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**EVALUATION OF ANTI OBESITY ACTIVITY OF** ***CITRUS SINENSIS* & *CITRUS LIMON*** **BY HIGH FAT DIET INDUCED MODEL USING *DANIO RERIO***

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**ABSTRACT**

Obesity, defined by the World Health Organization (WHO) as an abnormal or excessive fat accumulation that may impair health, is becoming one of the greatest challenges to global health in this millennium, with more than 300 million men and nearly 450 million women obese globally in 2018. Obesity is becoming a silent worldwide epidemic, with a steady increase in both adults and children.

Orange (*Citrus sinensis*) and lemon (*Citrus limon*) is a rich source of secondary metabolites which contribute to the pharmacological activities attributed to this plant. Several types of chemical compounds have been identified in fruits. Oranges and lemons are a good source of fiber and potassium, both of which can support heart health.

A large group of high fat diet induced adult zebra fishes were taken, weighed & divided into control, test (15, 30 and 50 mg/dl concentration), and standard groups. After 42 days, compared the total cholesterol level with control group of fishes (without treated test drug). *Citrus sinensis* & *Citrus limon* shows the anti-obesity activity on *Danio rerio*. Different concentration of test sample (15, 30 and 50 mg/dl) are showing the significant anti-obesity activity on zebra fish model where different standard obesity induced model (by using high fat diet induced) was used.

**1. INTRODUCTION**

Obesity, defined by the World Health Organization (WHO) as an abnormal or excessive fat accumulation that may impair health, is becoming one of the greatest challenges to global health in this millennium, with more than 300 million men and nearly 450 million women obese globally in 2018. Obesity is becoming a silent worldwide epidemic, with a steady increase in both adults and children. A suitable and economic experimental model mimicking the human condition would therefore be extremely useful to evaluate preventive measures and novel treatments. Zebrafish (*Danio rerio*) has become a popular model organism for several lines of biomedical research, including developmental biology, genetics, and neurobiology.[1]

There are anti-obesity drugs, affecting the fundamental processes of the weight regulation; however, they have shown serious side effects, which outweigh their beneficial effects. Most recent studies on the treatment of obesity and its complications have focused on the potential role of different plants preparation that can exert a positive effect on the mechanisms involved in this pathology. For instance, anti-obesity effects of green tea and its isolated active principles have been reported in both in vitro (cell cultures) and in vivo (animal models) that possess healthy effects, decreasing adipose tissue through reduction of adipocytes differentiation and proliferation. A positive effect in lipid profile, and lipid and carbohydrates metabolisms were demonstrated as well. In addition, anti-inflammatory and antioxidant activities were studied. [1][3]

Orange (*Citrus sinensis*) and lemon (*Citrus limon*) is a rich source of secondary metabolites which contribute to the pharmacological activities attributed to this plant. Several types of chemical compounds have been identified in fruits. Oranges and lemons are a good source of fiber and potassium, both of which can support heart health. [4][5]

*Citrus sinensis* & *Citrus limon* is a rich source of secondary metabolites which contribute to the pharmacological activities attributed to this plant. Several types of chemical compounds have been identified in fruits. The peel, leaves, juice, and roots of C. sinensis, which include the following groups: flavonoids, steroids, hydroxyamide, alkanes and fatty acids, coumarins, peptides, carbohydrates, carbamates and alkylamines, carotenoids, volatile compounds, and nutritional elements such as potassium, magnesium, calcium, and sodium. [13] [27]

Atorvastatin is a synthetic HMG-CoA reductase inhibitor which lowers plasma cholesterol levels by inhibiting endogenous cholesterol synthesis. It also reduces triglyceride levels. Low density lipoprotein (LDL)-cholesterol and triglyceride levels have been observed with atorvastatin in patients with hypercholesterolemia. Atorvastatin produced greater reductions in total cholesterol, LDL-cholesterol levels than lovastatin, pravastatin and simvastatin. As with other HMG-CoA reductase inhibitors, the most frequently reported adverse events associated with atorvastatin are gastrointestinal effects. In comparative trials, atorvastatin had a similar adverse event profile to that of other HMG-CoA reductase inhibitors. [12]

**2. MATERIAL AND METHODS**

**2.1. Plant Material**

Fresh and healthy *Citrus sinensis* & *Citrus limon* were procured from the locality of Hooghly district, West Bengal in the month of August. The leaf was identified and authenticated from Acharya Jagadish Chandra Bose Indian Botanic Garden, Kolkata, India.

**2.2. Chemicals**

Atorvastatin was obtained from college laboratory (GNIPST), Kolkata, India. Assay kit for HDL, LDL, VLDL were purchased from Apollo Diagnostics Ltd, Kolkata, India. All the reagents (laboratory grade), chemicals (laboratory grade) and instruments (weighing machine, Ph meter, UV spectroscopy & Centrifuge) were used for extraction were obtained from college laboratory (GNIPST), Kolkata, India.

**2.3. Preparation of lemon and oranges extracts**

Fresh lemon and oranges were brought from the market for the experiments, they were thoroughly washed. In case of lemon, it was sliced, and in case of oranges it was peeled off to extract the juices out of it. After the juice collection, it was filtered using a filter paper and funnel. Then it was diluted with distilled water with concentration of 0.5 % v/v. [27-28]

**2.4. Zebra fish maintenance**

Zebra fishes (*Danio rerio*) are kept with lab environment for 7 days. After collection, separated the fishes according to male and female. Then provided proper food and aeration. Zebrafish are kept in a circulating system that continuously filters and aerates the system water to maintain the water quality which is required for a healthy aquatic environment. The circulating system filters needs to be checked and changed regularly to ensure their proper function. These filters need to be changed regularly to ensure proper function and ensure the fresh water supply to all the aquarium. The pH of the system (between 6.8 to 7.5) water should be checked daily. Ideal temperature is 280C with a light: dark cycle of 14:10 hrs. Fishes are divided into different experimental groups. [16-17]

**2.5. LC50 Study of test compound (as per OECD guideline 2019)**

Zebrafishes are maintained under standard laboratory Conditions. Breeding process takes place in a breeding chamber by taking male & female in the ratio 2:3. Proper aeration and temperature is maintained and are kept undisturbed for 24 hrs. After 24 hrs. fish are separated and eggs are collected. Prepare different concentrations of test compound from a stock solution. One control and 8 different concentration of test solution (15 mg/dl, 20 mg/dl, 30 mg/dl, 50 mg/dl, 80 mg/dl, 120 mg/dl, 140 mg/dl, 160 mg/dl) is required. After preparing different concentrated solution, 20 embryos put into each petri plate using 1000 micro litter micropipette. We observe the percentage mortality and defects (Tail bend, neck bend) of embryo after 24-hour, 48-hour, 72-hour, 96-hour, and 120-hour. [37]

**2.6. Zebra fish Anesthesia**

At first, take the number of experimental fish from the aquarium. Then transfer the fish into the anesthetic’s solution using net. For anesthesia, fish are immersed in an ice–water bath (5 parts ice/1-part water at ≤4 °C) for ≥20 min. Then, observe the fish. After some time, fish lay on the bottom of the case and finally stop swimming. When the fish stops to gasp and the operculum movements are slow, the anesthesia procedure can be performed.[34] [36]

**2.7. Blood Collection technique from adult zebrafish**

**2.7.1. *Blood collection technique using*** ***micro-needle*-** It is a type of repeated blood collection technique. Micro-needle made using thin capillaries. Needle tip cut diagonally by using fine scissors. Fishes are anesthetized. Dissolved the needle into the trisodium citrate solution (5 mg/mL) to avoid the blood clotting. When the needle tip is immersed in the trisodium citrate solution, the liquid flows into the needle via capillary action, then expelled by mouth suction through the end of the capillary tube. Needle inserted at 30-450 angle into blood collection site. Blood rising into needle. [40]

**2.8. Induction of obesity**

**2.8.1. *High fat diet induced obesity****-*Zebrafishes are maintained under standard laboratory Conditions. At first, a group of fishes are taken for control group (n=12) and weighed, then a group of adult zebra fishes are taken for treated group (n=12) and weighed. After a few days when the fishes will be habituated in the environment, we started the protocols to obese them. We have fed them a very high cholesterol diet for at least 4-6 weeks. We prepared the high cholesterol diet food by blending the butter & the dry food granules in the motor and pestle. The weight ratio of the butter & food granules was 1:1. While feeding them a high calorie & high cholesterol diet, we have kept the regularity, time intervals, and a calculated, weighted amount of diet. We have regularly checked the before & the after weights of the fishes. We saw the diet is properly working as the fish weights increased gradually. For 80 kg of body mass of animals required maximum 75 grams of fat. So, we fixed our fat content by taking the value of 80 grams fat daily. Now, for a fish weighting 0.008 kg, fat contain in the obesity diet should be 0.008 grams. Normal marketed butter has 80% fat content, so we would need 0.01 grams of butter per 0.008-gram fish body weight. The ratio of the butter & dry food in the special diet was 2:1[1][3]

**2.9. *Determination of total cholesterol level by UV Spectroscopy-***

After carefully the blood was collected by using micro-needle, it was immediately sealed with heat (the blank side of the capillary was sealed using a candle). The sealed capillary tubes filled with blood were the centrifuged for 5 mints. (at 2000 rpm). And after the centrifuge was over, we observed a separate layer of plasma containing all the HDL, LDL, VLDL, and plasma. We collected the plasma in small test tubes or sample tubes. The test tubes were cleaned and marked as standard, control and blank using marker pens. Using the micropipettes 1micro-litre standard reagent and 1 micro-liter plasma was taken and diluted with 1 ml of solution from the test kit. The change of color with time maintaining concentration as a factor was very noticeable, as written in the assay protocol we gave it 30 minutes standby time before measuring the absorbance in the UV machine. The change of color in the standard, test and in the blank were much different. After 30 minutes, we measured the absorbance (505 nm) of the three solutions, the blank, the test and the standard in UV spectrophotometer. We can get the total cholesterol by using this formula- Test absorbance /Standard absorbance × 200. [28]

**2.10. *Treatment with standard drug (Atorvastatin) by using high fat diet induced model****-* Zebrafishes are maintained under standard laboratory Conditions. At first, a group of high fat diet induced adult zebra fishes are taken for control group (n=12) and weighed. Then a group of adult zebra fishes are taken for treated group (n=12) and weighed. After 42 days, a group (n=12) zebra fishes are taken from control group. Then check the total cholesterol level by UV spectroscopy. After 42 days, a group (n=12) of zebra fishes are taken from each concentration (2 mg/2000ml) of standard drug solution (treated group). Then check the total cholesterol level by UV spectroscopy. Then compared the total cholesterol level with control group of fishes (without treated test drug. [12]

**2.11. *Application of Citrus sinensis on Adult Zebra Fish by using high fat diet induced model***- Zebrafishes are maintained under standard laboratory Conditions. At first, a group of high fat diet induced adult zebra fishes are taken for control group (n=12) and weighed. Then a group of adult zebra fishes are taken for treated group (n=36) and weighed, that means a number (n=12) of fishes are taken for each concentration of test solution (15, 30 and 50 mg/dl concentration). After 42 days, a group (n=12) zebra fishes are taken from control group. Then check the total cholesterol level by UV spectroscopy. After 42 days, a group (n=36) of zebra fishes are taken from each concentration of test solution (treated group). Then check the total cholesterol level by UV spectroscopy. Then compared the total cholesterol level with control group of fishes (without treated test drug).[21]

**2.12. *Application of Citrus limon on Adult Zebra Fish by using high fat diet induced model*** Zebrafishes are maintained under standard laboratory Conditions. At first, a group of high fat diet induced adult zebra fishes are taken for control group (n=12) and weighed. Then a group of adult zebra fishes are taken for treated group (n=36) and weighed, that means a number (n=12) of fishes are taken for each concentration of test solution (15, 30 and 50 mg/dl concentration). After 42 days, a group (n=12) zebra fishes are taken from control group. Then check the total cholesterol level by UV spectroscopy. After 42 days, a group (n=36) of zebra fishes are taken from each concentration of test solution (treated group). Then check the total cholesterol level by UV spectroscopy. Then compared the total cholesterol level with control group of fishes (without treated test drug). [13] [27] [21]

**3. RESULTS AND STATISTICAL ANALYSIS**

Statistical analysis of the data was performed using MS Excel and GraphPad Prism software. The data was assessed using one-way ANOVA parametric test followed by Tukey’s multiple comparison test which compared data in respect to control and among themselves.

The value *p< 0.05* was considered to be statistically significant.

\* indicates *p<0.05*, \*\* indicates *p <0.001* and \*\*\* indicates *p<0.0001*.

**TABLE- 1 REPRESENT THE BODY WEIGHT AT DAY 42**

|  |  |  |  |
| --- | --- | --- | --- |
| **Control (gm)** | **Standard (gm) (Atorvastatin)** | ***Citrus sinensis* treated (gm)** | ***Citrus limon***  **treated (gm)** |
| **0.55** | 0.46 | 0.42 | 0.42 |
| **0.53** | 0.43 | 0.38 | 0.39 |
| **0.57** | 0.38 | 0.36 | 0.43 |
| **0.58** | 0.42 | 0.40 | 0.41 |
| **0.63** | 0.52 | 0.47 | 0.47 |
| **0.60** | 0.49 | 0.44 | 0.48 |

**TABLE- 2 REPRESENT THE CHOLESTEROL CONTENT AT THE DAY 42**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Negative Control (mg/dl)** | **Positive Control (mg/dl)** | **Standard drug (Atorvastatin) (mg/dl)** | **Lemon treated (mg/dl)** | **Orange treated (mg/dl)** |
| **265.06** | 367.97 | 272.9 | 281.9 | 286.2 |
| **257.21** | 320.45 | 278.93 | 290.2 | 302.5 |
| **250.7** | 340.6 | 280.45 | 275.1 | 310.8 |
| **261.9** | 351.57 | 260.78 | 296.5 | 305.9 |
| **275.45** | 372.54 | 296.3 | 285.6 | 270.2 |
| **241.8** | 330.2 | 285.1 | 289.3 | 295.7 |

Figure 02- Cholesterol content between negative control, positive control, standard (Atorvastatin), lemon treated and orange treated group

**After 42 days**

**0.8**

**0.6**

\*\*\*

\*\*\*

\*\*\*

**0.4**

**0.2**

**0.0**

**Drug**

Figure 01- After 42 days, the body weight between control, standard (Atorvastatin), *Citrus sinensis* treated and *Citrus limon* treated group

**Cholesterol content of the different classes of fishes from the measurements In the UV-Spectrophotometer**

**400**

\*\*\*

\*\*\*

**300**

\*\*\*

\*\*\*

**200**

**100**

**0**

**Drugs**

Figure 02- Cholesterol content between negative control, positive control, standard (Atorvastatin), lemon treated and orange treated are statistically significant in comparison to negative control group. The P value of unpaired t test was significant at <0.001

Figure 01- After 42 days, the body weight between control, standard (Atorvastatin), lemon treated and orange treated are statistically significant in comparison to control group. The P value of unpaired t test was significant at <0.001

**4. DISCUSSIONS**

Zebrafish is a valuable model in recent biomedical research, and in our comparative anti-obesity model we have firstly created a special dietary plan to obese the fishes and increase their blood cholesterol level. After the dietary protocol was finished, we divided the fishes in separate groups for anti-obesity trails. We have kept the fishes in two different groups and treated them with the oranges & lemons. (With two different concentrations for each). We have used two kinds of lemon-1. *Citrus limon* 2. *Citrus sinensis*

To measure the cholesterol content, we used an assay kit for HDL, LDL, VLDL. We took the blood from the fishes and centrifuged them to obtain the plasma, which was added to Assay kit solutions, then by measuring absorbance, (at 505 nm) we got the cholesterol content values.

After comparing the data of the standard drug and test compounds, the lemons and orange showed very good anti-obesity results.

**5. CONCLUSIONS**

As zebrafish is a cheaper model due to lowered cost in its maintenance and breeding, it is an important advantage to developing countries in Asia, Africa, and South America to invest more in zebrafish research. It is a good exploration model for obesity research as they have a similarity with cholesterol pathophysiology of human beings. The important findings were that both the lemons are significantly reduces body weight as well as the body cholesterol index in the fishes.

It is observed that the different concentration of test sample (15, 30 and 50 mg/dl) are showing the significant anti- obesity activity on zebra fish model where we have used different standard obesity induced model (by using high fat diet induced model).

**6. ACKNOWLEDGEMENT: Nil**

**7. CONFLICTS OF INTEREST: Nil**

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