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EVALUATION OF ANTIHYPERLIPIDIMIC ACTIVITY OF LEAVES EXTRACT OF *DACTYLORHIZA INCARNATA*

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ABSTRACT

High serum cholesterol levels leading to aterosclerosis can cause coronary heart disease (CHD).Hyperlipidemia is a main risk element for the expansion of coronary heart disease and is the most usual source of mortality and morbidity worldwide. presently available synthetic drug of hyperlipidemia are associated with a number of side effects. In recent times, a large volume of work aimed at the efficacy of herbal products, as they are safe and effective alternatives to synthetic drugs.The aim of the present study is to explore the phytochemical profile and anti-hyperlipidemic activity of entire plant of hydroalcoholic extract Dactylorhiza *incarnata* high-fat diet induced hyperlipidemic rats ata dose of 100 and 200mg/kg. Qualitative analysis of various phytochemical ingredient and quantitative analysis of total phenolic and flavonoids were determined by the well-known test agreement available in the text. The activity was evaluate by assessment of serum lipid profile viz. total cholesterol (TC), triglycerides (TG) and high density lipoprotein cholesterol of control and drug-treated animals. Phytochemical analysis revealed the appearance of phenols, proteins and amino acids, carbohydrates, saponins and diterpenes. The extract exhibited a dose dependent anti-hyperlipidemic activity and at dose level 200 mg/kg p.o. the extract indicated a significant decrease in the levels of serum TC, TG and HDL-C. The present study exhibit that the extract exhibits a potent lipid lowering activity in diet induced hyperlipidemia which account for some of the medical claims attributed to this plant.

KEYWORDS: - Dactylorhiza *incarnate,* Antihyperlipidimic activity, Extract

1.INTRODUCTION

1.1Hyperlipidemia

Hyperlipidemia is considered one of the major risk factors causing cardiovascular diseases (CVDs). CVDs accounts for one third of total deaths around the world, it is believed that CVDs will turn out to be the main cause of death and disability worldwide by the year 2020 (Ginghina *et al.,* 2011; Jorgensen *et al.,* 2013). Hyperlipidemia is an increase in one or more of the plasma lipids, including triglycerides, cholesterol, cholesterol esters and phospholipids and or plasma lipoproteins including very low-density lipoprotein and low-density lipoprotein, and reduced high-density lipoprotein levels. Hypercholesterolemia and hyper triglyceridemia are the main cause of atherosclerosis which is strongly related to ischemic heart disease (IHD) . There is a strong relation between IHD and the high mortality rate. Furthermore elevated plasma cholesterol levels cause more than four million deaths in a year (Kumar *et al.,* 2012). Atherosclerosis is a process of arteries hardening due to deposition of cholesterol in the arterial wall which causes narrowing of the arteries. Atherosclerosis and atherosclerosis-associated disorders like coronary, cerebrovascular and peripheral vascular diseases are accelerated by the presence of hyperlipidemia (Wells *et al.,* 2007).Hyperlipidemia relates to increased oxidative stress causing significant production of oxygen free radicals, which may lead to oxidative modifications in low-density lipoproteins, which present a significant function in the initiation and progression of atherosclerosis and associated cardiovascular diseases (Mishra *et al.,* 2011).

1.2 Hyperlipidemia classification

Hyperlipidemiain general can be classified to:

Primary

It is also called familial due to a genetic defect, it may be monogenic: a single gene defect or polygenic: multiple gene defects. Primary hyperlipidemia can usually be resolved into one of the abnormal lipoprotein patterns. (Tripathi, 2008).

Secondary

It is acquired because it is caused by another disorder like diabetes, nephritic syndrome, chronic alcoholism, hypothyroidism and with use of drugs like corticosteroids, beta blockers and oral contraceptives. Secondary hyperlipidemia together with significant hypertriglyceridemia can cause pancreatitis (Joseph, 2011). The main cause of hyperlipidemia includes changes in lifestyle habits in which risk factor is mainly poor diet in which fat intakeform saturated fat and cholesterol exceeds 40 percent of the total calories uptake (Joseph, 2011).

Type	Lipoprotein Elevated	Cholesterol	Triglyceride	Risk of Atherosclerosis
I	Chylomicrons	+	+++	Not elevated
IIa	LDL	++	Normal	High
IIb	LDL + VLDL	++	++	High
III	IDL	++	++	Moderate
IV	VLDL	+	++	Moderate
V	Chylomicrons + VLDL	+	++	Not elevated

Fig. 1: Classification of Hyperlipidemia

1.3 Causes and Risk Factors of Hyperlipidemia

Dietary Causes Dietary Fats and Fatty Acids: Dietary fatty acids are divided into three major classes (saturated, monounsaturated and polyunsaturated fatty acids). The foods that contribute to saturated fatty acids (e.g. myristic acid, palmitic acid, stearic acid, etc) meats (e.g. beef, pork, processed meat products, poultry), 2) milk and other dairy products (e.g. butter, cheese, ice cream, yoghurt), 3) tropical fats (e.g. Coconut, palm oils) and 4) egg (contain proportionately less saturated fat compared to other animal food sources). Monounsaturated fatty acids are present as oleic acid in olive oil, avocado, animal fats, etc. Polyunsaturated fatty acids are the omega-3 fatty acids (e.g. linoleic acid) and omega-6 fatty acids (e.g. linolenic acid) (Fauci *et al.*, 2008).Food choices made by individuals can influence intake of the different saturated fatty acids. Selecting leaner cuts of meat are high in palmitic acid and limiting the amount of lean meat would help in lowering saturated fat intak (Sereday *et al.*, 2004). Milk and other dairy products are high in myristic acid content. Substituting skim milk and non-fat dairy products for whole milk products will result in a reduction of saturated fat such as myristic acid intake.

Dietary Cholesterol: Like other sterols, cholesterol is a sterol i.e. a combination of steroid and alcohol) and lipid (a type of fat). It is found in foods such as eggs and dairy products and is also manufactured in the body, especially the liver. Cholesterol also stabilizes a cell against temperature changes. It is a major part of the membranes of the nervous system, the brain, the spinal cord and the peripheral nerves. In particular, it is incorporated into the myelin sheath that insulates the nerves from the surrounding tissue. Cholesterol is also the forerunner of important hormones such as the female sex hormone, oestradiol and the male sex hormone, testosterone and of vitamin D. Cholesterol is also used to produce the bile which is required to digest the fats in food. Nearly most of the body tissues are capable of making cholesterol, but the liver and intestines make the most.

1.3.1 Other Dietary Factors

Carbohydrates: Dietary recommendations to lower the total fat intake include increasing dietary carbohydrate intake because favorable plasma lipid and lipoprotein levels have been reported for populations and individuals whose habitual diet is rich in carbohydrates. High carbohydrate consumption being associated with a decrease in HDL cholesterol levels. Plasma triglyceride levels are not elevated in these individuals, possibly because obesity is rare (Charney, 1999).

Fiber: Studies have shown that only water-soluble fiber plays a role in lipoprotein metabolism in humans. A meta-analysis of 20 studies found that intake of oat products reduces serum cholesterol levels. The mechanism

by which dietary fiber affects plasma lipid levels is unknown. Insoluble fibers in wheat and vegetables do not to reduce cholesterol, but they do have other beneficial effects.

Protein: Soy protein also lowers serum cholesterol levels in animals and in hypercholesterolemic individuals when compared with casein (a dairy protein) and beef proteins. The mechanism underlying these changes is unknown but it has been stated that soy protein affects cholesterol absorption, bile acid absorption, the insulin-glucagon ratio, serum thyroxine levels and hepatic LDL-receptor activity.

Obesity: For a given level of body mass index (BMI), obesity is associated with hyperlipidemia, insulin resistance and hypertension and independent predictor of coronary artery disease (CAD). A meta-analysis of 70 studies indicated that weight reduction was related to increases in HDL cholesterol levels and significant decreases in total, LDL and VLDL cholesterol and triglyceride levels (Woollett *et al.,* 1992). Although they are not always coincident, obesity is also often accompanied by hyperlipidemia. Both obesity and hyperlipidemia are independently associated with atherosclerosis, non-alcoholic fatty liver disease and insulin resistance (Cortse *et al.,* 1983).

Diabetes and Insulin Resistance: Insulin resistance (type II diabetes) is associated with a number of lipid and lipoprotein abnormalities (Keys *et al.,* 1985). The lipid abnormality is associated with insulin resistance and hyperinsulinemia is hypertriglyceridemia. VLDL and total triglycerides are elevated in individuals with type II diabetes although the exact roles of insulin resistance and hypertriglyceridemia are disputed.

Physical Exercise/Activity: Sedentary lifestyles contribute to the development and maintenance of obesity (Keys *et al.,* 1985). Diet can also change in plasma lipoprotein concentrations that occur with exercise.

Alcohol Intake: Low dose ethanol consumption in healthy volunteers modestly activates hepatic de novo lipogenesis and that the major quantitative fate of ethanol is acetate produced in the liver. The acetate released into the plasma which inhibits lipolysis in peripheral tissues by 53% and whole body lipid oxidation is decreased by 73%.

Contraceptives and Other Pharmacologic Agents: Premenopausal women, using oral contraceptives containing a relatively low dose of estrogen combined with a medium or high dose of progestin had a 24 % higher median concentration of LDL cholesterol than who are not using hormones. Glucocorticoids and estrogens elevate triglycerides and raise levels of HDL cholesterol (Hegsted *et al.,* 1985).

1.4 Treatment of hyperlipidemia

In 1987 the National Institute of Health (NIH) established the National Cholesterol Education Program (NCEP) to be directed by the Adult Treatment Panel (ATP) for the purpose of issuing information for health professionals and the general public concerning testing, evaluating, monitoring and treating hyperlipidemia. An important criterion of ATP guidelines is the development of treatment goals for hyperlipidemia based on patient's risk of CHD.

Therapeutic lifestyle changes

Diet modification, regular physical activity, smoking cessation, and weight reduction should be tried as initial treatment, especially in mild cases of hyperlipidemia and in persons without CHD or CHD risk equivalent and<2 risk factors. It should be kept in mind that when dieting, cholesterol intake is reduced. At the same time, production of cholesterol, especially by the liver, increases. It is recommended that the intake should be restricted to 25%-35% of energy intake and that saturated fatty acids make up less than 7% of energy intake and that cholesterol intake should be less than 200 mg daily. The intake of plant sterol esters and soluble fibre is advisable. A healthy diet can result in 10% to 15% reduction of cholesterol blood level.

Drug therapy

High LDL, the presence of risk factors, and documentation of CHD should qualify initiating drug therapy along with TLC. During the early stages of the hyperlipidemia, blood monocytes and platelets attach to a vessel wall at the sites of endothelial damage. The release of the mediators such as platelet derived groth

factors leads to a proliferation of smooth cells in the intimal and medial lining of the vessel, collagen synthesis, cholesterol uptake and the beginning of the hyperlipidemic plaque results. Plaque ruptures are resulting in the acute syndromes of unstable angina, myocardial infarction and sudden cardiac death (Scott, 1991).

Diagnosis of hyperlipidemia

Hyperlipidemia typically shows no symptoms and can only be detected by a blood test. Screening for hyperlipidemia is done with a blood test called a lipid profile. According to National Cholesterol Education Program (NECP) screening (National cholesterol education program, 1994) should start at age 20, and if the report is normal, it should be repeated at least every five years. Normal levels for a lipid profile (AAFP, 2013) are listed below table 1.1.

Table 1.1: Normal levels for a lipid profile

Lipids	Desirable value	Borderline	High Risk
Cholesterol	Less than 200 mg/dl	200-239 mg/dl	240 mg/dl
Triglycerides	Less than 140 mg/dl	150-199 mg/dl	200-499 mg/dl
HDL cholesterol	60 mg/dl	40-50 mg/dl	Less than 40 mg/dl
LDL cholesterol	60-130 mg/dl	130-159 mg/dl	160-189 mg/dl
Cholesterol/HDL ratio	4.0	5.0	6.0

2. MATERIAL AND METHOD

2.1 Collection of plant material

Leaves of *Dactylorhiza incarnata* was collected from Vindhya Herbal Nursery Bhopal (M.P), India in the months of September, 2020.

2.2 Preparation of plant material for study

Plant materials selected for the study were washed thoroughly under running tap water and then were rinsed in distilled water; they were allowed to dry for some time. Then these plants materials were shade dried without any contamination for about 3 to 4 weeks. Dried plant materials were grinded using electronic grinder. Powdered plant materials were observed for their colour, odour, taste and texture. Dried plant material was packed in air tight container till any further use.

2.3 Extraction procedure

50 gm dried powdered *Dactylorhiza incarnata* has been extracted with Water: Alcohol solvent (30:70) using maceration process for 48 hrs, filtered and dried using vaccum evaporator at 45^0C.

2.4 Determination of percentage yield

The percentage yield of each extract was calculated by using following formula:

$$\text{Percentage yield} = \frac{\text{Weight of Extract}}{\text{Weight of powder drug Taken}} \times 100$$

2.5 Phytochemical Screening

The Dactylorhiza *incarnata* extract acquire was subjected to the precursory phytochemical analysis following standard methods by Khandelwal and Kokate. The extract was screened to identify the presence of various active principles of alkaloids, glycosides, Diterpenes, saponins, flavonoids and phenol.

2.6 Total Phenolic content estimation

Procedure: The total phenolic content of the extract was determined by the modified Folin-Ciocalteu method.10 mg Gallic acid was dissolved in 10 ml methanol, various aliquots of 5- 25µg/ml was prepared in methanol. 10mg of dried extract of plant material was extracted with 10 ml methanol and filter. 2 ml (1mg/ml) of this extract was for the estimation of Phenol. 2 ml of each extract or standard was mixed with 1 ml of Folin-Ciocalteu reagent (previously diluted with distilled water 1:10 v/v) and 1 ml (7.5g/l) of sodium carbonate. The mixture was vortexed for 15s and allowed to stand for 15min at 40°C for colour development. The absorbance was measured at 765 nm using a spectrophotometer.

2.7 Total flavonoids content estimation

Procedure: Determination of total flavonoids content was based on aluminium chloride method (Olufunmiso *et al.,* 2011). 10 mg quercetin was dissolved in 10 ml methanol, and various aliquots of 5- 25µg/ml were prepared in methanol.10mg of dried extract of plant material was extracted with 10 ml methanol and filter. 3 ml (1mg/ml) of this extract was for the estimation of flavonoid. 1 ml of 2% $AlCl_3$ methanolic solution was added to 3 ml of extract or standard and allowed to stand for 15 min at room temperature; absorbance was measured at 420 nm.

2.8 *In vivo* anti-Hyperlipidemic activity of *Dactylorhiza incarnata* extract

Animals:-Wistar rats (180–220 g) were group housed (n= 6) under a standard 12 h light/dark cycle and controlled conditions of temperature and humidity (25±2 °C, 55–65%). Rats received standard rodent chow and water *ad libitum*. Rats were acclimatized to laboratory conditions for 7 days before carrying out the experiments. All the experiments were carried in a noise-free room between 08.00 to 15.00 h. Separate group (n=6) of rats was used for each set of experiments. The animal studies were approved by the Institutional Animal Ethics Committee (IAEC), constituted for the purpose of control and supervision of experimental animals by Ministry of Environment and Forests, Government of India, New Delhi, India.

Acute toxicity studies

Acute oral toxicity was conducted according to the method of Organisation for Economic Co-operation and Development (OECD) (OECD, 2001). Animals were kept fasting providing only water, hydroalcoholic extract of leaves of *Dactylorhiza incarnata* (50,100,150,200,300 mg/kg/day) was administered orally for 4 days of six groups of rats (n=6) and the animals were kept under observation for mortality as well as any behavioral changes for evaluation of a possible antihyperlipidimic effect.

Induction of hyperlipidemia

Rats with an average body weight were made hyperlipidemic by giving high-fat diet (HFD) for 15 days. The HFD contained Cholesterol (2%), Cholic acid (1%), Dalda (20%), and Coconut oil (6%) as major constituents. Hyperlipidemia was confirmed by measuring the levels of serum lipids and lipoproteins in the rats.

Experimental designs

Group –I: Normal (vehicle alone)

Group –II: Hyperlipidemic rats treated with vehicle alone

Group -III: Hyperlipidemic rats treated with hydroalcoholic extract of *Dactylorhiza incarnata* (100mg/kg, p.o.)

Group –IV: Hyperlipidemic rats treated with hydroalcoholic extract of *Dactylorhiza incarnata* (200mg/kg, p.o.)

Group –V: Hyperlipidemic rats treated with Orlistat (60 mg/kg/day p.o.)

Animals were divided into five groups of 6 animals each. The first group treated normal vehicle alone. The group II received hyperlipidemic rats treated with vehicle alone (positive control). The groups III, IV and V received 100 mg/kg and 200 mg/kg of hydroalcoholic extract of *Dactylorhiza incarnata* and Orlistat (60 mg/kg/day p.o.) respectively for 15 days.

Biochemical Evaluation in Serum

Serum Triglycerides (TG), total cholesterol (TC), and HDL-cholesterol (HDL-C) were estimated by using commercial kits as per the manufacturer instructions. Blood was collected from the animals and centrifuged. The serum samples were collected in separate containers for biochemical estimations.

Statistical analysis

The results were expressed in mean±standard deviation. Statistical analysis was carried out by using one way ANOVA.

3. RESULTS AND DISCUSSION

3.1 Result of Percentage Yield

Table 3.1: % Yield of *Dactylorhiza incarnata*

S. No.	Part	% Yield (W/W)
1.	Leaves	7.2

3.2 Result of Phytochemical screening of extract

Table 3.2: Phytochemical screening of hydroalcoholic extract of *Dactylorhiza incarnata*

S. No.	Constituents	Hydroalcoholic extract
1.	**Alkaloids**	
	Dragendroff's test	-ve
	Hager's test	-ve
2.	**Glycoside**	
	Libermann's test	-ve
	Keller Kilani Test	-ve
3.	**Flavonoids**	
	Lead acetate	+ve
	Alkaline test	+ve
4.	**Phenolics**	
	$Fecl_3$	+ve
5.	**Proteins and Amino acids**	
	Xanthoproteic test	-ve
6.	**Carbohydrates**	
	Fehling's test	+ve
7.	**Saponins**	

	Foam test	+ve
8.	**Diterpenes** Copper acetate test	 -ve

3.3 Results of Estimation of Total Phenolic Contents and Total flavonoid content estimation

Table 3.3: Total Phenolic and Total flavonoid content of *Dactylorhiza incarnata*

S. No.	Extracts	Total Phenol (GAE) (mg/100mg)	Total flavonoid (QE) (mg/100mg)
1.	Hydroalcoholic extract	0.891	0.918

3.4 Results of antihyperlipidemic effect of extract of *Dactylorhiza incarnata*

Antihyperlipidemic effect of the hydroalcoholic extract *Dactylorhiza incarnata* on the high fat diet induced rats. The mean body weight as shown in Table 3.4. The activity levels of serum total cholesterol (TC), triglycerides (TG) and Serum high density lipoprotein (HDL) were observed in normal and experimental animals. In group II animals, the activity levels of serum total cholesterol (TC) and triglycerides (TG) were significantly elevated when compared to that of normal groups (Table 3.5). On the other hand the serum level of Serum high density lipoproteins (HDL) were significantly depleted in the HFD fed rat. In group III, IV and V animals, the activity levels of serum total cholesterol (TC) and triglycerides (TG) were significantly decreased when compared to that of normal groups also HDL level was significantly increased in the same groups.

Table 3.4: Mean Body Weight Change

Group	Drug	Dose	Body weight (g)	
			Onset of study	End of study
I	Normal	Normal saline	180.10±7.50	200.00±7.50
II	Control	HFD	190.05±8.50	251.10±8.50
V	Extract *Dactylorhiza incarnata*	100 mg/kg p.o.	200.00±7.00	193.00±7.00
VI	Extract of *incarnata*	200 mg/kg p.o.	200.05±8.00	188.00±8.00
IV	Orlistat	60 mg/kg p.o.	200.00±8.00	180.50±8.00

Values are expressed as the mean ± SEM of six observations. *** $P<0.001$ vs. control treatment (One-way ANOVA followed by Dunnett's test)

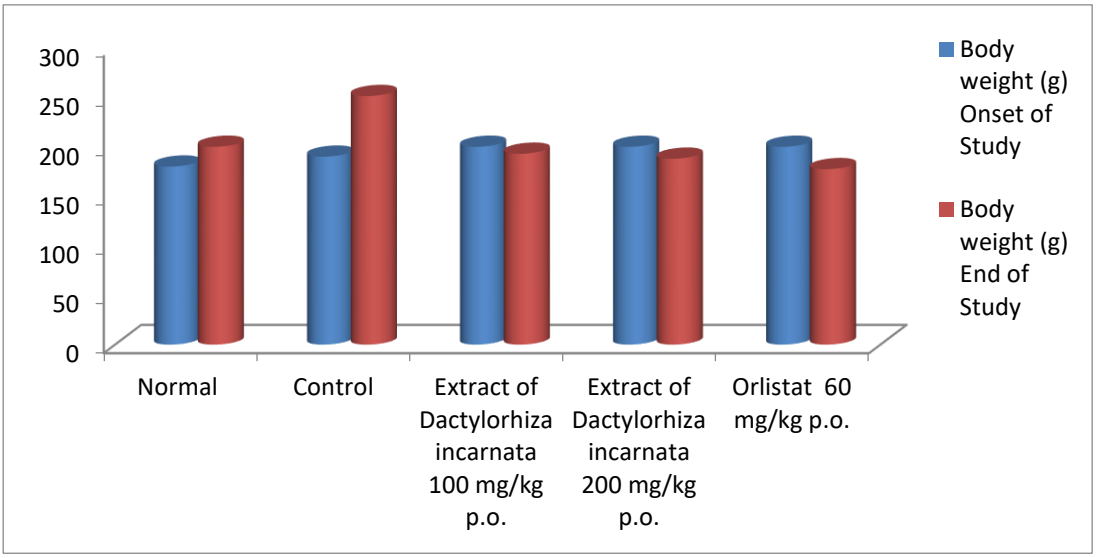

Figure 3.1: Effect of the hydroalcoholic extract of *Dactylorhiza incarnata* on body weight in HFD induced rat

Table 3.5: Effect of the hydroalcoholic extract of *Dactylorhiza incarnata* on serum lipid profile levels (mg/dL) in HFD induced rats

Treatment	Dose	Total cholesterol (mg/dL)	Triglycerides (mg/dL)	High density lipoproteins (mg/dL)
Normal	Normal saline	80.00 ± 5.00	82.00 ± 4.50	36.00 ± 4.00
Control	HFD	140.10 ± 5.00	150.0 ± 4.22	25.00 ± 4.60
Dactylorhiza incarnata	100 mg/kg p.o.	91.20 ± 3.70**	92.30 ± 3.10**	28.40 ± 2.50**
Dactylorhiza incarnata	200 mg/kg p.o.	83.10 ± 7.10***	85.10 ± 9.50***	30.50 ± 7.60***
Orlistat	60 mg/kg p.o.	81.40 ± 2.60***	80.20 ± 6.70***	28.10 ± 1.50***

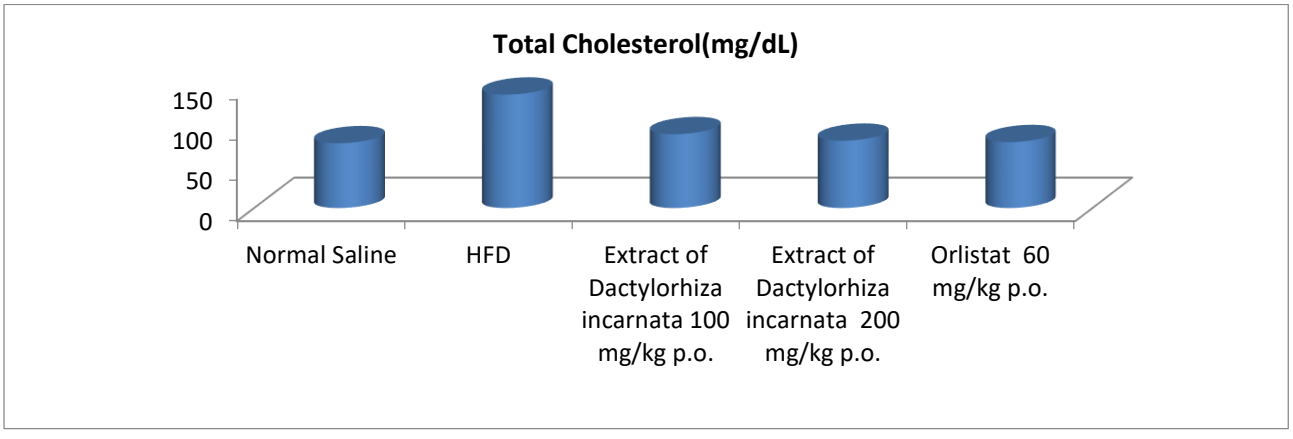

Figure 3.2: Effect of the hydroalcoholic extract of *Dactylorhiza incarnata* on serum lipid profile levels- Total cholesterol (mg/dL) in HFD induced rat

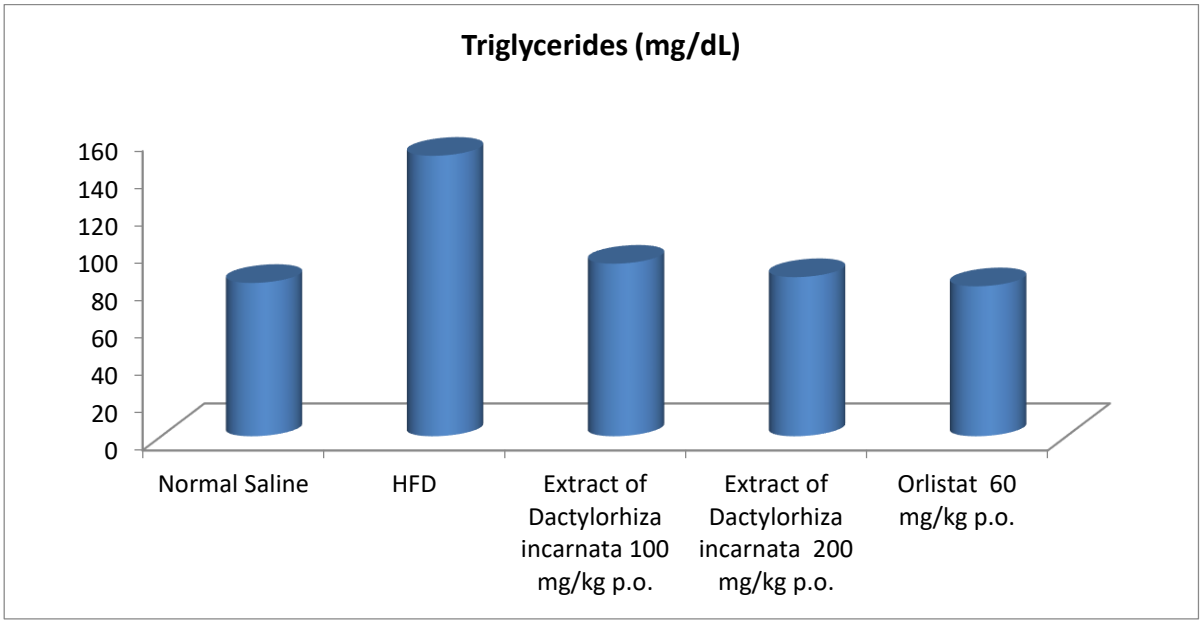

Figure 3.3: **Effect of the hydroalcoholic extract of *Dactylorhiza incarnata* on serum lipid profile levels-Triglycerides (mg/dL) in HFD induced rat**

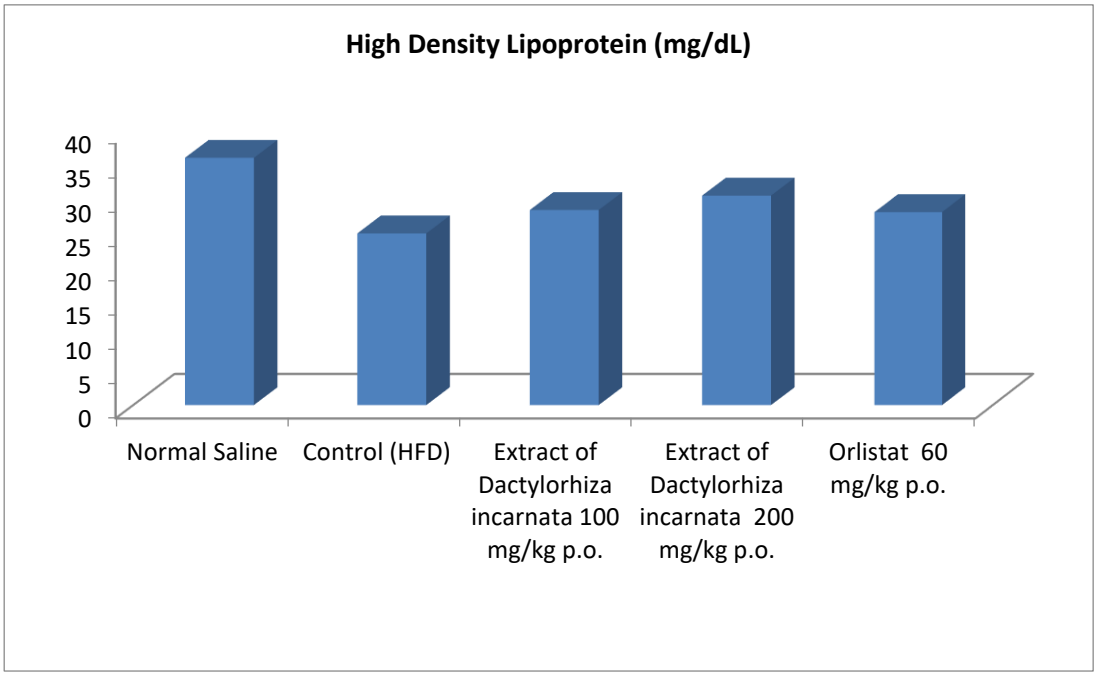

Figure 3.4: **Effect of the hydroalcoholic extract of *Dactylorhiza incarnata* on serum lipid profile levels-High density lipoproteins (mg/dL) in HFD induced rat**

*Dactylorhiza incarnata*a well known traditional medicinal plants possesses diverse biological activities and pharmacological function including reducing blood glucose and serum lipids. It has long been used to treat diabetes mellitus and related hyperlipidemia. Hypercholesterolemia, a high cholesterol diet and oxidative stress increase serum levels resulting in increased risk for development of atherosclerosis. Cholesterol is synthesized in all animal tissue. It is important to relate to its role in the stabilization of membrane structures because of its rigid planar structure. It also as a precursor for the synthesis of steroid hormones. In the present study, feeding rats with diets rich in cholesterol resulted in increased TC and TG levels. This model was used to study the potential of hypolipidemic effect of hydroalcoholic extract of whole plant of *Dactylorhiza*

incarnata that contained significant amounts of antioxidants properties. From this study, we found that daily oral administration hydroalcoholic extract of leaves of *Dactylorhiza incarnata* shows significantly reduced total cholesterol levels in plasma after 15 days of administration. This result agrees with literature where depleted level of HFD fed hyperlipdemia. HDL is directly anti-androgenic and it is believed to remove cholesterol from the developing lesions. The intense interest in this area results in part from the generally low toxicity of antioxidants and the hope that treatment with antioxidants might be additive with cholesterol lowering regimes. In the present study serum TG levels were significantly elevated in HFD rat. The excess of fat diet increased the TG level which is one of the causes of hardening of arteries. In conclusion, it could be said that the hydroalcoholic extract of *Dactylorhiza incarnata* exhibited a significant hypolipidemic activity. Administration of HFD produced a highly significant increase in weight mesenteric fat pads. A reduction in the raised weight in the fat pads as observed in the groups of animals treated with hydroalcoholic extract of *Dactylorhiza incarnata* may be attributed to increased thermogenesis and decreased lipogenesis.

CONCLUSION

Dactylorhiza incarnata belongs to the family Orchidaceae, with medicinal properties as per folk medicine. Orchidaceae is used for treating in inflammation of the gum & teeth, poultice on cuts and wounds and extract is given in intestinal disorders. Phytochemicals present in whole plant of *Dactylorhiza incarnata* was studied in the current study by biochemical tests. It was found that various phytochemical were present in high proportion Flavonoids, Phenolics,Carbohydrates, Saponins. In conclusion, from the observation, the protective effect of the hydroalcoholic extract of *Dactylorhiza incarnata* on high fat induced hyperlipidemia may be attributed to a decrease in cholesterol synthesis and increase in cholesterol excretion.

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