24 hour, 48 hour, 72 hour, and 96 hour germinated seeds, were diluted with 990µl carbonate/bicarbonate buffer (0.05M, pH 9.6) [12]. 100µl of these solutions were coated on the wells of microtitre plates (96 well Maxisorp type, Nunc, Roskilde, Denmark), and incubated at 4°C overnight. The wells were washed with Phosphate buffered saline, containing 0.05% Tween 20 (PBS-T), for 1h. 300µl of blocking solution (2% non-fat dry milk in PBS-T) was added to each of the wells, and incubated for 1 hour at room temperature. The wells were washed with PBS-T and 50 µl of anti-insulin IgG, raised in guinea pig (1:5000) solution, prepared with a blocking buffer, was added to each of the wells and incubated for 1 hour at room temperature. The wells were again washed with PBS-T and 50µl of anti- guinea pig IgG raised in goat - HRP conjugate (1:3000) solution prepared with a blocking buffer was added to each of the wells, and incubated for 1 hour at room temperature [9, 13]. The wells were washed with PBS-T and 100µl of TMB (3, 3', 5, 5' tetramethyl benzidine) / H2O2 (hydrogen peroxide) (Bangalore GeNei, India), which is the substrate of HRP, was added to each of the wells and incubated at room temperature for 15-30 min, untill sufficient blue color develops. The reaction was stopped

by adding 100µl of 2M sulfuric acid, and the absorbance was read at 450nm [13] using the ELISA plate reader (ELx800, BioTek, U.S).

Human insulin of recombinant DNA origin (INSUGEN-R Regular soluble short acting insulin, BIOCON) was used as the standard. (The composition of each ml is Human insulin IP 40 IU (International Unit), m-Cresol USP 0.25% w/v, water for injection IP q.s). Concentration of insulin was made to 10µg / 100µl and serially dilution to 1 x 10-8µg / 100µl with a carbonate / bicarbonate buffer. Controls for each of the samples employing the secondary antibody without the addition of primary antibodies were performed.

ELISA Experimental design and data analysis: Six experiments of ELISA were carried out. ELISA 1, 2 and 3 were performed with the freshly precipitated protein solution from the dried germinated seeds, and ELISA 4, 5 and 6 were performed with the protein samples which were stored for 48 hours in the refrigerator. Each ELISA was designed in a way to accommodate the samples of raw seeds, and seeds germinated for 24 hours, 48 hours, 72 hours, and 96 hours, of all the three beans under the study. All samples of the individual seeds were tested in triplicate. These were technical replicates; no biological replicates were used here. Three wells were taken as controls for each of the germinated seed samples, and one as the control for each raw seed sample. The average value was taken for further analysis. The net absorbance for each of the samples was calculated by deducting the absorbance of the controls from that of the observed absorbance of the test samples. The level (concentration) of insulin was represented in terms of the net absorbance values at 450nm. The concentrations of insulin in the test samples were determined from the standard graph. The concentrations of insulin in 1g of the dried seeds were also calculated. The standard graph and the determination of the concentrations of insulin in the test samples were done using the software GraphPad Prism 5.

Nutritional Analyses: Nutritional analysis was made using the information provided by USDA Nutritional Database. All ingredients were purchased from local grocery stores. Ingredients for the salads included: kidney beans, beets, carrots, vegetable oil (soybean oil), canola oil, onions, potatoes, sauerkraut, sprouted soybeans, parsley, salt, and black peppers.

RESULTS:

Fresh protein precipitates, and 48-hour-old protein precipitates of dry dormant seeds (raw seeds) and germinated for 24 hours, 48 hours, 72 hours and 96 hours of *G.max* (*Glycine max*), *V.radiata* (*Vigna radiata*) and *V.unguiculata* (*Vigna unguiculata*) were subjected to six ELISA tests. The presence of insulin was analyzed with anti-insulin antibodies. Insulin-like proteins were not detected in any of the raw seeds, whereas detectable amounts of insulin-like proteins, that showed immuno-reactivity towards the anti-insulin antibodies, were present in all of the germinated seeds, as evident in Table 1. The absorbance in 48-hour-old protein samples was found to be little lesser than that of the freshly prepared samples. The net absorbance in three test samples of six ELISA tests of raw seeds of all the seeds under investigation was observed to be zero (Table 1). We report the presence of insulin-like proteins that are immuno-reactive to anti-insulin antibodies in all the germinated seeds under study, whereas these were absent in the raw seeds.

Table 1. Levels of insulin-like proteins in raw and germinated seeds of *G.Max*, *V.radiata*, *V.unguiculata* as measured by ELISA and represented as Absorbance at 450 nm after rounding off to two decimal places.

	Net Absorbance at 450nm (observed absorbance minus the control absorbance)										
Test sample	**Elisa 1**	**Elisa 2**	**Elisa 3**	**Average of Elisa 1,2&3.**	**Elisa 4**	**Elisa 5**	**Elisa 6**	**Average of Elisa 4,5&6**	**Averages of 6 Elisa's**	**% Absor-bance***	**% Difference in Absorbance+**
G.max											
Raw	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0	0
24 h G‡	0.19	0.19	0.19	0.19	0.16	0.15	0.15	0.15	0.17	17	+17
48 h G‡	0.16	0.31	0.31	0.26	0.19	0.19	0.19	0.19	0.23	23	+6
72 h G‡	0.49	0.54	0.54	0.52	0.21	0.37	0.37	0.32	0.42	42	+19
96 h G‡	0.20	0.20	0.20	0.20	0.13	0.13	0.12	0.13	0.16	16	-26
V. radiata											
Raw	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0	0
24 h G‡	0.07	0.07	0.07	0.07	0.08	0.07	0.07	0.07	0.07	7	+7
48 h G‡	0.15	0.15	0.15	0.15	0.10	0.10	0.11	0.10	0.13	13	+6
72 h G‡	0.07	0.07	0.07	0.07	0.08	0.08	0.07	0.08	0.07	7	-6
96 h G‡	0.17	0.17	0.17	0.17	0.10	0.09	0.10	0.10	0.13	13	+6
V.unguiculata											
Raw	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0	0
24 h G‡	0.08	0.08	0.08	0.08	0.06	0.06	0.06	0.06	0.07	7	+7
48 h G‡	0.12	0.12	0.12	0.12	0.13	0.12	0.13	0.13	0.12	12	+5
72 h G‡	0.06	0.06	0.06	0.06	0.08	0.08	0.07	0.08	0.07	7	-5
96 h G‡	0.27	0.27	0.27	0.27	0.16	0.15	0.15	0.15	0.21	21	+14
**Standard µg insulin / 100 µl solution											
1 x 10^{1}	1.93	1.87	1.76	1.85	1.45	1.44	1.50	1.46	1.66		
1 x 10^{0}	1.61	1.39	1.22	1.41	1.61	1.37	1.12	1.37	1.39		
1 x 10^{-1}	1.43	1.12	1.12	1.22	1.17	1.16	0.91	1.08	1.15		
1 x 10^{-2}	1.24	1.24	1.24	1.24	1.34	1.12	1.01	1.16	1.20		
1 x 10^{-3}	0.64	0.64	0.63	0.64	0.93	0.89	1.06	0.96	0.80		
1 x 10^{-4}	0.52	0.64	0.64	0.60	0.87	0.56	0.56	0.66	0.63		
1 x 10^{-5}	0.31	0.31	0.33	0.32	0.42	0.45	0.48	0.45	0.39		
1 x 10^{-6}	0.21	0.21	0.21	0.21	0.33	0.35	0.37	0.35	0.28		
1 x 10^{-7}	0.11	0.11	0.11	0.11	0.22	0.33	0.30	0.28	0.20		
1 x 10^{-8}	0.08	0.08	0.08	0.08	0.11	0.11	0.11	0.11	0.10		

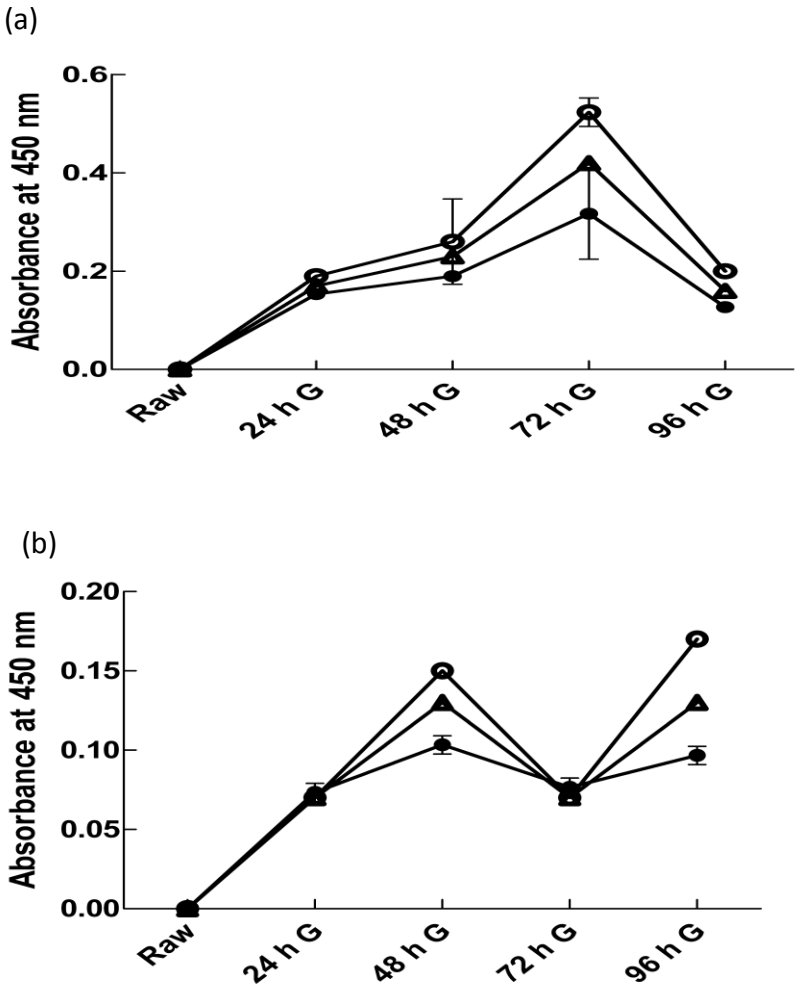
* Percentage absorbance is expressed as a fraction of 100; † '+' Sign indicates the increase in absorbance and '-' sign indicated the decrease in absorbance; ‡ 'G' indicates germinated; **Human insulin of rDNA origin

Levels of insulin in germinating seeds: The net absorbance values were rounded off to two decimal places, and the trend in the changes of insulin levels in the germinated seeds for various time periods was analyzed. Net expression of insulin varied with the various intervals of time. The variation pattern was similar in all of the experiments of ELISA for respective beans. In *G.max*, the dry dormant seeds expressed a 0% absorbance at 450nm while an increase of 17% insulin was observed at 24 hours of germination, followed by a further increase of 6% and 19% at 48 hours and 72 hours of germination; then there was a drop by 26% at 96 hours of

germination. It is clearly observed that insulin is expressed only during germination. In *V.radiata*, the dry dormant seeds expressed 0% absorbance at 450nm while an increase of 7% insulin was observed at 24 hours of germination, followed by an increase of 6% at 48 hours. There was then a decrease of 6% insulin at 72 hours, and again an increase of 6% at 96 hours of germination. In *V.unguiculata*, the dry dormant seeds expressed 0% absorbance at 450nm, while an increase of 7% insulin was observed at 24 hours of germination, followed by an increase of 5% at 48 hours. There was then a drop of insulin level at 5% at 72 hours, and again an increase of 14% at 96 hours. In *G.max*, the levels of insulin were observed to increase with an increasing period of germination with the maximum at 72 hours, and then there was a decrease while in *V.radiata* and *V.unguiculata*. Afterward, insulin levels increased till 48 hours, and then a fell in the level of insulin, which was observed at 72 hours, followed by an increase at 96 hours. Maximum levels of insulin were observed at 48 hours and 96 hours of germination in *V.radiata* and *V.unguiculata*, respectively (Table 1).

Trend in the levels of insulin in germinating seeds

The level of insulin in *Glycine max* smoothly increased during embryo development until 72 hours of germination, and then dropped. So, the pattern in Glycine max is uniform. This was not the case with *Vigna radiata* and *Vigna unguiculata* (Figure 1).



(c)

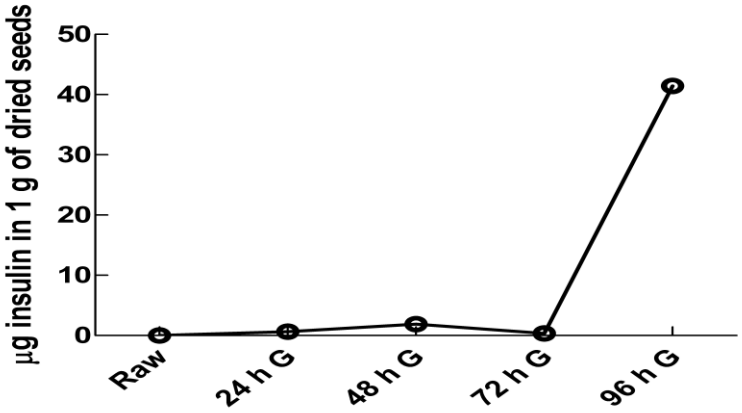


Figure 1. Levels of insulin-like protens in raw and germinated seeds of (a) *Glycine max*, (b) *Vigna radiata* and (c) *Vigna unguiculata*.

The concentrations of insulin-like proteins are represented as the Absorbance at 450nm. ELISA 1,2, and 3 corresponds to the freshly prepared samples and ELISA 4,5, and 6 corresponds to 48-hour-old samples. 'G' stands for germinated seeds. In these two beans, a zigzag pattern of insulin expression was observed. In them the exact same levels of insulin expression at 24 hours of germination is observed. It is further increasing at approximately equal levels at 48 hours of germination, and again dropping approximately equally at 72 hours and then again going up at 96 hours of germination.

Table 2: Concentration of insulin present in 100 µl of the protein samples and in 1 g of dried seeds

Test Sample	**Amount of Insulin ($x10^{-10}$µg) present in 100µl of freshly prepared protein sample**	**Amount of Insulin ($x10^{-10}$µg) present in 100µl of 48 hr old protein sample**	**Insulin levels ($x10^{-3}$µg) per g of seeds(dried) calculated from the data observed for freshly prepared protein samples**
Raw (*G.max)*	0.00	0.00	0.00
24 h germinated	4644.00	156.08	9.29
48 h germinated	17418.00	452.68	34.84
72 h germinated	1000000.00	6285.90	2000.00
96 h germinated	5684.90	84.83	11.37
Raw (*V. radiata)*	0.00	0.00	0.00
24 h germinated	242.34	8.15	0.48
48 h germinated	1956.00	29.64	3.91
72 h germinated	242.34	12.97	0.48
96 h germinated	3050.30	29.64	6.10
Raw (*V. unguiculata)*	0.00	0.00	0.00
24 h germinated	325.63	4.91	0.65
48 h germinated	955.17	84.83	1.91
72 h germinated	178.11	12.97	0.36
96 h germinated	20709.00	156.08	41.42

The observation of the study clearly indicates that the presence of insulin like proteins in the legume seeds is characteristic of the germination process. Insulin present in protein concentrate, as well as Insulin present in 1gm of the sprout was calculated Table-2, Figure-2).

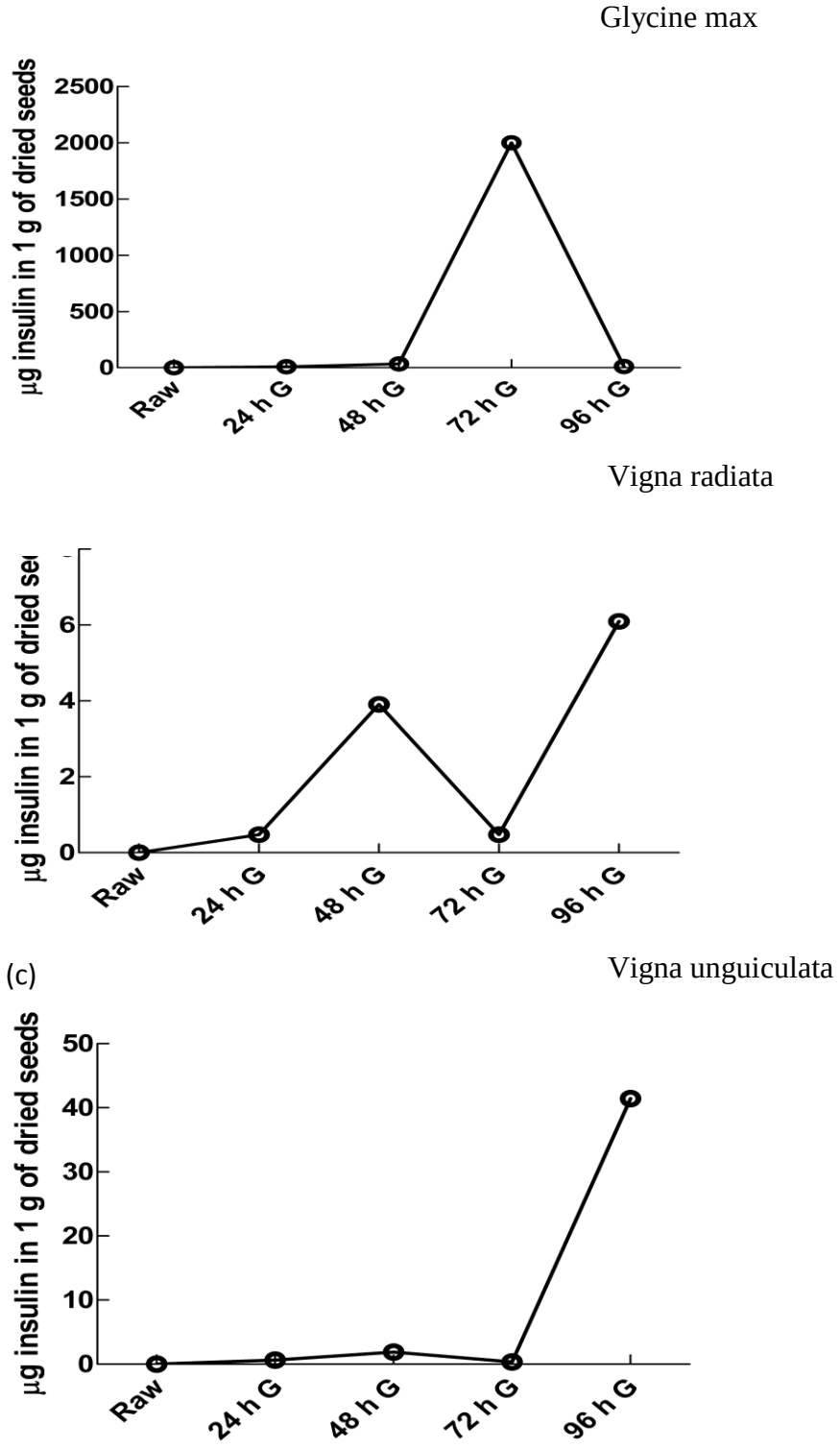


Figure 2. Insulin levels per gram of dried seeds of (a) *G.max*, (b) *V.radiata* and (c) *V.unguiculata.* ELISA data observed in freshly prepared protein samples were used.

This investigation shows that soybean sprout is a source of insulin. Therefore, development of new functional food products with soybean sprouts would be beneficial for people who suffer from type 2 diabetes, or who are in the pre-diabetic stage. On the base of recent functional product formulation VGT.CO (Figure 3, we created a new formulation (Figure 4 and Figure 5). of VGT.CO.Spr.SB50 which had substitution of 50% of kidney be by sprouted soybeans (Figure 4) and in the formulation of VGT.CO.Spr.SB100 we have substituted 100% of kidney beans by sprouted soybean (Figure 5).

Figure 3. Nutrition Facts of VGT.CO (Salad with kidney bean)

Nutrition Facts

Serving Size 100 g
Servings Per Container 5

Amount Per Serving	
Calories 130	Calories from Fat 70
Calories from Saturated Fat 5	
	% Daily Value*
Total Fat 8g	**13%**
Saturated Fat 0.5g	**3%**
Trans Fat 0g	
Polyunsaturated Fat 2.5g	
Monounsaturated Fat 5g	
Cholesterol 0mg	**0%**
Sodium 120mg	**5%**
Potassium 300mg	**9%**
Total Carbohydrate 11g	**4%**
Dietary Fiber 2g	**10%**
Soluble Fiber 0g	
Insoluble Fiber 0g	
Sugars 2g	
Protein 3g	**6%**

Vitamin A 20%	•	Vitamin C 15%
Calcium 2%	•	Iron 6%
Vitamin D 0%	•	Vitamin E 8%
Vitamin K 40%	•	Thiamin 6%
Riboflavin 2%	•	Niacin 2%
Vitamin B6 6%	•	Folate 15%
Vitamin B12 0%	•	Biotin 0%
Iodine 0%	•	Magnesium 6%
Manganese 10%	•	Phosphorus 6%
Selenium 0%	•	Zinc 2%
Pantothenic Acid 2%		

* Percent Daily Values are based on a 2,000 calorie diet. Your daily values may be higher or lower depending on your calorie needs.

	Calories	2,000	2,500
Total Fat	Less than	65g	80g
Sat Fat	Less than	20g	25g
Cholesterol	Less than	300mg	300mg
Sodium	Less than	2,400mg	2,400mg
Potassium	Less than	3,500mg	3,500mg
Total Carbohydrate		300g	375g
Dietary Fiber		25g	30g

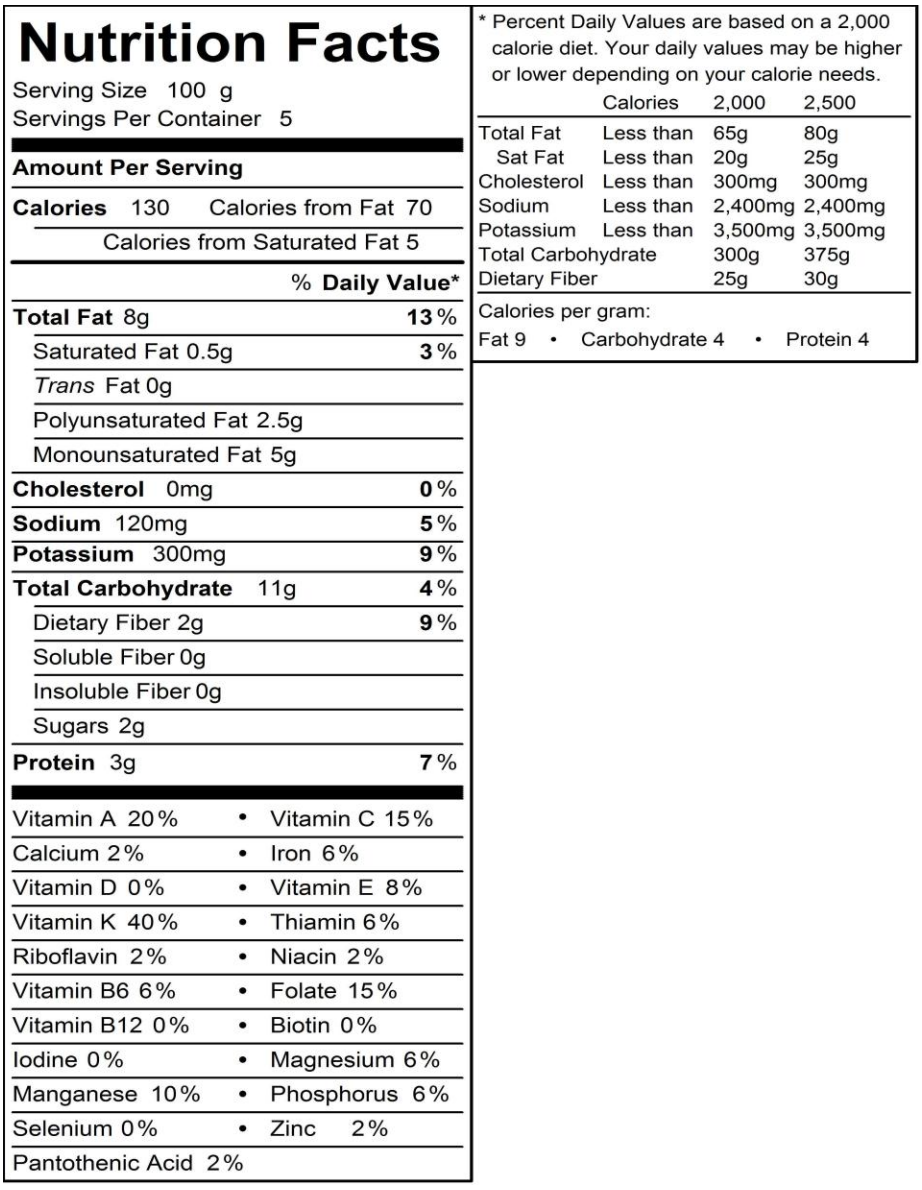
Calories per gram:
Fat 9 • Carbohydrate 4 • Protein 4

Ingredients: kidney beans, beets, carrots, vegetable oil (soybean oil), canola oil, onions, potato, salt, sauerkraut, parsley, and black peppers.

Computerized analysis shows that VGT.CO is cholesterol free, low in saturated Fat (3%), a source of folate (15%), vitamin C (15%), vitamin A (20%) and a good source of vitamin K (40%). It is an excellent source of omega-3 (0.690 g per serving). The ratio of omega 3 to Omega 6 was 1:2.16.

Nutritional composition of new product with 50% sprouted soybean provided in the Figure 4.

Figure 4. VGT.CO.Spr.SB50 Nutrition facts



Nutrition Facts

Serving Size 100 g
Servings Per Container 5

Amount Per Serving	
Calories 130	Calories from Fat 70
	Calories from Saturated Fat 5
	% Daily Value*
Total Fat 8g	**13%**
Saturated Fat 0.5g	**3%**
Trans Fat 0g	
Polyunsaturated Fat 2.5g	
Monounsaturated Fat 5g	
Cholesterol 0mg	**0%**
Sodium 120mg	**5%**
Potassium 300mg	**9%**
Total Carbohydrate 11g	**4%**
Dietary Fiber 2g	**9%**
Soluble Fiber 0g	
Insoluble Fiber 0g	
Sugars 2g	
Protein 3g	**7%**

Vitamin A 20%	•	Vitamin C 15%
Calcium 2%	•	Iron 6%
Vitamin D 0%	•	Vitamin E 8%
Vitamin K 40%	•	Thiamin 6%
Riboflavin 2%	•	Niacin 2%
Vitamin B6 6%	•	Folate 15%
Vitamin B12 0%	•	Biotin 0%
Iodine 0%	•	Magnesium 6%
Manganese 10%	•	Phosphorus 6%
Selenium 0%	•	Zinc 2%
Pantothenic Acid 2%		

* Percent Daily Values are based on a 2,000 calorie diet. Your daily values may be higher or lower depending on your calorie needs.

	Calories	2,000	2,500
Total Fat	Less than	65g	80g
Sat Fat	Less than	20g	25g
Cholesterol	Less than	300mg	300mg
Sodium	Less than	2,400mg	2,400mg
Potassium	Less than	3,500mg	3,500mg
Total Carbohydrate		300g	375g
Dietary Fiber		25g	30g

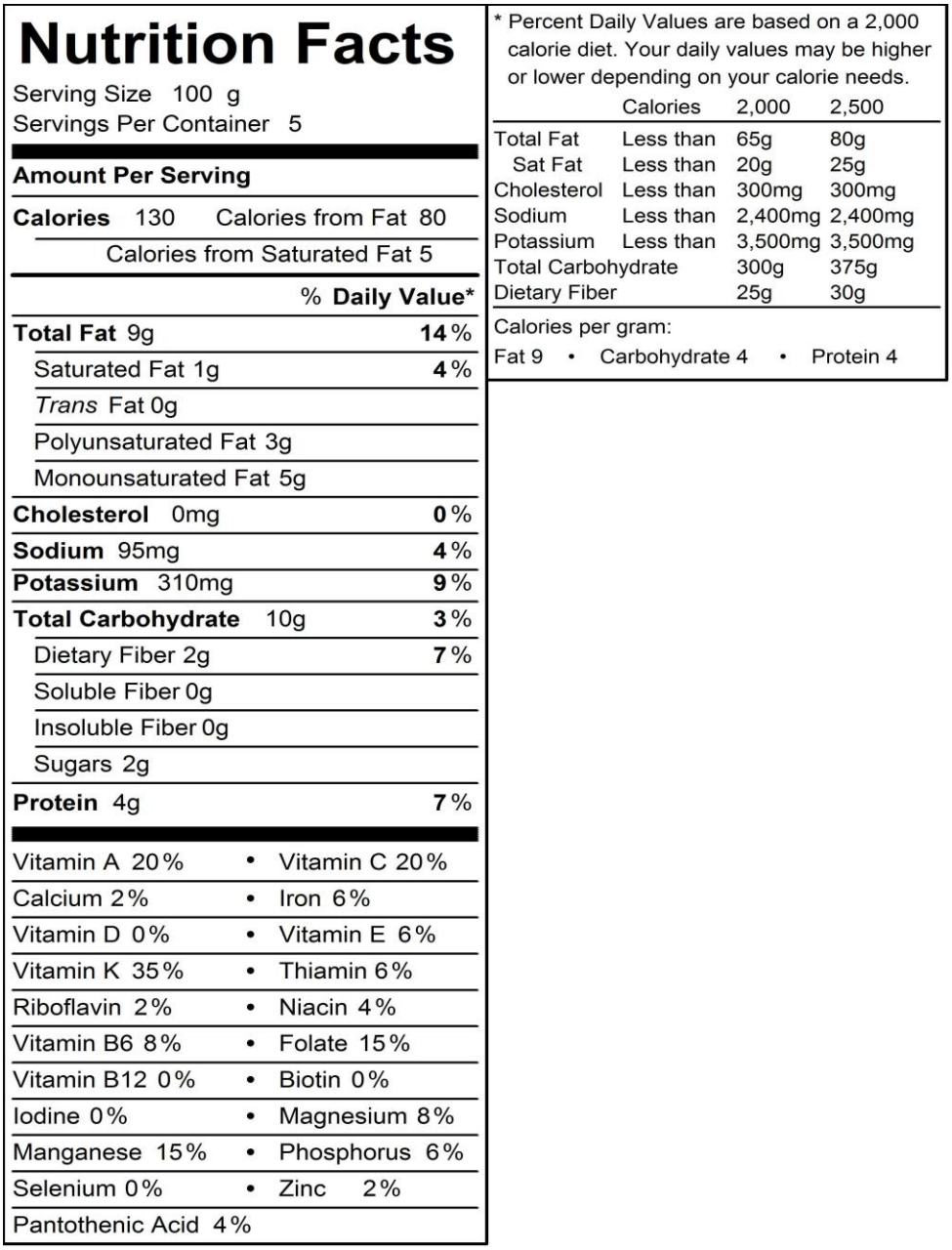
Calories per gram:
Fat 9 • Carbohydrate 4 • Protein 4

Ingredients: kidney beans, beets, carrots, vegetable oil (soybean oil), canola oil, onions, potato, salt, sauerkraut, sprouted soybeans, parsley, and black peppers.

This food is cholesterol free, low in saturated fat (3%), a source of folate (15%), vitamin C (15%), vitamin A (20%) and a good source of vitamin K (40%). It is an excellent source of omega-3 (0.690 g per serving). The ratio of omega 3 to omega 6 is (1:2.63).

Nutritional composition of new product with 100% sprouted soybean provided in the Figure 5.

Figure 5. VGT.CO.Spr.SB.100 Nutrition facts



Nutrition Facts

Serving Size 100 g
Servings Per Container 5

Amount Per Serving	
Calories 130	Calories from Fat 80
	Calories from Saturated Fat 5
	% Daily Value*
Total Fat 9g	**14%**
Saturated Fat 1g	**4%**
Trans Fat 0g	
Polyunsaturated Fat 3g	
Monounsaturated Fat 5g	
Cholesterol 0mg	**0%**
Sodium 95mg	**4%**
Potassium 310mg	**9%**
Total Carbohydrate 10g	**3%**
Dietary Fiber 2g	**7%**
Soluble Fiber 0g	
Insoluble Fiber 0g	
Sugars 2g	
Protein 4g	**7%**

Vitamin A 20%	•	Vitamin C 20%
Calcium 2%	•	Iron 6%
Vitamin D 0%	•	Vitamin E 6%
Vitamin K 35%	•	Thiamin 6%
Riboflavin 2%	•	Niacin 4%
Vitamin B6 8%	•	Folate 15%
Vitamin B12 0%	•	Biotin 0%
Iodine 0%	•	Magnesium 8%
Manganese 15%	•	Phosphorus 6%
Selenium 0%	•	Zinc 2%
Pantothenic Acid 4%		

* Percent Daily Values are based on a 2,000 calorie diet. Your daily values may be higher or lower depending on your calorie needs.

	Calories	2,000	2,500
Total Fat	Less than	65g	80g
Sat Fat	Less than	20g	25g
Cholesterol	Less than	300mg	300mg
Sodium	Less than	2,400mg	2,400mg
Potassium	Less than	3,500mg	3,500mg
Total Carbohydrate		300g	375g
Dietary Fiber		25g	30g

Calories per gram:
Fat 9 • Carbohydrate 4 • Protein 4

Ingredients: beets, carrots, vegetable oil (soybean oil), canola oil, onions, potato, salt, sauerkraut, sprouted soybeans, parsley, and black peppers.

This food is cholesterol free, low in saturated fat (4%), a source of manganese (15%), folate (15%), vitamin A (20%), vitamin C (20%) and a good source of vitamin K (35%). It is an excellent source of omega-3 (0.690 g per serving). The ratio of omega 3 to omega 6 is 1:3.1

Fat composition in VGT.CO.Spr.SB100 provided on the Table 3.

Table 3. Fat Composition in VGT.CO.Spr.SB100

Total Fat	9.027 gr
Saturated Fat	0.769 gr
Monounsaturated Fat	5.102 gr
Polyunsaturated Fat	2.939 gr
Omega-3 Fat	**0.690 gr**
Omega-6 Fat	**2.147 gr**
Trans Fat	0.030 gr
Cholesterol	0.000 mg
Other Fats	8.061 mg

Vitamin Composition in VGT.CO.Spr.SB 100 provided on the **Table 4.**

Table 4. Vitamin Composition in VGT.CO.Spr.SB100

Nutrient	**Amount**	**Units**
Vitamins:		
Vitamin A	44.912	µg RAE
Retinol	0.000	µg RAE
Carotene	44.716	µg RAE
Beta-Carotene	36.962	µg RAE
Alpha-Carotene	7.395	µg RAE
Beta-Cryptoxan...	0.360	µg RAE
Lycopene	0.000	µg RAE
Lutein+Zeaxan...	4.612	µg RAE
Thiamin (B1)	0.103	mg
Riboflavin (B2)	0.048	mg
Niacin (B3)	0.619	mg
Vitamin B6	0.142	mg
Vitamin B12	0.000	µg
Betaine	22.054	mg
Biotin	0.000	µg
Choline	6.290	mg
Total Folate	62.032	µg
Food Folate	62.032	µg

Folic Acid	0.000	µg
Inositol	0.000	mg
Pantothenic	0.322	mg
Vitamin C	11.727	mg
Vitamin D	0.000	µg
Total Vitamin E	1.394	mg
d-Alpha-Tocoph...	0.000	mg
dl-Alpha-Tocoph...	0.000	mg
Beta-Tocopherol	0.002	mg
Gamma-Tocoph...	2.073	mg
Delta-Tocopherol	0.075	mg
Alpha-Tocotrienol	0.000	mg
Beta-Tocotrienol	0.000	mg
Gamma-Tocotrie...	0.000	mg
Delta-Tocotrienol	0.000	mg
Vitamin K	29.732	µg
Other Vitamins	0.000	mg

Sterols composition in VGT.CO.Spr.SB100 provided on the Table 5.

Table 5. Sterols Composition in VGT.CO.Spr.SB100

Sterols:	
Betasterol	31.211 mg
Campesterol	18.213 mg
Cholesterol	0.000 mg
Phytosterol	8.06 mg
Stigmasterol	0.227 mg

DISCUSSION:

The changes in levels of insulin with regards to changes in the germination period indicate that the expression of these proteins is strongly regulated during germination (Table 1). Metabolism commences in the seeds as soon as their cells are hydrated. Respiration and protein synthesis starts after imbibition, using components conserved in the dry seed. This is followed by synthesis of RNA and DNA repair and synthesis. The final event is the expansion of the cells of the radical, which precedes cell division and mobilization of cell reserves. In soybean, PI3K synthesis has been reported in 72 hours old seedlings [9], which can be correlated with the maximum levels of insulin in 72 hour germinated seeds of soybean observed in our study. This is a phase of cell division and elongation of radicals that can be associated with insulin-activated signaling. Insulin activated signaling is suggested to be required in the protein synthesis and in cell proliferation. Also, it might play a role in sugar metabolism and transport during

germination. So, insulin synthesis can be said to be highly regulated in the process of germination, which is reflected in the observations of our study in which the levels of insulin are varying with various germination periods according to the requirement of these proteins for the various physiological processes that take place during germination.

Expression of insulin only in germinating seeds

It is very clear from the observations obtained from six ELISA experiments that there is absolutely no insulin expression at the dormancy phase of the three leguminous seeds under investigation. The insulin is expressing only when embryo development occurs. The expression of insulin during germination is in a very defined pattern. Intermediate kinases of insulin signaling pathway like identification of phosphatidyinositol-3-kinase (PI3K) in soybeans during nodule organogenesis that is associated with membrane proliferation[10] and is the target of Rapamycin kinase (ZmTOR)[17] and S6 ribosomal protein kinase (ZmS6)[11,12] in maize during germination suggests the induction of insulin signal transduction during germination. Seed germination is a critical process in plant growth and development, during which the quiescent embryonic cells shift into a metabolically active state in which complex biochemical and physiological changes occur. The absence of proteins in raw seeds with positive immuno-reactivity to anti-insulin antibodies and the presence of such proteins in the seeds after various periods of germination would suggest that insulin-like proteins are expressed only during seed germination, and appears to be particularly associated with this process. Expression of these proteins during germination, which involves metabolism reactivation, translation of stored mRNAs followed by transcription and rapid growth, and cell division, strongly indicates that insulin-like proteins have an association with some of these processes. *Arabidopsis thaliana* SHAGGY-like kinases (AtSK), which are the homologues of the mammalian Glycogen synthase kinase (GSK-3), were found in tissues during gametophyte and embryo development [12]. These kinases play an important role in cell growth and differentiation and protein synthesis in mammals.

Comparison of three formulations, (Figures 3,4,5) show that VGT.CO.Spr.SB.100 product with 100% of soybean sprouts alongwith base formulstion provided the best yield of Vitamin C, (20% of daily recommended value), and Manganese, (15% of daily recommended value). It was found to be a very good source of Vitamin K, as well as omega 3 fatty acid. But, the content of omega 6 is more in formulation of VGT.CO.Spr.SB.100, and the ratio of omega 3 to omega 6 was increased. So, for better ratio of omega 3 to omega 6 product VGT.CO.Spr.SB.50 looks more attractive. Data provided on the Table 5 shows that VGT.CO.Spr.SB100 is cholesterol free. The sterol content of VGT.CO.Spr.SB.100 was 31.211mg for betasterol and 18.213 mg campesterol which are known to improve plasma cholesterol levels via regular dietary intake [13]. Thus the nutritional composition of the developed formulations emphasizes its immense health benefits. The beneficial effect involves improved bone health [14] due to the presence of Vitamin K and manganese, reduced blood plasma sugars due to its insulin-like activity and most important effect in improving cardiovascular health) due to the presence of excellent amount of omega 3 fatty acid [15] as well as ratio of omega 3 to omega 6 fatty acid and plant sterols.

Future directions: We have to find out the levels of insulin-like proteins in germinated seeds of soybean: (a) *Glycine max*, (b) *Vigna radiata* and (c) *Vigna unguiculata* untilgarmination process is stopped, to see the pick of insulin-like proteins synthesis.

CONCLUSIONS:

1. The present study provides valuable insight for exploring the possibility of sprouts as an alternate source of insulin, and for the development of functional foods for treating diabetes.
2. The development of two new products VGT.CO.Spr.SB.50 and VGT.CO.Spr.SB.100 are cholesterol free, and very low in Saturated Fat. They are also a good source of Vitamin A and Vitamin C, a very good source of Vitamin K, and an excellent source of Omega-3.

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