e-ISSN: 2248-9126
Vol4|Issue 1| 2014 |26-33. Print ISSN: 2248-9118

Indian Journal of Pharmaceutical Science & Research

www.ijpsrjournal.com

MALUS DOMISTICA AS AN INHIBITOR OF GLYCATION

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ABSTRACT

Diabetes mellitus is a metabolic disorder that is principally characterized by insulin resistance, relative insulin deficiency and hyperglycemia. The present research work was designed to study the inhibition of glycation with natural Inhibitor *"Malus domistica"*. For this purpose, normal plasma was collected from healthy volunteers. To study glycation inhibition, thirty two combinations were made and all these combinations were placed at 37°C at same time for five weeks. Human normal plasma was used as a protein. Four milliliter of samples was drawn after 1st, 2nd, 3rd, 4th and 5th week of incubation to perform the experiments. Browning production was recorded by taking absorbance at 370nm of all combinations. Dialysis was performed to remove the excess/ free glucose. Glucose and protein estimation was done to check the glucose and protein concentration before and after dialysis. Glycation inhibition was measured with TBA assay and also by ELISA. The activity of I_2 (10 times diluted) inhibitor was approximately high mainly in the 2nd week of incubation. *"Malus domistica"* is effective and efficient in lowering the glycation level in conditions when the level of glucose is high i.e. in diabetes. So the results indicate that in future *"Malus domistica"* can be used for lowering glucose level in the body.

Keywords: Advanced Glycation End products, Non-Insulin Dependent Diabetes Mellitus, Enzyme linked Immunosorbent Assay, Thiobarbituric Acid.

INTRODUCTION

Glycation is a haphazard process that impairs the functioning of biomolecules, Glycation (sometimes called non-enzymatic glycosylation) is the result of a sugar molecule, such as fructose or glucose, bonding to a protein or lipid molecule without the controlling action of an enzyme. [1]. It has been known for a long time that human blood proteins like hemoglobin [2] and serum albumin [3, 4] may undergo a slow non-enzymatic glycation, mainly by formation of a Schiff base between gamma amino groups of lysine (and sometime arginine) residues and glucose molecules in blood (Milliard reaction). This reaction can be inhibited in the presence of antioxidant agents [5] although this reaction may happen normally elevated glycoalbumin is observed in diabetes mellitus [4].

Advanced Glycation End products (AGEs) are formed as a result of a chain of chemical reactions after an initial glycation reaction [6, 7, 8]. AGEs may be formed external to the body (exogenously) by heating (e.g., cooking) sugars withfats or proteins [9]or inside the body (endogenously) through normal metabolism and aging. [10].

The genesis of free radicals in diabetes include autoxidation processes of glucose, the non-enzymatic and progressive glycation of proteins with the consequently increased formation of glucose-derived advanced glycosylation end products (AGEs), [11] and enhanced glucose flux through the polyol pathway [12] Elevated generation of free radicals resulting in the consumption of antioxidant defense components may lead to disruption of cellular functions and oxidative damage to membranes and may enhance susceptibility to lipid peroxidation [13]. Under physiological conditions, a widespread antioxidant defense system protects the body against the adverse effects of free radical production [14]. The antioxidant defense system represents a complex network with interactions, synergy and specific tasks for a given antioxidant [15]. The efficiency of this defense mechanism is altered in diabetes and, therefore, the ineffective scavenging of free radicals may play a crucial role in determining tissue damage [16].

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Recent decades have shown a rising interest in traditional plant treatments for diabetes. Plants often contain substantial amounts of antioxidants including tocopherol (vitamin E), carotenoids, ascorbic acid (vitamin C), flavonoids and tannins [17] and it has been suggested that antioxidant action may be an important property of plant medicines used in diabetes

The apple is the pomaceous fruit of the apple tree, species *Malus domistica* in the rose family (Rosaceae). It is one of the most widely cultivated tree fruits, and the most widely known of the many members of genus Malus that are used by humans. [18]. Apple is one of the few medicinal plants that have maintained its popularity for a long period of time. They help with diabetes, heart disease, weight loss, and controlling cholesterol. The fiber contained in apples reduces cholesterol by preventing reabsorption. [19] Laboratory studies have also found that components in apple have anticancer activity [20]. Clinical evaluations have revealed that the pharmacologically active ingredients are concentrated in both the extract. Most experimental results were highly encouraging as they revealed that level of blood glucose was significantly lower after oral administration of ethanolic extract of apple in glucose load condition and in STZ-induced diabetes [21].

Apple treatment showed 49% decrease in blood sugar levels and a 52% decrease in triglycerides at day 42 and no change in cholesterol on using along with glibenclamide [22]. Oral administration of processed *"Malus domistica"* prevented the progression of NIDDM-related symptoms in high-fat diet-fed mice, and suggested that PAG could be useful for treating NIDDM [23]. Use of *"Malus domistica"* is being promoted for a large variety of conditions. Oral administration of *"Malus domistica "* might be a useful adjunct for lowering blood glucose in diabetic patients as well as for reducing blood lipid levels in patients with hyperlipidemia [24]. *"Malus domistica"* extract showed hyperglycemic activity on NIDDM rats [25]. The ethanolic extract of *"Malus domistica"* appeared to be more effective than glibenclamide in controlling oxidative stress [26].

The present project was designed to study the effect of aqueous extract of *Malus domistica* on production of advanced glycation end products (AGEs).For this purpose the most active concentration of glucose was optimized and investigated. The most active concentration of inhibitor was also optimized and investigated.

MATERIALS AND METHODS

To study the effect of Apple (*Malus domistica*) on the formation of advance glycation end product *in-vitro* thirty-two combinations (Table 1) were made and all these combinations were placed at 37^{o}C for five weeks. Plasma was used as a protein sample. 0.1 milliliter of samples was drawn after 1st, 2nd, 3rd, 4th and 5thweeks of incubation to perform the experiments for advance glycation end product inhibition. At temperature (37^{o}C) different concentrations of glucose and inhibitor were used. Dilution of plasma and solution of glucose and inhibitor were made in phosphate saline buffer (PBS). (75Mm, pH: 7.4 containing Sodium Azide).

In-vitro Inhibition

In-vitro glycation of plasma (Preparation of Plasma-AGE). Plasma was incubated with all glucose concentrations with and without inhibitor in phosphate buffer saline (containing Sod. Azide) at 37^{o}C for 1-5 weeks simultaneously [27].

In-vitro Inhibition with Plasma

Three different concentrations of inhibitors were incubated with plasma (10mL) and 3 different concentration of glucose for detecting advance glycation end product inhibition.

Glucose Concentrations:

Glucose 1: 50mmol, Glucose 2: 25mmol, Glucose 3: 5.5mmol

Inhibitor Apple (*Malus domestica*) Concentrations:

Inhibitor 1= I_1= Extract (as such), Inhibitor 2 = I_2 =10 times diluted, Inhibitor 3 = I_3= 20 times diluted

Protein (Plasma) Concentrations: Protein: 25mg/ml

Samples were drawn after 1st, 2nd, 3rd, 4th and 5th weeks of incubation to perform the experiments for advance glycation end product inhibition. In above each week 0.1ml sample was taken from original and remaining again placed at 37^{o}C. Then added 3.9ml distilled water in it and made volume 4ml. Then absorbance was taken at 370nm. Sample blanks will be run with each condition of glucose and inhibitor concentration. Later on samples were dialyzed to get rid of free glucose [28] by using dialyzing membrane.

Total proteins in all samples after dialysis were determined by Biuret method using biuret reagent [29]. Measuring protein before and after dialysis monitored sensitivity and validity of method. Glycation (enzymatic and non-enzymatic) was estimated by TBA technique [30] taking absorbance at 370nm using spectrophotometer. Advance Glycation End Product(AGEs) was determined by using ELISA following the procedure of Zhang *et al.* (2005)[31], using alkaline phosphatase enzyme and paranitrophenyl phosphate as a substrate, Data will be analyzed according to statistical methods like means, standard error of mean and regression techniques [32].

RESULTS AND DISCUSSION

Browning Production Determination at different concentrations of glucose with inhibitor at 37^{o}C in plasma our results indicated that in case of plasma incubated with glucose concentration of G_1 (50mM) showed maximum absorbance (fluorescence) (1.99) at 2nd week of incubation and minimum absorbance at 4th week (0.271). I_1 showed minimum absorbance (fluorescence) at 3rd week (0.217) as compared to I_2 and I_3 at 37 ^{0}C (Fig.1). It indicated that suitable amount of inhibitor (I_1) most effectively decreased browning in 3rd week as compared to other combinations.

In case of plasma incubated with glucose concentration of G_2 (25mM) showed maximum absorbance (fluorescence) (0.938) at 1st week of incubation and minimum absorbance at 3rd week (0.196). I_1 showed minimum absorbance (fluorescence) in 2nd week (0.128) as compared to I_2 and I_3 at 37 ^{0}C in hot extract (Fig. 2). It indicated that suitable amount of hot inhibitor (I_1) most effectively decreased browning in 2nd week as compared to other combinations.

In case of plasma incubated with glucose concentration G_3 (5.5mM) showed maximum absorbance (fluorescence) of (0.89) at 1st week of incubation while value of absorbance decreased to its minimum (.222) in 4th week of incubation. I_2 showed minimum absorbance (fluorescence) in 3rd week (0.072) as compared to I_1 and I_3 at 37 ^{0}C in extract (Fig.3). It indicated that suitable amount of inhibitor (I_2) most effectively decreased browning in 3rd week as compared to other combinations

Malus domistica extract effect on the basis of browning

$I_1$of extract showed minimum browning at glucose concentration of G_1 and G_2 than I_2 and I_3. $I_{2\ showed}$ minimum browning at glucose concentration of G_3 than I_1 and I_3 in extract. Minimum browning is shown by I_2 in *Malus domistica* extract. Overall water extract of inhibitor (*Malus domistica*) showed that compound worked effectively and hence I_2 of extract showed better results.

THIOBARBITURIC ACID TEST

Thiobarbituric acid test is used to measure the Glycation level. Here glycation levels were checked by this test for different concentration of glucose (G_1, G_2 and G_3) and inhibitor (I_1, $I_2$and I_3) at 37^{o}C. Our result indicated that plasma incubated with G_1 (50mM) showed gradual increase in glycation and maximum glycation(10.14 mol/mol) occurs at 5th week of incubation. I_1 showed maximum inhibition (1.6 mole/mole) in 3rd week as compared to I_2 and I_3 at 37 ^{0}C in extract (Fig. 4). It indicated that suitable amount of hot inhibitor (I_1) most effectively inhibited glycation in 3rd week as compared to other combinations.

Plasma incubated with G_2 (25 mM) showed maximum level of glycation(8.182 mol/mol) at 5th week of incubation while decreased glycation level to (4.08 mole/mole) at 2nd week. I_1 showed maximum inhibition (1.571 mole/mole) in 3rd week as compared to I_2 and I_3 at 37 ^{0}C in extract (Fig. 5). It indicated that suitable amount of inhibitor (I_1) most effectively inhibited glycation in 3rd week as compared to other combinations. Plasma incubated with G_3 (5.5 mM) showed maximum level of glycation (6.87 mol/mol) at 5th week of incubation while decreased glycation level to (3.28 mole/mole) at 1st week. I_3 showed maximum inhibition (0.89mole/mole) in 1st week as compared to I_1 and I_2 at 37 ^{0}C in extract (Fig. 6). It indicated that suitable amount of inhibitor (I_3) most effectively inhibited glycation in 1st week as compared to other combinations.

Malus domistica extract effect on the basis of glycation

- I_1 (extract as such) showed minimum glycation (maximum inhibition) at glucose concentration of G_1 and G_2 both in 3rd week than I_2 and I_3.
- I_3 of extract showed minimum glycation (maximum inhibition) at glucose concentration of G_3 in 1st week.
- Maximum glycation inhibition is shown by I_1in*Malus domistica* extract.

On thorough study we came to conclude that samples having low browning (fluorescence) had also low level of glycation.

Enzyme linked Immunosorbent Assay (ELISA)

The results plotted, were obtained from ascorbic acid after performing ELISA at 37^{0}C temperature and 5 weeks incubated in *in vitro* conditions.

The results indicate that G_1 (50mM) showed maximum Advance Glycation End Products (AGEs) formation (0.021μg) in 5th week of incubation while decreased Advance Glycation End Products (AGEs) formation (0.006μg) in 1st week. I_2 (200mmol) showed maximum inhibition (0.004μg) in 2st week as compared to I_1 and I_3 at 37 ^{0}C (Fig. 7). It indicated that suitable amount of inhibitor (I_2) most effectively inhibited Advance Glycation End Products in 2st week as compared to other combinations. Our result indicated G2(25mM) showed maximum Advance Glycation End Products (AGEs) formation (0.023μg) in 5th week of incubation while decreased Advance Glycation End Products (AGEs) formation (0.008μg) in 2st week. I_2 (200mmol) showed maximum inhibition (0.006μg) in 2st week as compared to I_1 and I_3 at 37 ^{0}C (Fig. 8). It indicated that suitable amount of inhibitor (I_2) most effectively inhibited Advance Glycation End Products in 1st week as compared to other combinations. Our result indicates that G_3 (5.5mM) showed maximum Advance Glycation End Products (AGEs) formation (0.022μg) in 5th week of incubation while decreased Advance Glycation End Products (AGEs) formation (0.007μg) in 1st week. I_2 (200mmol) showed maximum inhibition (0.005μg) in 1st week as compared to I_1 and I_3 at 37 ^{0}C (Fig. 9). It indicated that suitable amount of inhibitor (I_2) most effectively inhibited Advance Glycation End Products in 1st week as compared to other combinations.

Malus domistica extract effect on the basis of ELISA

I_2 (10 times diluted thanI_1) of extract showed maximum inhibition of advanced glycation end product at glucose concentration of G_1 and G_2 than I_1 and I_3.

I_3 showed minimum AGE at glucose concentration of G_3 than I_1 and I_2 in extract.

Minimum AGE is shown by I_2 in *Malus domistica* extract.

Table 1. Different combinations for Advance glycation end product inhibition

Sr.No	**Combinations**				
	Temperature 37°C				
1.	**Buffer**				
2.			**Plasma**		
3.			Glucose-1		
4.			Glucose-2		
5.			Glucose-3		
6.			Inhibitor I_1		
7.			Inhibitor I_2		
8.			Inhibitor I_3		
9.	Glucose-1	+	Inhibitor I_1		
10.	Glucose-1	+	Inhibitor I_2		
11.	Glucose-1	+	Inhibitor I_3		
12.	Glucose-2	+	Inhibitor I_1		
13.	Glucose-2	+	Inhibitor I_2		
14.	Glucose-2	+	Inhibitor I_3		
15.	Glucose-3	+	Inhibitor I_1		
16.	Glucose-3	+	Inhibitor I_2		
17.	Glucose-3	+	Inhibitor I_3		
18	Plasma	+	Glucose-1		
19.	Plasma	+	Glucose-2		
20.	Plasma	+	Glucose-3		
21.	Plasma	+	Inhibitor I_1		
22.	Plasma	+	Inhibitor I_2		
23.	Plasma	+	Inhibitor I_3		
24.	Plasma	+	Inhibitor I_1	+	Glucose-1
25.	Plasma	+	Inhibitor I_2	+	Glucose-1
26.	Plasma	+	Inhibitor I_3	+	Glucose-1
27.	Plasma	+	Inhibitor I_1	+	Glucose-2
28.	Plasma	+	Inhibitor I_2	+	Glucose-2
29.	Plasma	+	Inhibitor I_3	+	Glucose-2
30.	Plasma	+	Inhibitor I_1	+	Glucose-3
31.	Plasma	+	Inhibitor I_2	+	Glucose-3
32.	Plasma	+	Inhibitor I_3	+	Glucose-3

Fig. 1. Determination of browning at 37°C for 50 mM glucose (G1) in *Malus domistica*

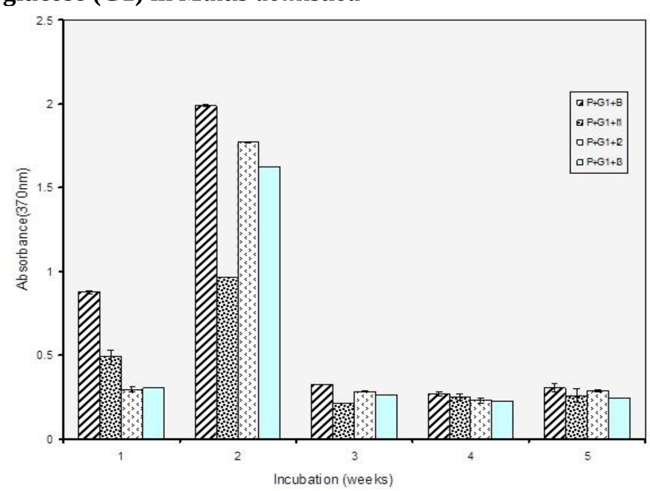


Fig. 2. Determination of browning at 37°C for 25 mM glucose (G2) in *Malus domistica*

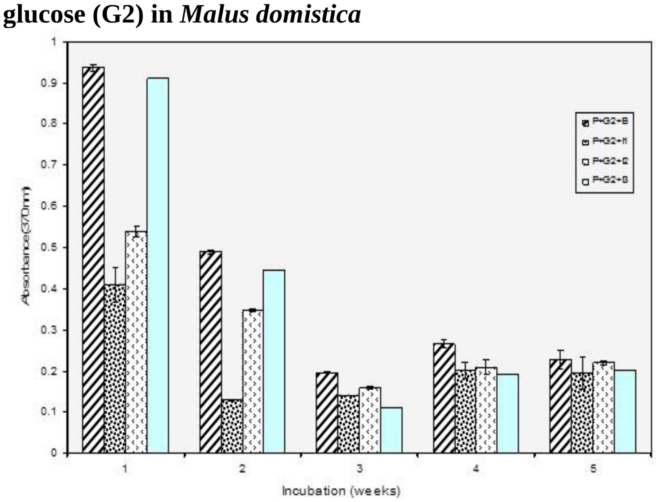


Fig. 3. Determination of browning at 37°C for 5.5 mM glucose (G3) in *Malus domistica*

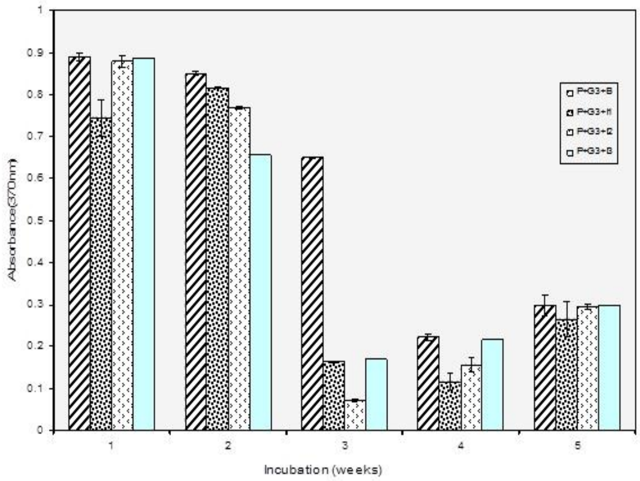


Fig. 4. Determination of Glycation at 370C for 50 mM Glucose (G1) in *Malus domistica*

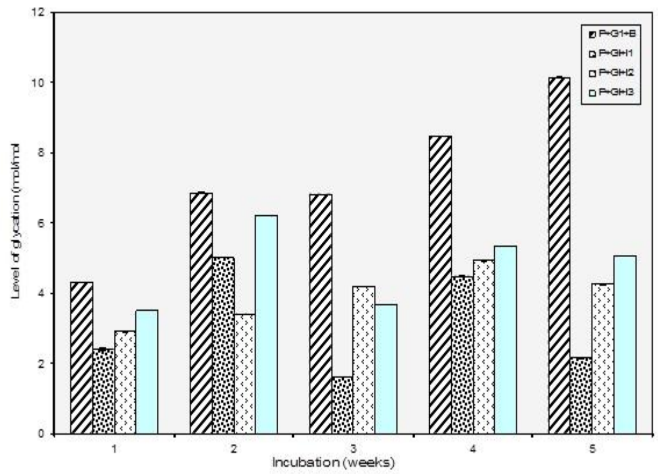


Fig. 5. Determination of Glycation at 370C for 25 mM Glucose (G2) in*Malus domistica*

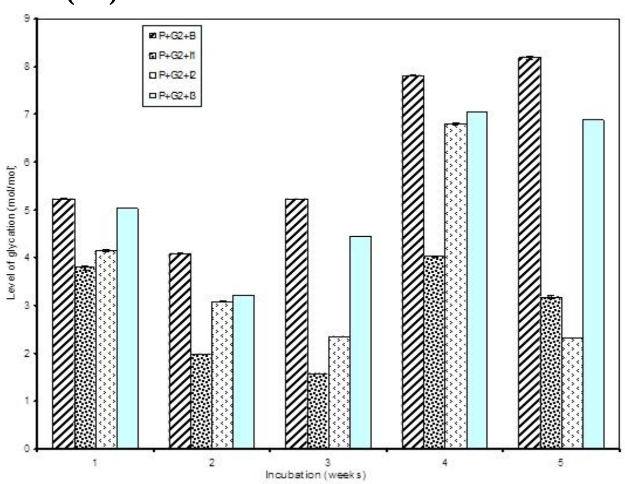


Fig. 6. Determination of Glycation at 370C for 5.5 mM Glucose (G3) in*Malus domistica*

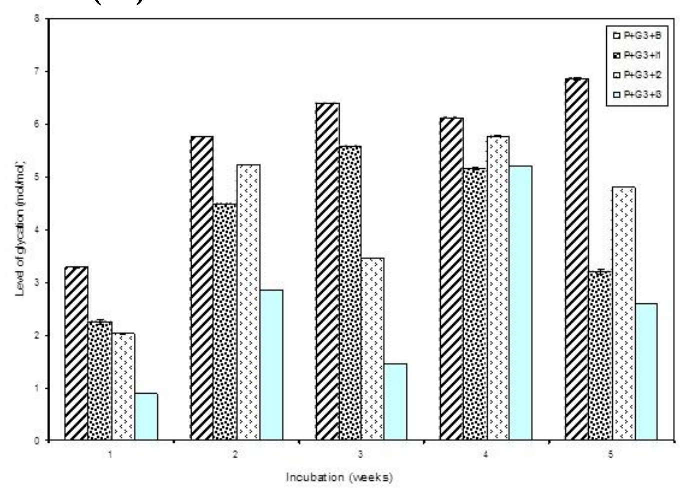


Fig. 7. Enzyme linked Immunosorbent Assay (ELISA) at 37^{0}C for 50mM Glucose (G1)

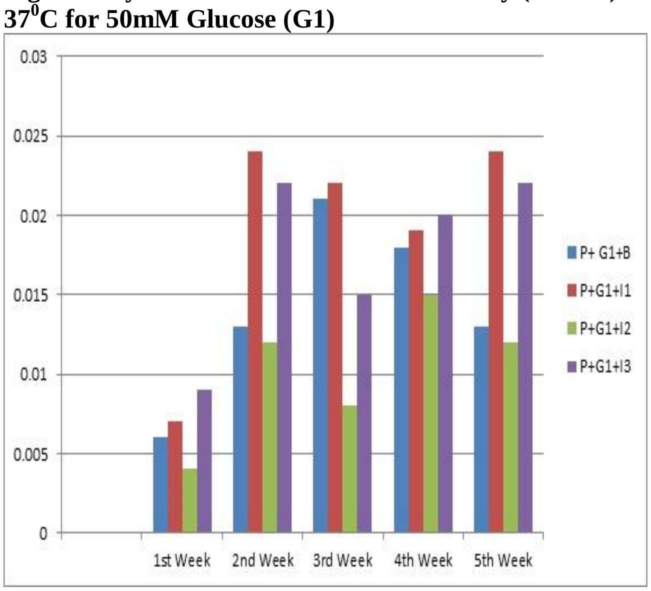


Fig. 8. Enzyme linked Immunosorbent Assay (ELISA) at 37^{0}C for 25mM Glucose (G2)

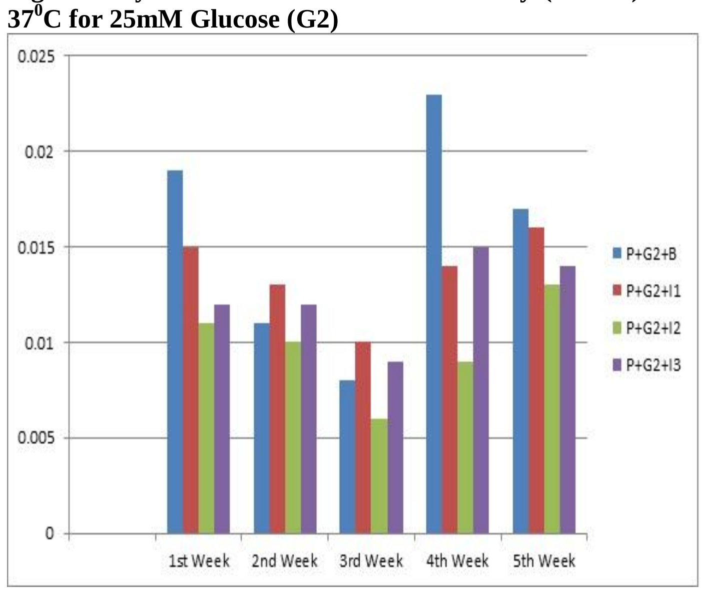
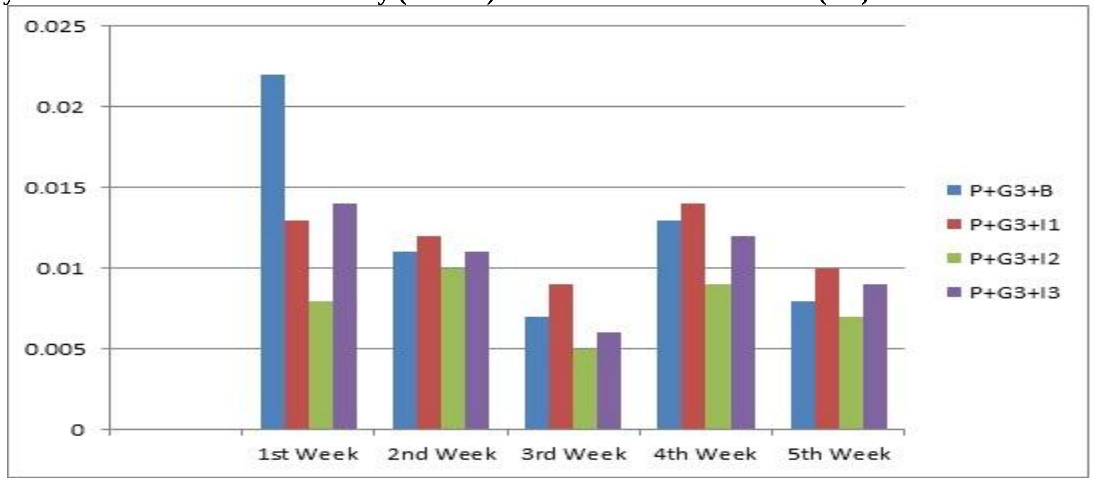


Fig. 9. Enzyme linked Immunosorbent Assay (ELISA) at 37^{0}C for 5.5mM Glucose (G2)



Overall water extract of inhibitor (*Malus domistica*) showed that compound worked effectively and hence I_2 of extract showed better results.

Our results coincided with those of Nishigaki(2010) [33]who reported that Effect of fresh *Malus domistica* extract on glycated protein. In line with our results, it has also been proved that *Aloe vera* is a Hypoglycemic Drugs in Diabetes Mellitus (34-37).

Pearson (1999) [38] findings indicating that *Malus domistica* juice inhibits low density lipoprotein oxidation and Song (2005) [39] found Associations of dietary flavonoids with risk of Type 2 Diabetes. In accordance with our findings (40, 41, 42) also investigated Antioxidant and antiproliferative activities of common fruits. Our results are in accordance with Rendell (1986) [43] who reported the Inhibition of glycation of albumin and hemoglobin by acetylation *invitro* and *invivo*.

CONCLUSION

"Malus domistica" is effective and efficient in lowering the glycation level in conditions when the level of glucose is high i.e. in diabetes. So the results indicate that in future *"Malus domistica"* can be used for lowering glucose level in the body.

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