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SILK WORM AS A MODEL TO EVALUATE HYPOGLYCEMIC ACTION

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ABSTRACT

The Diabetes mellitus (DM) is the major global issue, even though the peoples are applying insulin therapy and several oral formulations which are prepared by ayurvedic as well as synthetic methods. There are enormous numbers of plants are possessing anti diabetic property. By considering this in present study was conducted to demonstrate how locally available medicinal plants is found useful in resolving the diabetes on silk worm as an animal model. The plant extract of *Costus igneus* was employed as a hyperglycemic agent to suppress the hyperglycemic effect caused due to the feeding of the excess of glucose coated leaves. The results demonstrated effective in reducing the levels of glucose, total sugars &glycogen content in hemolymph of the silkworm. Pertaining to the growth those silkworms were restored their growth that was effected due to the elevated levels of glucose, total sugars.

Keywords:*Costus igneus*, Epidemic, Hypoglycemia, Silkworm

Diabetes mellitus (DM) a metabolic disorder, exhibiting hyperglycemic condition either by impaired deficiency insulin secretion or a resistance to insulin action, or combination of both Elberry et al., (2015). DM is rapidly gaining the status of a potential epidemic especially in India; more than 62 million individuals are diagnosed with this disorder. In 2000, India (31.7 million) topped the world with the highest number of people with diabetes mellitus followed by China (20.8 million) with the United States (17.7 million) in second and third place respectively (Kumar et al., 2013). Almost 60% of the world's diabetic population is from Asia (Chan et al., 2009);it affects mortality, treatment cost and quality of life remarkably (Yeung et al., 2017).

The current studies in India indicate that there is an alarming rise in prevalence of diabetes which has gone behind epidemic form. The occurrence and consequences of DM found to be high in countries like India (31.7%), China (20.8%), and U.S.A (17.7%). It is predicted that by 2030, India, China, and United States will have largest number of people with diabetes. With a prevalence of 200,000 type 1 diabetics in India estimated that the cost of treatment could be as high as US$50 million (Shobhana et al, 2002; Piero et al, 2014).

The treatment of diabetes mellitus is main global problem even though insulin therapy and oral anti diabetic drugs are first line treatment for DM. Anti-diabetic oral drugs are used in management of DM they act by increasing insulin release from β cell of pancrease. They include sulfonylureas, biguanides, α-glucosidase inhibitors and glinides which can be used alone or combined with other drugs. Sulphonylureas are useful in treatment of DM which cannot be controlled by diet these are absorbed rapidly from intestine along with a number of medicinal plants and their formulation are used for treating diabetes in ayurvedic medicine system. In India indigenous remedies have been used in treatment of diabetes mellitus huge numbers of plants are used that posses anti diabetic potential (Patel et al, 2011). The Silkworm in Medicinal Research Purpose: study of hormones, brain structure physiology, genetics, discovery of drugs & anti – diabetic drug screening (Matsumoto et al, 2016).

In order to evaluate of anti-diabetic effect it is essential to adopt an appropriate animal model in which plasma glucose levels can be estimated. Thus mice and rats are widely used to screen anti-diabetic drugs, but maintenance of these animals is expensive as well as ethical issues from animal welfare make it inappropriate. To resolve this many models systems are developed using invertebrate animals such as fruit flies (*Drosophila melanogaster*) and nematodes (*Caenorhabditis elegans*). The small body sizes of these animals, however, make it difficult to inject test

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samples and collect hemolymph (Tipping & Perrimon, 2014; O'Reilly et al., 2014). Silkworms arealso widely used invertebrate as animal models to study therapeutic effect of anti-bacteria, anti-fungal & anti-viral (Matsumoto et al., 2015). The properties of silkworm are advantageous for biochemical experiments; they exhibits large size & slow moving behaviors adequate for handling.Hemolymph sampling methods for determining sugar levels in the silkworm are well established (Kurokawa et al., 2007).

In the present scenario diabetes is epidemic issues that need a lot of attention thus it is the most preferred area for research, considering this in present study was conducted to demonstrate how useful the locally available medicinal plant in resolving, utilizing a silk worm as an animal model system.

MATERIALS AND METHODS

Commercially available Recombinant human insulin from Torrent Pharmaceuticals Ltd, India. The leaves of *Costus igneus* were collected from Botanical garden, Karnatak UniversityCampus Pavatenagar, Dharwad, were also used for our study the chemicals used were of analytical grade.

Preparation of leaf disc & coating with glucose

The mature leaves were collected from mulberry farm, they were washed with distilled water and then the leaves discs of equal size were prepared. To the leaf disc glucose solution (1 ml) was coated on each leaf and was kept aside for drying. The sugar coated leaves were cut and feed to the individual silkworms separately in the compartments being prepared in the treys. For each silkworm two leaves of mulberry coated with appropriate sugar was fed.

Silkworm & Mulberry Leave

The silkworms and the mulberry leaves used for the present study were a kind gift from a local silkworm cultivar, Dharwad, Karnataka. The 5th instar second day silkworm larvae were collected from the commercial silkworm cultivars and they were placed in our silkworm rearing laboratory maintained at 23-28^0 C and humidity levels of 85-90%. The larvae were fed with mulberry leaves for 24 hours initially so that they get adapted to lab conditions, before they were used for the experimental purpose, the length and body weight was determined just before they were fed with high glucose diet. Then the larvae were divided into different groups each groups with 15-20 larvae. Group 1: mulberry leaves without glucose coating; group 2: leaves coated with 5% glucose; group 3: leaves coated with 10% glucose; group 4: leaves coated with 20% glucose; group 5: these larvae were kept on starvation. Each group was kept separate in individual trays wherein compartments were made with help of cardboard for each larva.

Preparation of Plant Extracts

Fresh leave of *Costus igneus* (Insulin Plant) were used to prepare extract; the leaves (5 gm) were taken and grind it in pestle and mortar by adding 10 ml of saline all these processes was carried out at 4^0 C. Then the extract is centrifuged at 5,000 rpm for 10 mins at 4^0 C. The supernatant were collected which was injected into the 3rd midgut of silkworm (20 µ) for different groups of larvae.

Insulin

The commercially available Recombinant human insulin (Torrent Pharma Ltd) was procured, and 20µl of insulin were injected into the 3rdmidgut of hyperglycemic silkworms to study its action on controlling the glucose level.

Injecting Test Samples

The silkworms were held firmly in the hand, with the help of a micro injector (20 µl) of the extract was administered into the 3rd midgut of hyperglycemic silkworms.

Hemolymph Extraction

As the Silkworm has open circulatory system thus in order to determine the glucose concentration in it, the hemolymph was collected. By holding the organism very carefully in hand, with the help of sharp scissor the 1st proleg was cut and the hemolymph comes out and it was collected in fresh micro-centrifuge tube and used for total sugar estimation.

Fat Bodies Extraction

Silkworms were held firmly and then it is pinned dorsally on dissection plate. The body of silkworm was dissected with the help of sharp blade and scissors, the forceps and needle are used to remove silk glands, malphigian tubules and other organs. Fat bodies were isolated from dorsolateral region of each

larva and collected in micro-centrifuge tube and used for glycogen estimation.

Processing of hemolymph for quantification of total sugar

It was carried out according to Matsumato et al, (2015); hemolymph (5 µl) was immediately mixed with 9 volumes (45 µl) of 0.6 N perchloric acid. Precipitated proteins were removed by centrifugation at 3,000 rpm for 10mins at 4^0 C. The supernatant (Hemolymph extract) was diluted with appropriate amount of distilled water for sugar quantification. Total sugar in the hemolymph was determined using Phenol sulfuric acid method and serially diluted glucose is used as a standard.

Processing of Hemolymph for Glucose Estimation: Before Injection

Hemolymph (20 µl) was mixed with 190 µl of $ZnSO_4$ and 190 µl of $Ba(OH)_2$. Precipitated proteins were removed by centrifugation at 5000 rpm for 15 minutes. The supernatant was diluted with appropriate volume of distilled water for glucose quantification, serially diluted glucose as a standard.

After Injection

Hemolymph (50 µl) was mixed with 475 µl of $ZnSO_4$ and 475 µl of $Ba(OH)_2$. Precipitated proteins were removed by centrifugation at 5000 rpm for 15 mins. The supernatant was diluted with appropriate volume of water for glucose quantification, serially diluted glucose as standard.

Processing Steps For Fat Body

The processing of the fat body was carried out according to Matsumoto et al, (2015). And the amount of sugarswas quantification by anthrone sulphuric acid method.

The Total Sugars Estimation

The Phenol sulphuric acid method was used for total sugars estimation, according to Dubois et al, (1956) with slight modifications. In hot acidic medium, Glucose is dehydrated to hydroxymethyl furfural. This forms a green colored product with phenol and has absorption maximum at 490 nm.

Glucose Estimation by Nelson Somogyi Method

The Sugars are quantities using the standard Nelson Somogyi assay.

Glycogen Estimation

Glycogen was quantified according to Anthrone sulphuric acid method as described by Hedge et al, (1962).

RESULTS AND DISCUSSION

Impairment in the Weight & Length

It was observed that there was an impairment of the growth viz. body weight and body size in all groups of silkworm except that of with Group 1. As evident the body weight and height of group 1it was 3.5 gm and 3.68 cm respectively.The larvae of group 5 on starvation were dormant and their body weight and length was 2.7 gm and 3.2 cm respectively. Larvae feed with mulberry leaves coated with 5% of sugar (group 2) the body weight and size was slightly altered, weight was 3.3gm and body length 3.5 cm.

The larvae of group 3,also demonstrated similar pattern of the body weight was 3 gm andlength 3.2 cm. And then the group 4 the growth rate was more drastically decreased,body weight 2.8 gm and body size3.1 cm(Fig. 1 and 2).

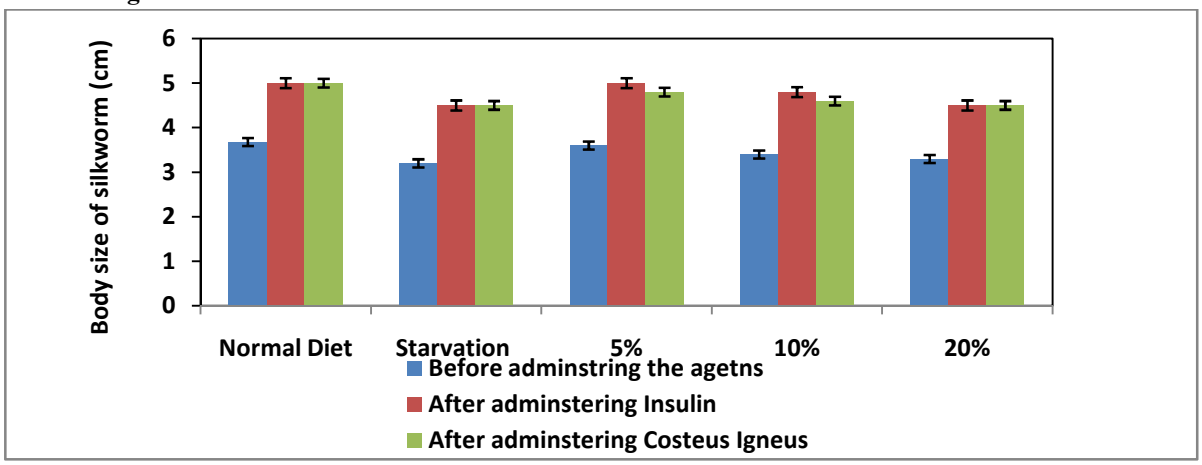

Fig. 1: The effect of *Costus igneus* extract and insulin on growth of the impaired silkworm (body length) previously fed with high- glucose diet

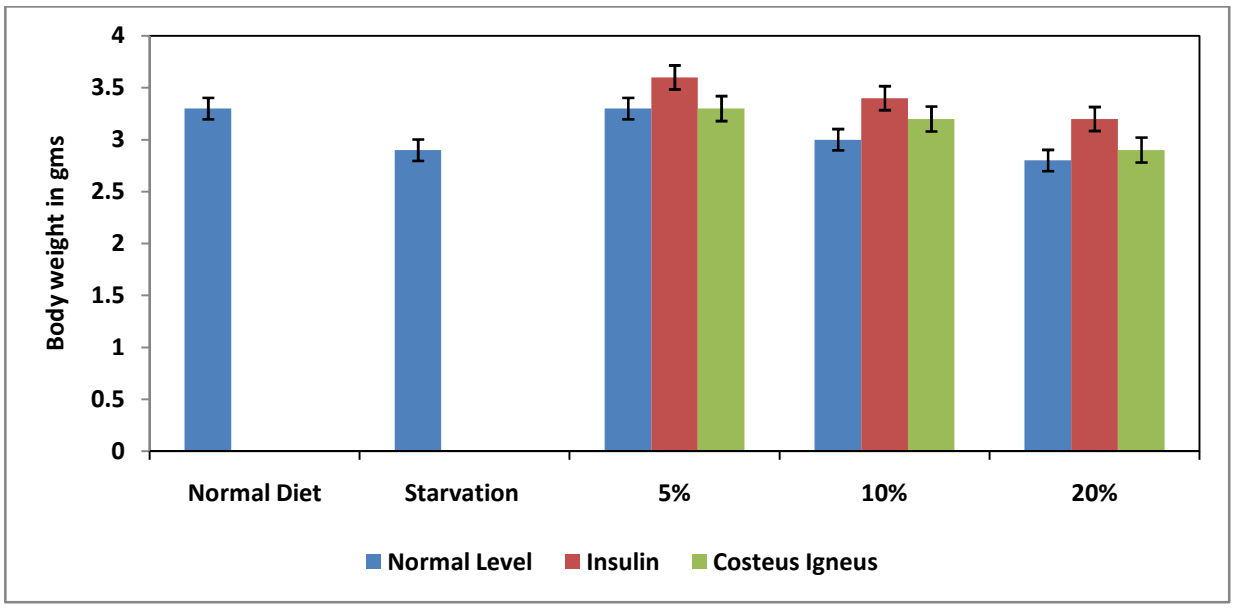

Fig. 2: The effect of *Costus igneus* extract and insulin on growth of the impaired silkworm (body weight) previously fed with high- glucose diet

Total Sugars, Glucose & Fat Body Estimation

Silkworm larvae of group 2;3;4 exhibited greater fold increase in hemolymph the total sugar levels, compared with that of group 1 & 5. In the larvae the hemolymph of group 2 the sugar level was raised by 125% (20 mg/ml); in group 3 it was it was 181% (29 mg/ml); group 4 it was increased by 250% (40 mg/ml), whereas the in group 1 it was (16 mg/ml [considering this as 100% the relative activity was calculated]) & group 5 showed almost half 60% (9 mg/ml)(Fig.3).

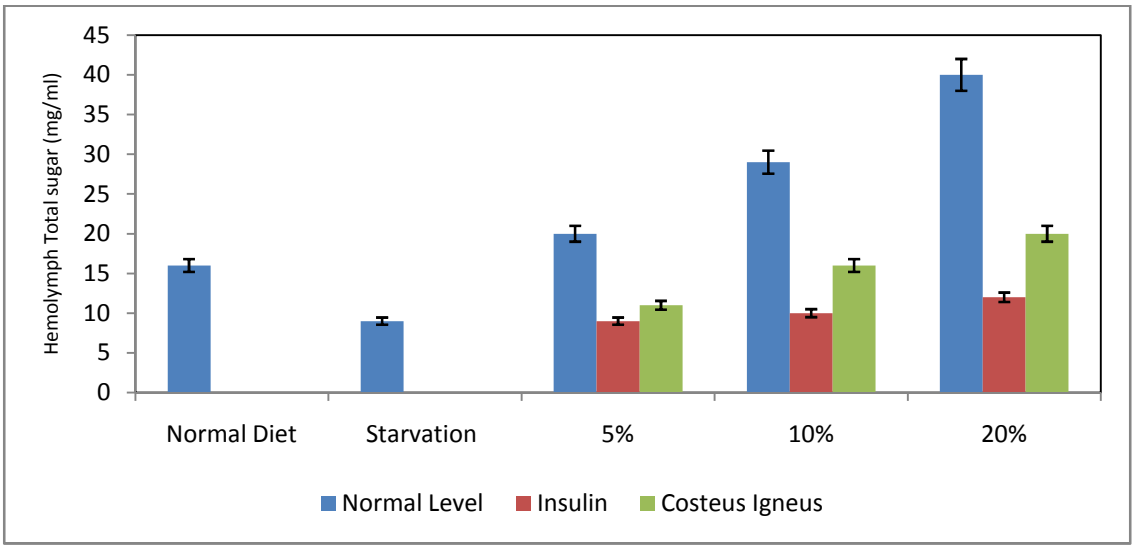

Fig. 3: Effect on Hemolymph total sugar before & after administering *Costus igneus* extract & Human Insulin on impaired silkworm fed with high- glucose diet

Similarly when the glucose levels were estimated using the hemolymph, the group fed high levels of glucose demonstrated elevated levels of glucose, where in group 5 showed least followed by group 1 and subsequently group 2, 3 & group 4 was highest (Fig. 4). These findings indicated that silkworms can be made hyperglycemic by feeding them with a high glucose diet for 12 hrs. Thus we assume that glucose is taken up in the silkworm midgut by transporter mediated system, thereby increasing hemolymph sugar level. Together these finding suggests that silkworms have a regulatory system for maintaining hemolymph sugar level.

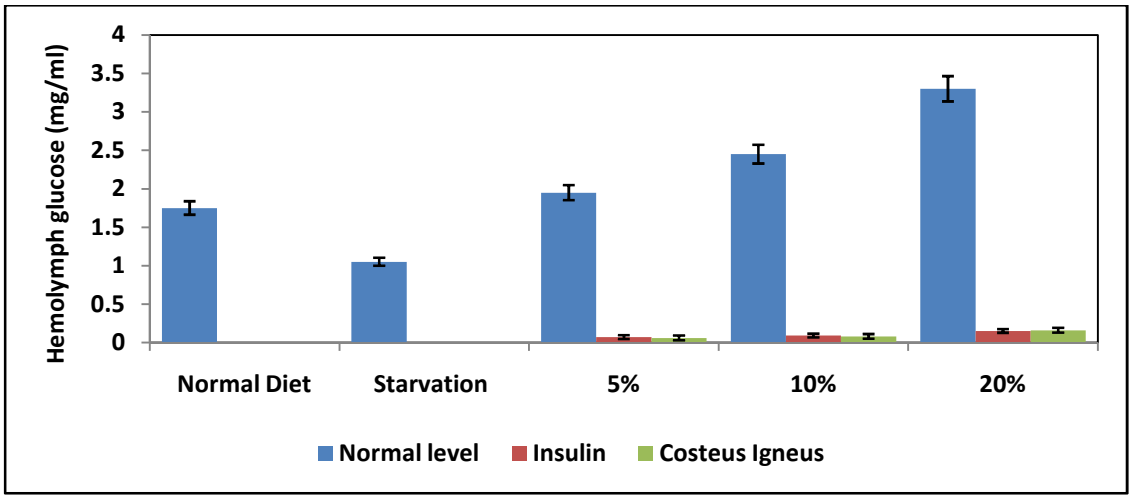

Fig. 4: Effect on Hemolymph glucose before & after administering *Costus igneus* extract & Human Insulin on impaired silkworm fed with high- glucose diet

The fat body in the invertebrate mimics that liver and adipose tissues in the mammals thus the glycogen content in the fat body were estimated. The amount of glycogen in the fat body was also higher in silkworm fed with a high glucose diet. In Group 4 silkworms, there was rapidly increased in glycogen content in the fat body i.e. 347% (0.8 mg/ml), in group 3 it was 195% (0.45 mg/ml) and lastly group 2 it was 134.78% (0.31 mg/ml) respectively. While the glycogen content in normal diet Group 1 it was100% (0.23 mg/ml) whereas in case of starvation condition group 5 silkworm are kept for fasting the glycogen is used up by the body system for energy and the amount was found tobe very less 20% (0.04 mg/ml) (Fig. 5). Therefore hemolymph and fat body sugar levels could be manipulated in silkworms by either feeding them a high glucose diet or by fasting them.

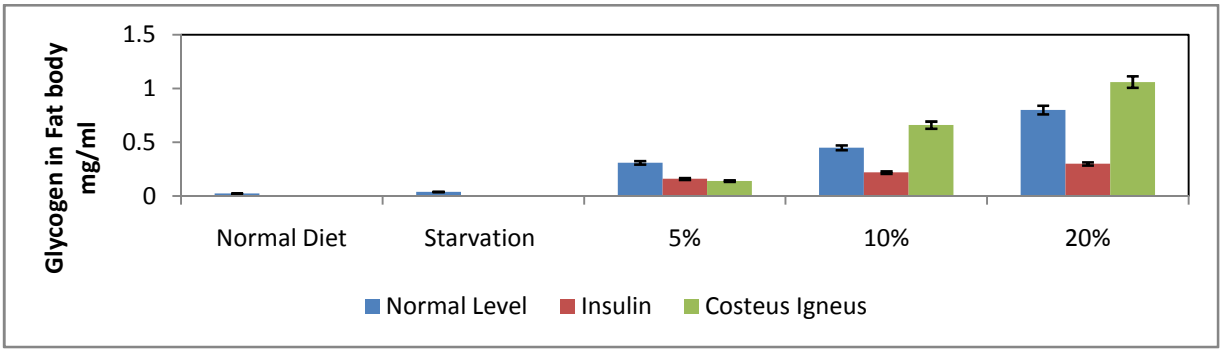

Fig. 5: Effect on glycogen content in fat body of silkworm; before & after administering *Costus igneus* extract & Human Insulin on impaired silkworm fed with high- glucose diet

The present results obtained from the present study are in agreement with the work done by (Matsumoto, et al 2011). They reported following set of results: sugar level in silkworm hemolymph increased immediately after feeding them high glucose diet, compared with silkworms fed a normal diet. The hemolymph sugar level of fasted silkworms was less than half that of silkworm fed a normal diet. The amount of sugar in the fat body of fasted silkworms was less than one-tenth that in silkworms fed a normal diet. The growth defect of hyperglycemic silkworms is caused by Advanced Glycation End products (AGE) accumulation in the hemolymph. Trehalose a dimer of two glucose molecules is a major sugar in insect's hemolymph and glucose is generally not detected in insect hemolymph. The sugar level in the hemolymph was elevated and also the glycogen level in the fat body thus sugar does not passively diffuse into the organ but it is transported by specific uptake system, moreover silkworms have a trehalose and glucose transporter.

In silkworms fed a normal diet, higher levels of Tret 1 are expressed in muscle and in fat body compared to midgut, silk gland or malphighian tubules. The high sugar accumulations detected in organs expressing high levels of Tret 1 indicates the possibility that sugar uptake is regulated by sugar transporters. Trehalose transporter mechanism: In insects, malpighian tubules are functionally analogues to mammalian kidneys. Among sugars, the disaccharide trehalose is highly important to insects because it is the main hemolymph sugar to serve as a source of energy and carbon. The trehalose transporters TRET 1 participate in the transfer of newly synthesized trehalose from the fat body across the cellular membrane into the hemolymph. Hence trehalose is major hemolymph sugar in most insects. It is predominantly synthesized inthe fat body and released into the hemolymph.

Growth

This study revealed that upon administering the leaf extracts, the body growth was restored stating that these leafs can act as an effective antidiabetic agents, In group 2 it was very slight alteration when compared with that of group 4 where in their was drastic alteration in terms of growth here the positive control was group 1 and negative control was group5. In present study human insulin was also used as control for comparing the efficiency of these leaf extract in restoring the growth as well as in regulation in the sugar levels.The body weight and length of silkworms with normal diet (group 1) was 3.5 gm and 3.68 cm respectively. And the starved silkworm larvae (group 5) were 2.9 gm and 3.2 cm. Group 2: A silkworm with 5% glucose diet the body weight was 3.3 gm and length was 3.6 cm. After injecting the extract of *Costus igneus* within 6 hours resulted in recovery in terms of body weight and length which was 3.3 gm and 4.8 cm respectively. Growth 3: At 10% glucose diet the body weight was 3.0 gm and length was 3.4 cm. After treating with *Costus igneus* extract resulted in moderate recovering of growth i.e. body weight was 3.2 gm and length was 4.6 cm. Growth 4: Where as in 20% body weight was 2.8 gm and length was 3.3 cm after treating extract of *Costus*

igneus resulted in rapid recovery i.e. body weight was 2.9 gm and length was 4.5 cm.

Costus igneus leaf extract was effective in controlling hyperglycemic condition. In case of hyperglycemic silkworm larvae of group 2 the hemolymph sugar level was decreased after injecting the leaf extract of *Costus igneus*.Initially Group 2 (5% glucose) total sugar level was 25% (20 mg/ml) after treatment resulted in remarkable decrease i.e. 55% (11 mg/ml).In Group 3(10 % glucose) total sugar in the hemolymph was 81.25% (29 mg/ml) after injecting *Costus igneus* extract total sugar level was decreased i.e. 55.17 % (16 mg/ml) and in higher concentration of Group 4 (glucose 20%) total sugar level was 150% (40 mg/ml). After supplementing with *Costus igneus* extract it showed drastic decrease in total sugar level which corresponds to 50 % (20 mg/ml).Whereasthe total sugar level in the hemolymph of silkworms fed with normal diet was 16 mg/ml and in starvation it was about half of that of normal i.e. 9 mg/ml. The hemolymph glucose level rose exponentially in the group 2, 3 & 4 as compare to Group 1: normal diet (1.75 mg/ml) as well as group 5: starvation (1.05 mg/ml).

After introducing leaf extract of *Costus igneus* in the hemolymph of hyperglycemic silkworms the glucose level was depleted. Earlier the glucose level in group 2 was 11.42 % (1.95 mg/ml) after supplementing with leaf extract *Costus igneus* the glucose level was step down to 3.07% (0.06 mg/ml). And in case of group 3 with excess glucose diet showed 40% increased (2.45 mg/ml) after treating with *Costus igneus* extract it showed remarkable decreased 3.27% (0.08 mg/ml). And lastly ingroup 4 the glucose level was 90.28% (3.3 mg/ml) sharply reduced i.e. 4.8% (0.16 mg/ml)and hence *Costus igneus* was effective in reducing hemolymph glucose level in hyperglycemic silkworms.

Similarly the glycogen level in the fat body was also high before, whereas after administering the leaf extract the glycogen level was also reduced remarkably in group 2, 3 whereas in group 4 initial value was very high but it was also reduced but not that remarkable.

Similar pattern of results were reported by (Manasi *et al*, 2016) they worked on Rat L6 myoblast cell lines from Streptozotocin (STZ) induced diabetic Swiss mice in which they studied hypoglycemic action of insulin like protein from *Costus igneus* and action of *Costus igneus* in controlling hyperglycemia, is in accordance with our results and they are as follows: an orally active insulin-like protein (ILP) from *Costus igneus* having potent hypoglycemic property in STZ-induced diabetic Swiss mice. The blood glucose level was reduced significantly within two hours after feeding ILP orally in an oral glucose tolerance test. It reveals that ILP acts via insulin signaling pathway and can be used as oral insulin mimetic.

When silkworm fed with high glucose diet (5%, 10%, and 20%) there was an accumulation of glycogen content in the fat body (Fig. 5). The amount of glycogen present in fat bodies of silkworm fed with 5% glucose diet was 34.78% (0.31 mg/ml) after administration of leaf extract of *Costus igneus* within 6 hours come down to 45.16% (0.14 mg/ml). At 10% glucose diet the glycogen amount in the fat bodies of silkworm was 95.65% (0.45 mg/ml) after injecting with leaf extract of *Costus igneus* resulted in moderate decreased in the glycogen amount in fat body i.e. 146% (0.66 mg/ml). Glycogen amount in silkworm fed with 20% glucose diet was higher 247.82% (0.8 mg/ml) which was come down to 132.5% (1.06 mg/ml). Glycogen amount in the silkworms with normal diet was 0.23 mg/ml whereas in case of underfed silkworms glycogen amount was 0.04 mg/ml.

The Human insulin was very effective restoring the normal conditions in hyperglycemic model (group 2; 3; 4) by enhancing the growth rate and also helpful in reducing the total sugars, glucose and glycogen levels in all the larvae administered with human insulin. Similarly, Matsumoto et al, (2011) have reported similar kind of experiments wherein they used silkworm as a model system for the evaluation of anti diabetic drugs: In which they have used Human insulin as an anti diabetic drugs: The concentration of the glucose in the hemolymph silkworm fed with 10% glucose diet was reported to be 8mg/ml whereas after administration of insulin depleted the hemolymph sugar level to 6mg/ml. And also suggested that the possibility of evaluating the therapeutic effect of anti diabetic drug in an invertebrate hyperglycemic animal model. They also reported the examination of the human insulin that enhances the uptake of sugar into the fat body of

silkworms by Protein Kinase B (AKt) phosphorylation via activation of phosphoionositide 3 kinase. Therefore the hypoglycemic effects of human insulin in hyperglycemic silkworms are due to activation of the insulin signaling pathway in silkworms similar to mammals.

A peptide hormone called bombyxin present in silkworms with a structural similarity to human insulin bombyxin increases phosphorylated AKt in silkworms. Moreover injection of glucose promotes the release of Bombyxin into hemolymph. Silkworms might control the hemolymph sugar level by activating the insulin signaling pathway with Bombyxin. Impairment in the growth is a characteristic feature of hyperglycemic silkworms due to the accumulation of AGEs. Which was restored to the normal level after injecting with a insulin. Several months are required to induce there complications. By comparisons, the growth defect of hyperglycemic silkworms was observed within 3 days and insulin restored the growth defect in hyperglycemic silkworm.

The administration of recombinant Human insulin decreased hemolymph sugar level in silkworms fed a high glucose diet. In mammals insulin enhances glucose uptake via Akt phosphorylation. Human insulin restored the growth defect of hyperglycemic silkworms. In silkworms fed a 10% glucose diet for 4 days both body size and weight were reduced compare to normal diet.

CONCLUSION

Based on the results obtained, it is concluded that the *Costus igneus* and diabetes powder significantly reduced glucose level in the hemolymph. Eventually, the zone of Inhibition of the growth was restored and total sugar and glycogen amount was decreased after injecting this anti diabetic plant extracts.

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