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A Study On The Pharmacological Effects Of A Flavonoid-Rich *Costus Igneus* And *Trigonella Foenum-Graecum* Foliage On A Diet-Induced Obese Zebrafish (*Danio Rerio*)

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Abstract

The prevalence rates of Obesity and diabetes are steadily increasing worldwide for the past several decades. Many commercial drugs are available for use in the management of diabetes. However, their side effects and high costs emphasize the need for herbal alternative drugs. The present study was designed to evaluate the effect of a flavonoid-rich extract of insulin plant (*Costus igneus*) and Fenugreek (*Trigonella foenum-graecum*) foliage extract on diet-induced zebrafish. **Methods:** Adult zebrafish were divided into four diet groups: (i) control fed (CF); (ii) overfed (OF); (iii) OF supplemented with insulin plant (OF+I); and (iv) OF supplemented with fenugreek foliage (OF+F). The euthanized zebrafish were analyzed for body weight, BMI, Blood glucose, and Triglyceride (TG) level at the end of the fourth week. **Results:** Fenugreek foliage significantly decreased BW, BMI, Blood glucose, and Triglyceride levels in OF zebrafish. Moreover, Histopathological evaluation showed excessive fat accumulation in obese zebrafish liver indicating Non-alcoholic Fatty Liver Disease (NAFLD) when compared with insulin plant. **Conclusion:** This study adds a new insight into the anti-obesity properties of insulin plant and fenugreek foliage and its flavonoids, as weight management agents.

Key words: High-fat diet, Obesity, Costus igneus, Trigonella foenum-graecum, zebrafish

1. Introduction

Obesity is a significant health concern [1]. According to the International Diabetes Federation, (2021) 537 million individuals had diabetes (2021) with Type2 *Diabetes mellitus* (T2DM) accounting for most cases. Obesity is known to cause many non-communicable ailments like cardiac diseases, high blood glucose levels, and musculoskeletal syndromes Meguro [2]. Insufficient insulin production by the pancreatic islet cells leads to T2DM is a chronic illness. It generates various micro and macrovascular problems, with diabetic nephropathy (DN) being one of the most serious microvascular consequences, which can lead to end-stage renal disease Sharchil [3].

Costus igneus, commonly known as an insulin plant in India, belongs to the family Costaceae and consists of four genera and approximately 200 species. The *Costus igneus* is the largest in the family with about 150

species, mainly tropical in distribution. It is believed that consumption of the leaves helps to lower blood glucose levels, and diabetes reported a fall in blood glucose levels. The *Costus igneus* shows various pharmacological properties like diuretic, antioxidant, antimicrobial, and anti-cancerous (OECD, 2015). Leaves of *C. igneus* were one among the plants known to be effectively used for treating diabetes by the tribal people of Kolli hills of Namakkal district, Tamil Nadu Elavarasi, and Saravanan [4]. It reduces fasting as well as postprandial blood glucose levels. But the exact mechanism of action behind the antidiabetic activity is yet to be understood. Along with the antidiabetic activity, insulin plant also reduces diabetes-associated complications; helps to maintain renal, and hepatic parameters to a controlled level, decreases the amount of glycosylated haemoglobin, corrects the lipid profile, increases body weight as well as insulin level and showed marked improvement in the histopathological examination Flowerlet and Bimi, [5]. The diabetes patients containing two leaves daily in the morning and evening for a month help maintain blood glucose levels. Allopathic doctors also recommend this and are found to be effective in bringing blood sugar levels completely under control. The catchphrase of insulin plants is "a leaf a day keeps diabetes away" Meti [6].
Fenugreek (*Trigonella foenum-graecum*), is a historically used herbal medicinal plant that is popular in Africa, India, South, and Central Asia Basch [7]. It is traditionally used to treat several conditions, such as kidney ailments, vitamin deficiency disease, tuberculosis, chronic coughs, chapped lips, baldness, cancer, Parkinson's disease anti-diabetic and anti-carcinogenic quality. It possesses antioxidant, antihyperlipidemic, antibacterial, antifungal, anti-inflammatory, and galactagogic properties Nagulapalli [8]. Fenugreek's pharmacological effects are attributed to a range of bioactive compounds such as polyphenols, steroids, lipids, alkaloids, saponins, flavonoids, hydrocarbons, carbohydrates, galactomannan fiber, and amino acids. Several scientific groups examined its anti-diabetic effect. A previous study showed that fenugreek increased glucose uptake in HepG2 cells is due to the overexpression of the glucose transporter (GLUT-2) Kumar [9] and sterol regulatory element-binding protein (SREBP1C) mRNA levels Naicker [10]. Pradeep and Srinivasan Pradeep and Srinivasan [11], demonstrated the effects of antidiabetic activity combined with 3% onion and fenugreek. A potential fenugreek-based drug was compared to Metformin in a clinical trial that showed that Fenugreek combined with Metformin gave better results than Metformin alone Verma [12]
Obesity and its treatments have been studied in several animal models. Adult zebrafish have been increasingly used to explore common chronic human metabolic diseases such as obesity Seth [13] Nguyen [14]. Scientifically zebrafish are known as *Danio rerio* which is belonging to the family *Cyprinidae* of the order Cypriniformes. Zebrafish is a vertebrate model having a high degree of physiological and genetically based similarities to humans. It is reported that this organism shares 70% of the human genotype with 80% of genes known to be associated with human disease Carnovali [15]. An investigation was undertaken using the zebrafish as a model for the analysis of phenotypic characteristics through induced metabolic disease, obesity in diet, and the potential pharmacological effects of *Costus igneus* and *Trigonella foenum-graecum*.

2. Materials and methods

2.1 Zebrafish husbandry

Zebrafish were maintained at a regulated temperature of 28℃ at a 14 h light: 10 h dark cycle. All animal experiments were performed following the standard methods of Guidance on the housing and care of Zebrafish, Barney Reed & Maggy Jennings, Research Animals Department, Science Group (2010).

2.2 Zebrafish feeding procedures

Adult male Zebrafish (n=60) were randomly divided into four dietary groups (n=15 fish each) as follows: (i) control fed (CF); (ii) overfed (OF) (iii) OF supplemented with insulin plant (OF+I); and (iv) OF supplemented with fenugreek foliage (OF+F). Body weight (BW) and body mass index (BMI) were measured at week 0, at the end of the fourth week. Euthanized zebrafish were analyzed for body weight, BMI, Blood glucose, and Triglyceride (TG) level as shown in Table 1.

Table 1: Zebrafish feeding composition

Feeding groups	Composition
(i) CF	20 mg of fish fed/cysts/fish/day of freshly hatched *Artemia nauplii*
(ii) OF	60 mg of fish fed/cysts/fish/day (20 mg cysts/fish thrice a day) of freshly hatched *Artemia nauplii*
(iii) OF+I	60 mg of fish fed/cysts/fish/day, 20 mg thrice a day + 2 mg Insulin powder/fish/day, fed thrice a day (20 min before *Artemia nauplii* feeding)
(iv) OF+F	60 mg of fish fed/cysts/fish/day, 20 mg thrice a day + 2 mg Fenugreek powder/fish/day, fed thrice a day (20 min before *Artemia nauplii* feeding)

CF- Control feed, OF-Over feed, I-Insulin feed, F-Fenugreek feed

The *Artemia nauplii* (brine shrimp) were purchased from a local pet shop and cultivated in salt water with a motor for aeration and focused with light for temperature maintenance. After 24 hours, the cysts hatched and grew as adults which were given as an overfed to zebrafish for 3-4 weeks to induce obesity as this model is in close resemblance to the reality of obesity in humans. After feeding, it was observed that the fish had consumed the supplied dose and were thoroughly randomly checked to assess for any possible sign of change in behavior throughout the day. The entire protocol took four weeks to complete. In the present study, zebrafish were maintained in different tanks (one zebrafish per 1 L tank; Oka [16] and Montalbano [17]. The course of treatment was followed by the protocol of Montalbano and the methods description partly reproduces their wording [18]. Each fish's phenotypic characteristics including Body weight, length, BMI, Blood glucose, Serum triglyceride, and a histological evaluation of the Liver were determined.

2.3 Determination of body weight, Length, and BMI of Zebrafish

The Body weight (BW), Length (L), and Body Mass Index (BMI) were measured at week 0 and end of Week 4 of feeding experiments and euthanizing the zebrafish using tricaine. The fish were monitored for stage III of anesthesia to prevent the loss of equilibrium, loss of opercular movements, and loss of reactivity. This stage usually will be reached in a minute, after which measurements were taken. For determination of body weight(g), anesthetized fish were dried on Whatman paper and measured to one decimal point in milligrams. Body length (cm) was measured from the anterior-most point of the mouth to the most posterior-most region of the caudal fin (Standard Length, SL) using millimeter paper (to 0.5 mm) (Leibold [19]. Body Mass Index (BMI) (mg/cm^{-2}) values were calculated using weight and body length parameters.

2.4 Estimation of Blood glucose level

Fasting glucose levels were estimated using a Glucose meter (Accu-Sure) from the dorsal artery. Fish were left starving overnight and sedated using tricaine before cutting the dorsal artery. Like the method used to measure blood sugar, blood was drawn with a pipette and transferred to a glucose meter strip.

2.5 Estimation of Serum Triglyceride (TG) level

For the analysis of Serum triglyceride levels, blood samples were collected from the dorsal artery and pooled from all dietary groups from each feeding experiment. Blood was collected and transferred to a microcentrifuge tube and incubated at room temperature for 30 min. The microcentrifuge tubes were then centrifuged at 5000 rpm at room temperature for 15 min after which serum was separated and subjected to triglyceride level analysis using Serum Triglyceride Determination Kit according to manufacturer's instructions.

2.6 Histopathological Analysis of Zebrafish

After a 4-week exposure period, the livers of zebrafish (10 fish for each concentration group) were dissected and fixed in 10 % formalin at 4 °C for 24h. Subsequently, the fixed liver tissues were dehydrated in gradient ethanol, hyalinized in xylene, and embedded in paraffin wax at 56 °C. Then, the paraffin blocks were sectioned at 4 μm thickness. The sections were collected on glass slides and stained with hematoxylin and eosin (H&E) using an H&E Staining Kit.

2.7 Statistical quantification

Statistical analysis was carried out using Student's paired t-test. The results of the study are expressed as mean ± SD, and statistical graphs were drawn with Microsoft Excel 2007. $P < 0.05$ is considered as statistically significant.

3. Results

At the end of the fourth week of the experiment, the Overfed group (ii) zebrafish had a significant increase in terms of both BW and BMI (Table 1). After a fourth week from the start of the experiment, the BW increased in OF+I (iii) treated and untreated OF (ii) groups, although (3.338 ± 1.702) displayed a greater BW and increase than OF (iii) supplemented with Insulin (1.382 ± 0.169) (Fig.1). However, BW showed statistically significant differences between OF vs OF+I from starting and the end of the fourth week. Moreover, starting from the first week of the experiment, compared with the matched untreated (i) group (0.624 ± 0.012) the OF treated with fenugreek foliage (1.123 ± 0.071) showed a modest decrease in BW as expected.

In the study, (Fig. 2) showed the length (L) parameter in the overfed group (ii) exhibited a length of (0.042 ± 0.0005) and fenugreek foliage (iv) group has grown up to (0.039 ± 0.0006) following which insulin plant fed group registered (0.042 ± 0.0001) accordingly.

The BMI showed the same trend an increase in the BW at the end of the fourth week (Fig. 3). The major values were reported in the over-fed (ii) group (0.177 ± 0.040), which differed statistically from those found in the group of fish supplemented with fenugreek foliage (iv) group (0.071 ± 0.001). Conversely, in the fenugreek treated (OF+F) and untreated control fish (i), found a negligible decrease in BW.

Table 2: Measurement of Body Weight, Length, and BMI of Zebrafish

Feeding Groups	Weight (g)		Length (cm)		BMI (mg cm⁻²)	
	Week 0	Week 4	Week 0	Week 4	Week 0	Week 4
CF	0.617±0.067	0.624±0.012	0.033±0.001	0.035±0.0006	0.054±0.004	0.049±0.005
OF	0.617±0.067	3.358±0.759	0.033±0.001	0.043±0.0002	0.054±0.004	0.177±0.040
IF+OF	0.617±0.067	1.382±0.075	0.033±0.001	0.042±0.0005	0.054±0.004	0.080±0.005
FF+OF	0.617±0.067	1.123±0.071	0.033±0.001	0.039±0.0003	0.054±0.004	0.071±0.001

CF- Control feed, OF-Over feed, IF-Insulin feed, FF-Fenugreek feed. Values are expressed as mean ± SEM.

After four weeks of treatment, the morphometric analysis showed varied growth among the four groups. At week 0, measurements for blood glucose level, there is no significant difference observed in the blood glucose levels among the four dietary groups (Table. 2). However, at week 4 (Fig. 4) endpoint, there was a significant increase in the blood glucose levels in the OF (ii) group (0.214 ± 0.025) as compared to the CF (i) group (0.068 ± 0.007). While observing the glucose level OF+F (iv) group showed an exceptionally lower value of (0.100 ± 0.021mg dl) followed by the OF+I (iii) group (0.115 ± 0.017mg/dl). This implies that fenugreek has greater pharmacological properties and the ability to control the glucose level in the zebrafish model (Table 3).

Table 3: Estimation of Blood glucose level

Feeding Groups	Blood glucose (mg/dl)	
	Week 0	Week 4
CF	0.066±0.007	0.068±0.007
OF	0.066±0.007	0.214±0.025
IF+OF	0.066±0.007	0.115±0.007
FF+OF	0.066±0.007	0.100±0.009

CF- Control feed, OF-Over feed, IF-Insulin feed, FF-Fenugreek feed. Values are expressed as mean ± SEM.

As Based on the lipid parameter, high-fat diet-induced obese zebrafish increased their Triglyceride (TG) level (Fig. 5). After week 4 induction with a high-fat diet TG concentration of CF (i) and obese group (ii) was (1326 ± 8.142) and (1856 ± 12.06). Fenugreek (iv) showed effective triglyceride (TG) lowering at week 4 endpoints (1344 ± 12.06) and insulin (iii) group has (1478 ± 5.821). Size differences were recorded between control and obese groups, a condition indicating fat accumulation, especially in the abdominal area.

Table.4 Estimation of Triglyceride level

Feeding Groups	Triglyceride level (mmol/L)	
	Week 0	Week 4
CF	1241±14.78	1326±8.142
OF	1241±14.78	1856±12.06
IF+OF	1241±14.78	1478±5.821
FF+OF	1241±14.78	1344±12.06

CF- Control feed, OF-Over feed, IF-Insulin feed, FF-Fenugreek feed. Values are expressed as mean ± SEM.

To evaluate whether lipids accumulated in the liver, hepatic tissue was stained with haematoxylin and eosin microscopic evaluation of a stained sample of OF (ii) group zebrafish liver tissue revealed the presence of hepatic steatosis (accumulation of fat vacuoles) in zebrafish. No changes were observed in the liver of the CF (i) group (Fig. 6 a) throughout the study period. In overfed zebrafish, most of the cell cytoplasm was occupied by large fat vacuoles (Fig. 6 b). In overfed groups, except for control feeding groups, a marked increase in Oil Red O was observed. This was accompanied by a significant enlargement of the hepatocyte area, with an exponential increase in the size of the hepatocytes, which was already present in each subgroup compared to the control-fed group. Consequently, the Fenugreek-fed group (Fig. 6 c) demonstrated a greater reduction in lipid accumulation (number and size of red spot) of liver tissues compared to the overfed and insulin-fed group (Fig. 6 d) which is in line with the results of the plasma TG lowering.

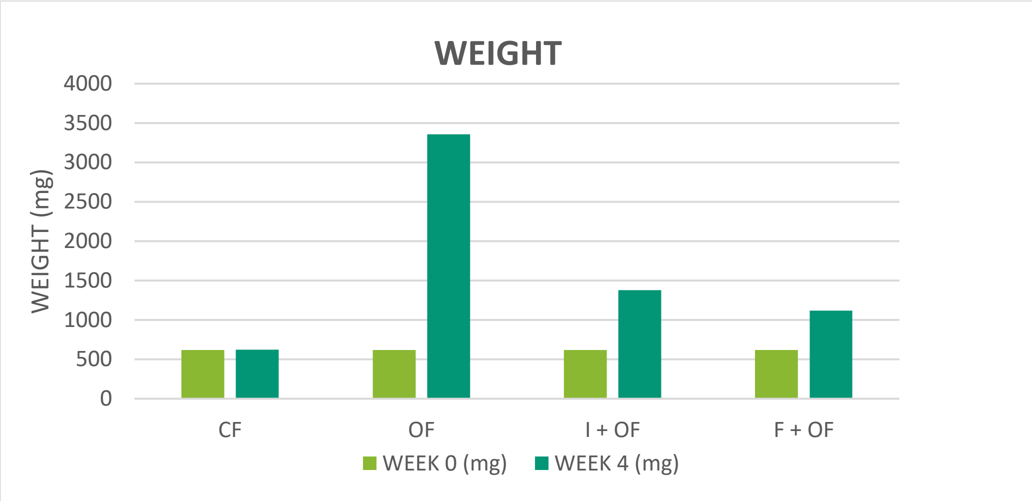

Fig. 1 Weight of the four feeding groups was measured at week 0 and week 4 end-points. Values are presented as mean ± SE.

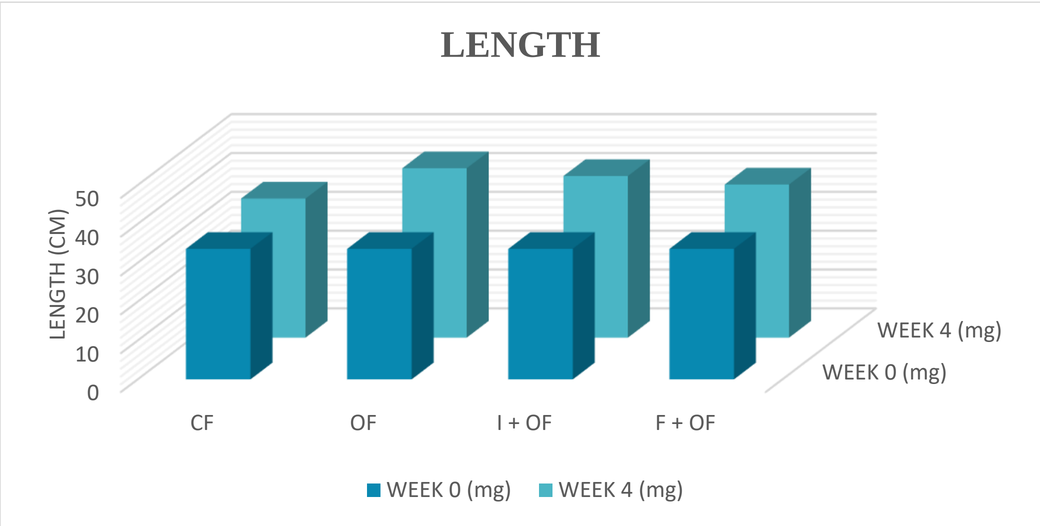

Fig 2. Body length was measured at week 0 and week 4 end-points. Values are presented as mean ± SE

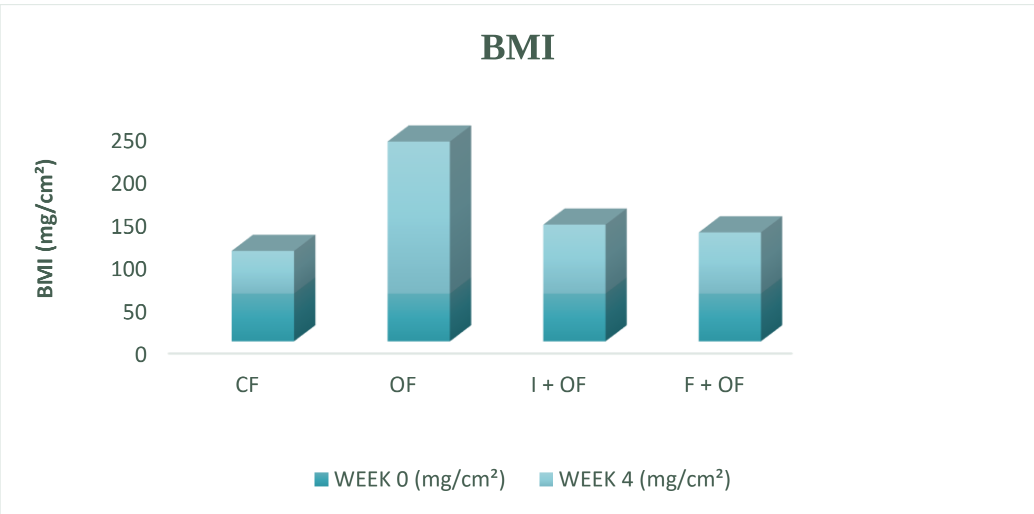

Fig 3. BMI values were calculated using weight and length parameters at week 0 and week 4 end points. Values are presented as mean ± SE

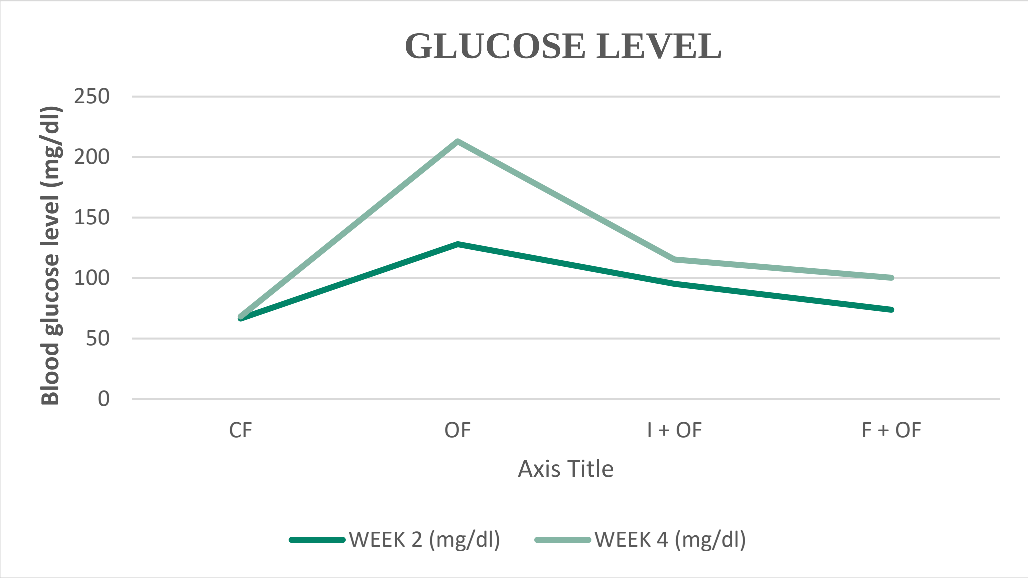

Fig 4. Fasting blood glucose levels were measured at week 2 and week 4 end-points.

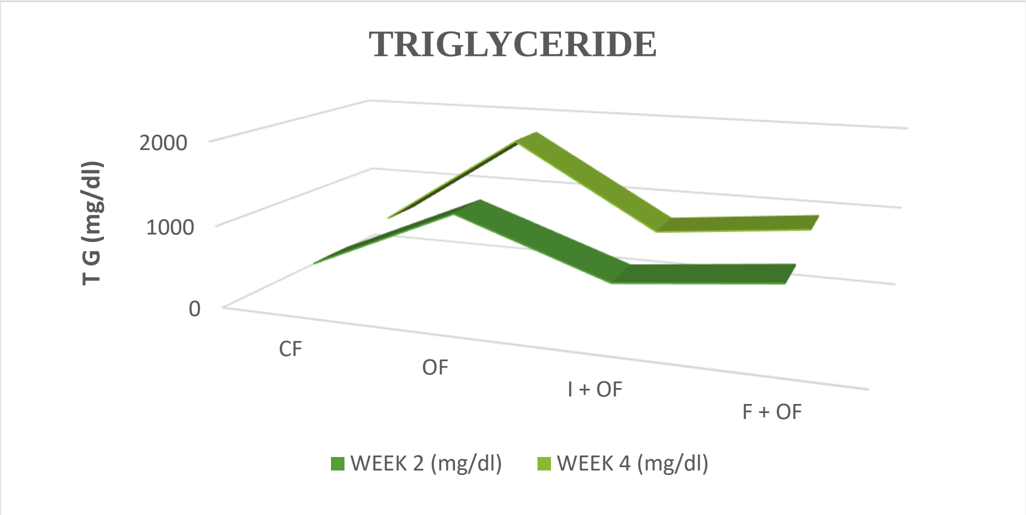

Fig 5. Phenotypic analysis of Serum TG level was measured at week 2 and week 4 end-points.

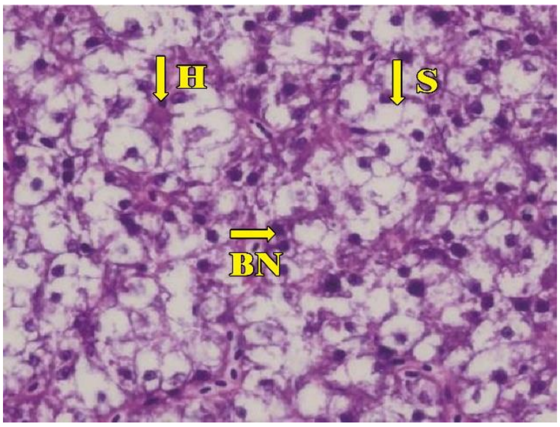

Fig. 6 a, The liver of the control (CF) group showing normal appearance of Hepatocytes (H), Sinusoids(S), Binucleated Hepatocytes (BN)

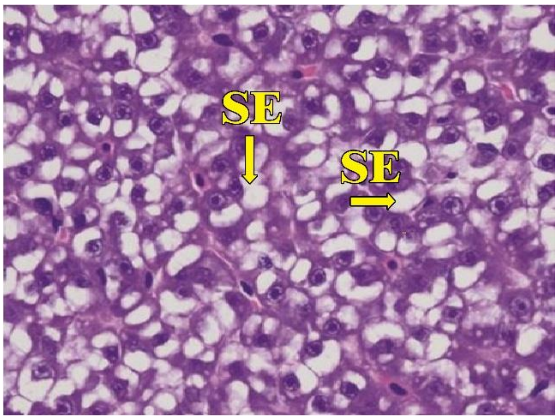

Fig. 6 b, Liver of OF group showing progreeive worsening of Sinusoid Enlargement (SE)

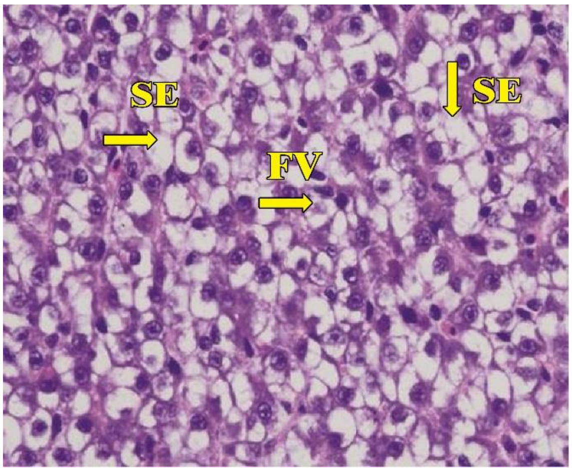

Fig. 6 c, Liver of Insulin (OF+I) treated showing the onset of sinusoidal enlargement (SE), a smallnumber of Fatty Vacuoles (FV)

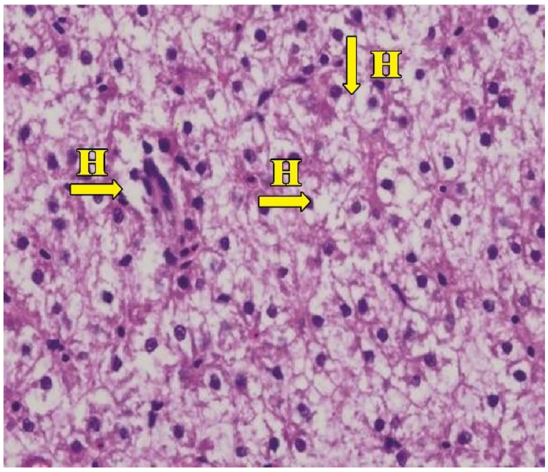

Fig. 6 d, Liver of Fenugreek (OF±F) treated showing control histology of liver hepatocyte was observed (H)

Discussion

Increased fat consumption has been associated with obesity condition. This condition is followed by elevated metabolic syndrome and the development of NAFLD, several researchers reported that a high-fat diet not only induced obesity in humans but also influenced obesity in animals. Therefore, fat accumulation in the animal as a response to high-fat diet administration is mostly used in obesity research applications Lozano [20] Nilsson [21]. Differences with in carbohydrate, protein, and fat diet can stimulate excess energy intake by its highly luscious nature, influencing the satiating power. Long-term exposure to a high-fat diet, especially in hungry conditions, might be sufficient to cause overconsumption of energy with obesity as a result Bray [22]. The present study reported that the flavonoids of insulin plants and fenugreek foliage have an anti-obesity effect on fat accumulation in diet-induced obese zebrafish. As is evident from our data fenugreek foliage exhibited significant blood glucose lowering, triglyceride level lowering, and lipid accumulation lowering effects at week 4 endpoints. Moreover, the insulin plant also remarkably decreased blood glucose, triglyceride, and lipid level. Kim [23], suggested that cinnamon extract has a regulatory role in blood glucose levels and lipids and it may also exert a blood glucose-suppressing effect by improving insulin sensitivity or slowing absorption of carbohydrates in the small intestine. The findings by Kannappan [24] indicated the improvement of glucose metabolism in vivo by cinnamon bark extract in fructose-fed rats. Sharma and Raghuram [25] stated that *Trigonella foenum-graecum* seed increased fecal bile acid and cholesterol excretion. It may be secondary to a reaction between the bile acids and fenugreek-derived saponins causing the formation of micelles too large for the digestive tract to absorb. Kassaian [26] and Gaddam [27] conducted and reported no significant effect of *Trigonella foenum-graecum* seed powder on TC, HDL-C, and LDL-C levels between treatment and control groups. These discrepancies might be possibly due to methodological issues such as differences in the method of preparation, dose, and species identification of *Trigonella foenum-graecum* seed. Hampden [28], reported that the effects of fenugreek oil on the pancreas in an Alloxan-induced diabetic rat model showed that pancreatic cell damage and renal function were slightly reversed after treatment with fenugreek oil. The findings by Bhat [29] Ethanolic extract of *Costus igneus* has a potent antidiabetic effect in alloxan-induced diabetic rats. It had lower body weights, and high blood glucose levels as compared to normal rats. However, orally administered ethanolic extract of *Costus igneus* significantly increased the body weight and decreased blood glucose levels in diabetic rats. This may be due to the improvement of the glycaemic control mechanisms and insulin secretions from remnant pancreatic β-cells in diabetic rats. The exact biological active constituents responsible for the said effect are neither reported nor was the exact mode of action of the antidiabetic activity reported earlier, the observation had it is used in folklore diabetic treatments. Titta [30], showed that orange juice (OJ) diminished high-fat diet-induced body weight gain in mice, reducing the fat mass in both abdominal and inguinal regions by 50%. The histological examination of the adipose tissue displayed a marked reduction in the size of the adipocyte cells and lipid accumulation in OJ-treated mice compared with untreated ones. Ami [31], concluded that combined effect of a high dosage of methanolic plant extract and a low dose of metformin proved more effective, additive, and safe because of the most negligible side effects. Hence, the combined effect of the methanolic extract of *C. pictus* and metformin can manage diabetes mellitus effectively. Hence, verify the data and findings are in strong correlation with the previous reports though this is the first comparative study on the effect of insulin plant and fenugreek foliage in zebrafish.

Conclusion

The result of this investigation revealed that flavonoid-rich insulin and fenugreek foliage possess significant anti-diabetic activity in diet-induced diabetic zebrafish, in a diet-dependent manner. In addition, insulin and fenugreek control glucose metabolism in liver tissue causing euglycemia. Therefore, the combination of insulin and fenugreek can manage diabetes mellitus effectively. Obesity can also be induced in other ways besides adverse diet, or other metabolic diseases like diabetes could be investigated.

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