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Anti-Obesity Efficacy of Selected Plants Against Progesterone Induced Obesity

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Abstract
The aetiology of obesity is multifactorial and is becoming a major health concern in the current scenario. Hormone imbalance is one the reasons for the development of obesity. Progesterone is a neurosteroid female reproductive hormone. Imbalance in the production of this hormone modulates lipid metabolism leading to obesity. Numerous trials have been conducted to find and develop new anti-obesity therapy through herbal approach to minimize side effects associated with the currently available anti-obesity drugs. The present study was designed to evaluate antiobesity efficacy of 3 plants selected; *Citrus limon*, *Nigella sativa* and *Cyperous rotundus* against progesterone induced obesity in female BALB/C mice. Progesterone was administered subcutaneously, and plant extracts was given orally for 28 days. Food intake and body weight gain were monitored regularly and lipid profile, neurotransmitters (serotonin and dopamine) which play key role in energy homeostasis were analysed. The results suggests among the 3 plants, *Citrus limon* and *Nigella sativa* showed better reduction in the levels of circulating free fatty acids, lipid profile resulting in decrease food intake and body weight gain in female BALB/C mice in comparison with progesterone treated group followed by *Cyperous rotundus* at dose of 400mg/kg b w. Thus, these plants can be considered as an effective treatment in management of obesity owing to their potential anti-obesity effect.

Keywords
Hormone lipid profile, Neuro transmitters, Obesity and Progesterone.

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1. INTRODUCTION
In the current scenario obesity has become an epidemic worldwide, with negative impact on health, increased fat accumulation which eventually leads to decreased life expectancy. Females are more prone for development of obesity due to changes in oestrogen and progesterone hormones which cause homeostasis imbalance which include decrease in insulin sensitivity and change in glucose and lipid metabolism resulting in complete reduction of energy expenditure [1]. Progesterone is a female reproductive hormone, its level increases during luteal phase of menstrual cycle and controls secretary phase of endometrium. It acts a potent neuro active steroid which exerts neuroprotective and neurogenic activity by regulating the release of various neurotransmitters [2]. Imbalance in neurotransmitters such as serotonin, dopamine and GABA lead to change in feeding behaviour. Prolonged use of progesterone containing contraceptive pills and hormone replacement therapies lead to obesity by hyperphagia and increase fat accumulation by interfering in carbohydrate and

lipid metabolism [3, 4]. Currently available synthetic drugs have tremendous side effects which mimic sympathetic nervous system causing body to feel under stress or nervous which causes headache, insomnia irritability, palpitation and agitation. Drugs which block enzyme activity causes diarrhoea, bloating and abdominal pain [5]. Since existing pharmaceuticals fall apart to come up with long term solutions, there is an ever pressing need to find and develop new strategies to overcome obesity. Since time immemorial herbal extracts have been tried for treating obesity. In the current study three plants were selected to evaluate anti obesity efficacy.

Cyperus rotundus is a species of sedge (*Cyperaceae*). It is a traditional medicinal herbal used widely as anti diabetic, anti diarrhoeal, cytoprotective, anti mutagenic, antimicrobial, antibacterial and antioxidant [6, 7]. *Cyperus rotundus* extract showed lipolysis in 3T3-F442 adipocytes *in vivo* study [8].

Nigella sativa, known as black cumin, is an annual flowering plant in the family *Ranunculaceae* is used in India [9], Arabian countries [10]. and Europe for culinary and medicinal purposes as a natural remedy for a number of illnesses and conditions that include asthma, hypertension, diabetes and inflammation [11]. And also, for lipid lowering and insulin sensitizing activity [12].

Citrus limon has many important natural chemical components, including citric acid, ascorbic acid, minerals and flavonoids. It has recently been shown that flavonoids also play a role in this respect. Some authors suggest that flavonoids have different biological functions, including antioxidative, anti-inflammatory, antiallergic, antiviral, antiproliferative, antimutagenic, and anticarcinogenic activities [13, 14, 15, 16].

Thus, the present study was aimed to investigate the anti-obesity effect of these selected plants in progesterone induced obesity in female Balb/C mice.

2. MATERIALS AND METHODS

2.1. Materials

Progesterone, Orlistat and neurotransmitter standards were obtained from Sigma-Aldrich (MO, USA) and all the other reagents were of analytical grade obtained from SRL (Mysore). Kits were procured from Agappe India Pvt Ltd. Ernakulum Kerala. To analyse plasma hormones Leptin and adiponectin kits were procured from cloud- clone Corp Houston, USA *Citrus limon*, seeds of *Nigella sativa*, and roots of *Cyperus rotundus* were collected from local suppliers, Mysore. The plant authentication was done in Plant Anatomy Research Centre west Tambaram, Chennai.

2.2. Preparation of plant extracts

Citrus Limon Peel, Seeds of *Nigella sativa*, and roots of *Cyperus rotundus* were collected from local market. Lemon fruits were cut, and its juice was removed, and the peels were shade dried for 2 days. Seeds of *Nigella sativa* and roots of *Cyperus rotundus* were ground to form course powder. The extracts were prepared using 70% ethanol by keeping on orbital shaker overnight and the extracts were filtered using Whatmann no.1 filter paper. The ethanol was evaporated using flash evaporator (Rotavac Schwabach, Germany). The plant extracts were lyophilized (Lyolab, Hyderabad, India).

2.3. Experimental animals

All the experimental procedures were approved by Institutional Animal Ethical Committee Reg No: (IAEC/2016/BN/9) Female BALB/C mice (in house animal stock colony, DFRL, India) weighing 20-25g. The experimental animals were maintained under standard laboratory conditions at temperature of 23 ± 2°C with relative humidity 55 ± 10%. 12 h light /dark cycles was maintained throughout the experiment. The animals were fed standard pellet diet and water *ad libitum*.

2.4. Experimental design

A total of fifty-four female BALB/C mice were randomly divided into nine groups (n=6) and were treated daily for 28 days. Plant extracts were orally fed prior 30 minutes of progesterone administration.

1. Group I: Control (CTL)
2. Group II: Progesterone (PG) 10 mg/kg body weight subcutaneously in dorsal neck region.
3. Group III: Standard drug Orlistat (STD) 10 mg/kg body weight orally + progesterone
4. Group IV: *Citrus limon* peel (*C.L*) 200 mg/kg body weight orally + progesterone.
5. Group V: *Citrus limon* peel (*C.L*) 400 mg/kg body weight orally + progesterone.
6. Group VI: *Cyperous rotundus* (*C.R*) 200 mg/kg body weight orally + progesterone.
7. Group VII: *Cyperous rotundus* (*C.R*) 400 mg/kg body weight orally + progesterone.
8. Group VIII: *Nigella sativa* (*N.S*) 200 mg/kg body weight orally + progesterone
9. Group IX: *Nigella sativa* (*N.S*) 400 mg/kg body weight orally + progesterone

2.5. Food intake

The effect of plant extracts on food intake was recorded on day 1, 14 and 28. The mice were deprived of food 1 hr prior to experiment. On these days 10g of sweetened corn was presented to groups of mice in glass petri dishes and food intake was recorded at 2 hr time interval. The result was recorded as average of all the weeks at respective time intervals and amount food consumed/20 g body weight was calculated [17].

2.6. Body weight, organ weight and relative liver weight

The body weight of mice was recorded every week for 28 days using an electronic balance and the % mean change in their body weight was calculated. Anthropometric parameters such as BMI and LEE's index were calculated at the 1st and 4th week of the experiment using the formula,

BMI = weight (kg)/(Height in meters) ^{2}

LEE INDEX = weight 0.33/Naso – anal length

At the end of the experiment mice were sacrificed and organs were isolated, weighed and relative weight was calculated.

Relative liver weight = (liver weight/ Body weight) × 100

2.7. Biochemical estimations

On 29th day blood was withdrawn by retro-orbital puncture from over-night fasted mice. Blood was centrifuged at 3000 rpm for 15 min and serum was separated and stored at -80^{0}C. Biochemical parameters like blood glucose, total cholesterol (TC), high density lipoprotein (HDL). Triglyceride (TG), Serum glutamate oxaloacetate transaminase (SGOT), Alkaline phosphatise (ALP) and serum glutamate pyruvate transaminase (SGPT) were estimated using commercially available diagnostic kits in Semi Auto analyser. Whereas very low-density lipoprotein (VLDL) levels were estimated using Friedwald's formula.

VLDL in mg % = Triglycerides/5

2.8. Free Fatty Acid Estimation

Free fatty acids were estimated according to the method of [18]. with slight modifications in sample volume and incubation time. The extraction of free fatty acid was carried out using chloroform-heptane-methanol mixture with a phosphate buffer (pH 6.4). This extraction mixture allows sufficient extraction of FFA from serum and contamination with interfering agents is avoided. The copper soaps of FFA are determined colorimetrically with diphenyl carbazide at 550 nm.

2.9. Lipid Peroxidation

The lipid peroxidation was estimated in terms of thio barbituric acid reactive substances (TBARS) formed in the liver homogenate as described by spectro photometry [19]. The liver homogenate was heated with TBA reagent for 20 min in boiling water bath. After cooling, the samples were centrifuged at 2000 rpm for 10 min. Supernatant was collected and the absorbance was measured at 532 nm against blank. The MDA equivalents of the serum samples were calculated using an extinction coefficient of 1.56×105M^{-1}cm^{-1}

2.10. Estimation of monoamines

Monoamine neurotransmitters serotonin and dopamine contents in mouse brain were measured by HPLC coupled with electrochemical detector [20]. The brain tissue (100mg) was homogenised in an ice-cold solution of 0.4 M percloric acid containing 5 mm sodium bisulfite and 0.04 mm EDTA for avoiding oxidation and then centrifuged at 30,000g for 15 min at 4°C. 10 µl of the resulting supernatant was chromatographed on a C_{18} RP column using Waters 1465 HPLC. The mobile phase consisted of 17.6% methanol (v/v) and 82.4% distilled water containing 0.0876 mm EDTA disodium, 1.512 mm triethylamine, 9 mm DL-10-camphorsulfonic acid, 20 mm $Na_2HPO_4.12H_2O$ and 15 mm citrate at a flow rate of 0.7 ml/min. The measurements were done at electrode potentials of a glassy carbon electrode +650 mV vs Ag/AgCl reference electrode with waters 1645 electrochemical detector. Serotonin and dopamine were identified and quantified by comparing their retention times and peak areas to those of standards. The concentrations of serotonin and dopamine were expressed in ng/g wet tissue**.**

2.11. Estimation of blood glucose level

The mice were fasted overnight prior to the experiment. Blood was obtained from a tail cut (by removing the distal 2 mm of the tail) and was assessed for baseline glucose levels using a One-touch Ultra 2 (Lifescan, Johnson & Johnson) glucometer. The glucose concentration was given as mg/dl of blood.

2.12. Plasma Hormones analyses

Leptin and adiponectin concentration was measured using leptin EIA kit (SEA084mu) and adiponectin EIA Kit (SEA 605mu) from cloud – Clone corp Houston, USA according to manufacturer's instructions. Adiponectin concentration was expressed in pg/ml. And leptin concentration was expresses in ng/ml.

2.13. Histology analysis

At the end of experiment mice were sacrificed by cervical dislocation, liver was isolated and stored in 10% formalin solution. The sections of liver were stained with haematoxylin and eosin and observed using light microscope under bright field 40 x (Olympus, Japan equipped with COOL SNAP digital camera).

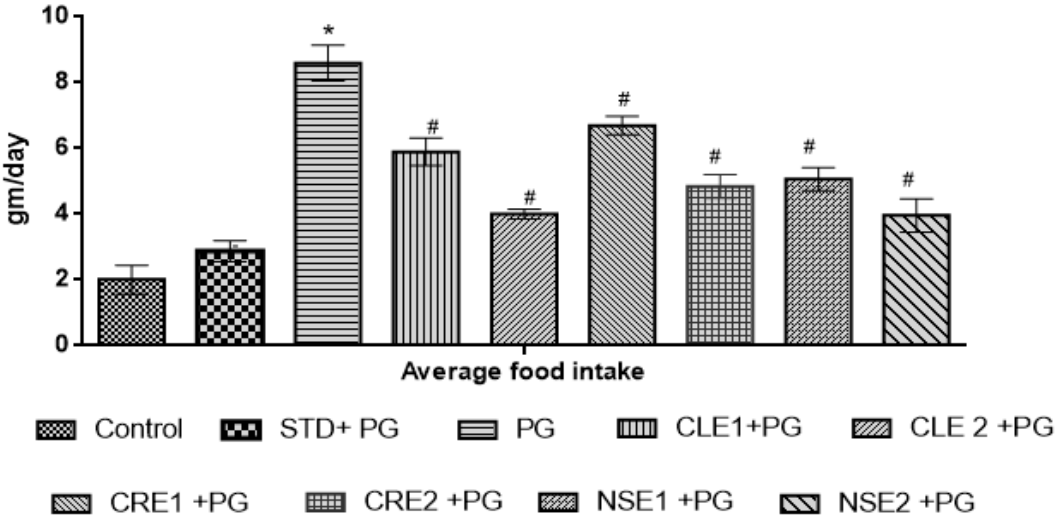
2.14. Statistical analysis

The results are expressed as mean ± SD, n= 6. Comparison between progesterone and herbal treatment groups by one-way ANOVA followed by Tukey's test. $P < 0.05$ (95% level). The analysis was performed using Graph Pad Prism 6.

RESULTS

3.1. Food consumption

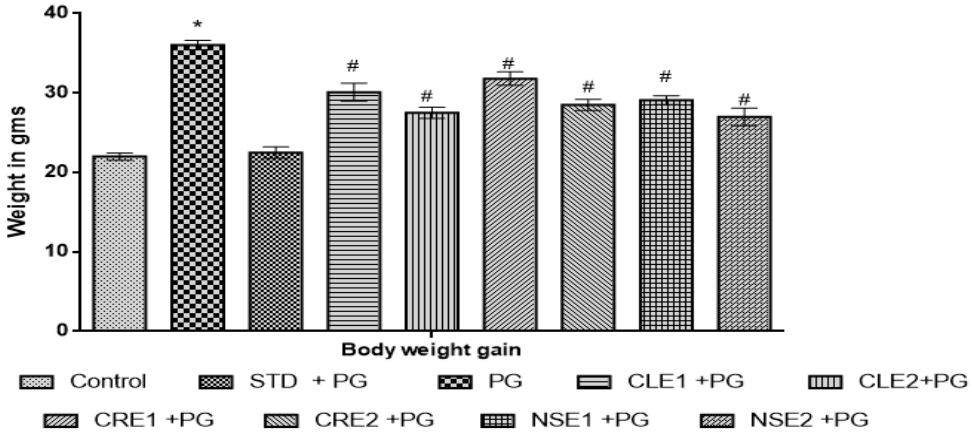
Excess food intake leads to obesity by accumulation of fat treatment with plant extracts there was significant decrease in food intake.



Assessment of food consumption behavior. Data represented ± SD *p < 0.05 versus control group and # p< 0.05 verses progesterone group.

3.2. Body weight change

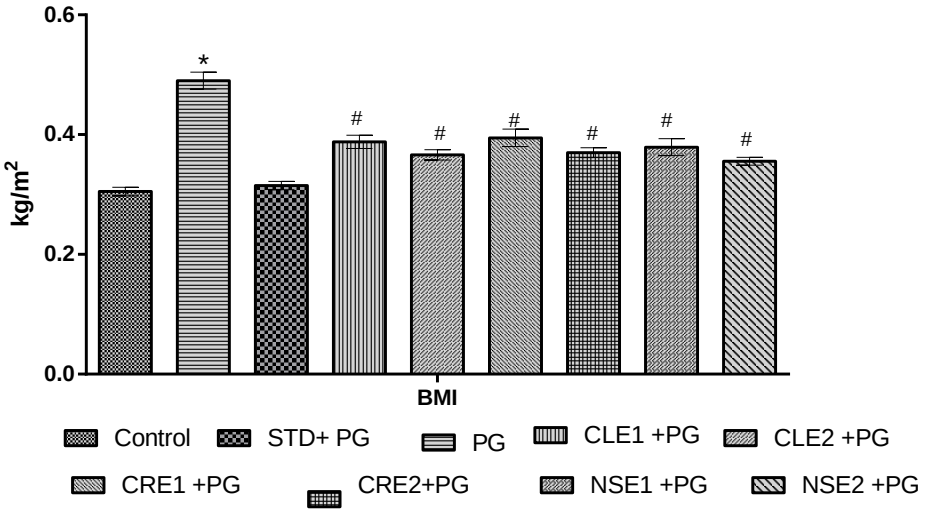
Obesity leads to excess accumulation of adipose tissue which leads increase in body weight.



Data represented ± SD *p < 0.05 versus control group and # p< 0.05 verses progesterone group.

3.3. Effect of progesterone on BMI by plant extracts

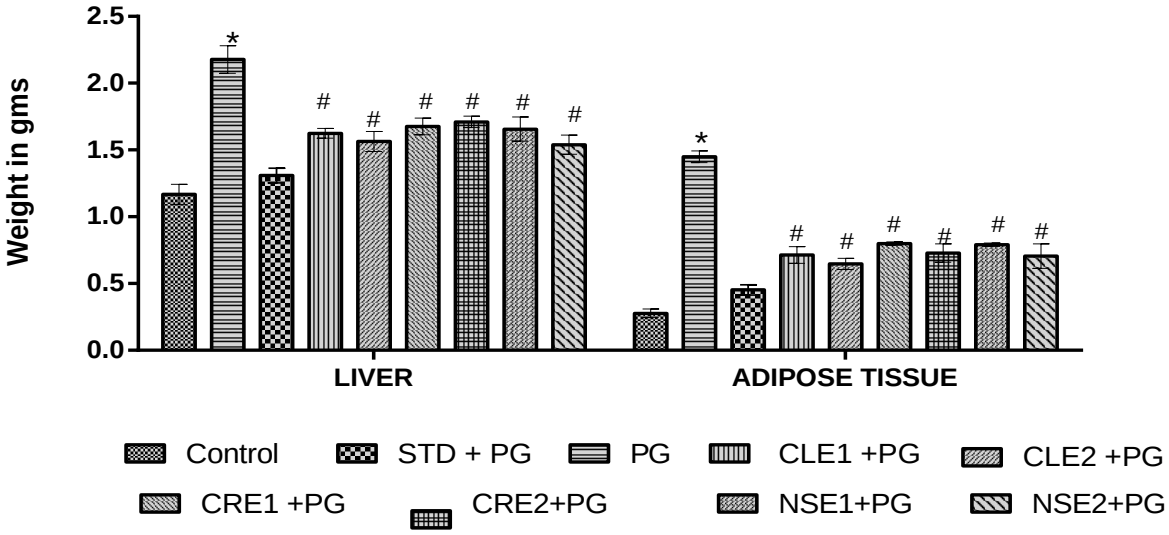
BMI is marker for determining obesity. Treatment with plant extracts there was significant decrease in these indices compared with control group.



Data represented mean ± SD *p < 0.05 versus control group and # p< 0.05 verses progesterone group.

3.4. Change in organ weights

Effect of progesterone on organ weight by plant extracts
Organ weight was taken on 29th day of the experiment after sacrifice of mice. During obesity there is increase in adipose tissue mass and infiltration of fatty acids in liver.



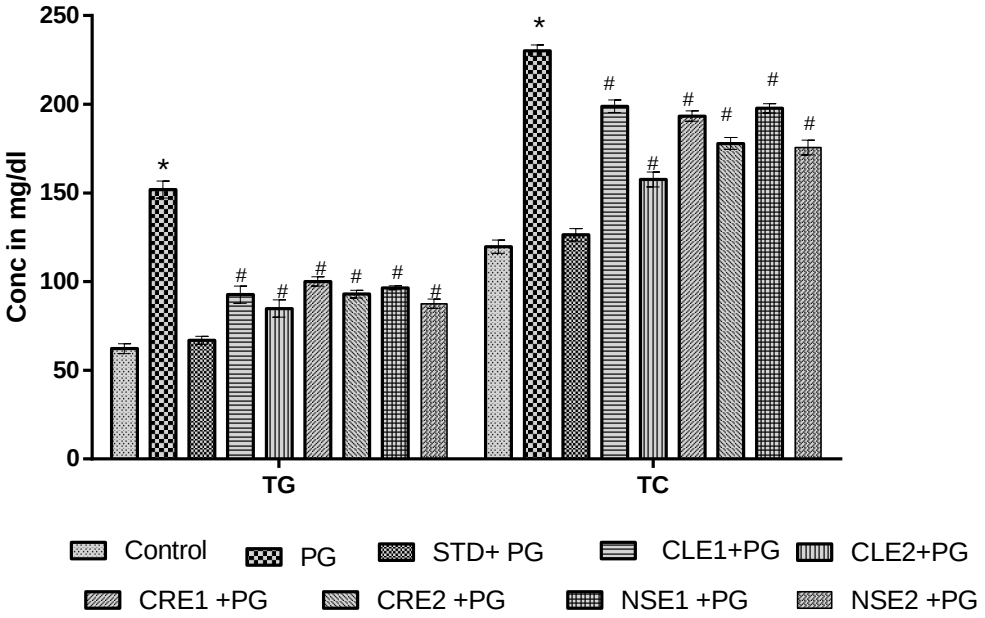
Data represented mean ± SD *p < 0.05 versus control group and # p< 0.05 verses progesterone group.

3.5. Lipid profile

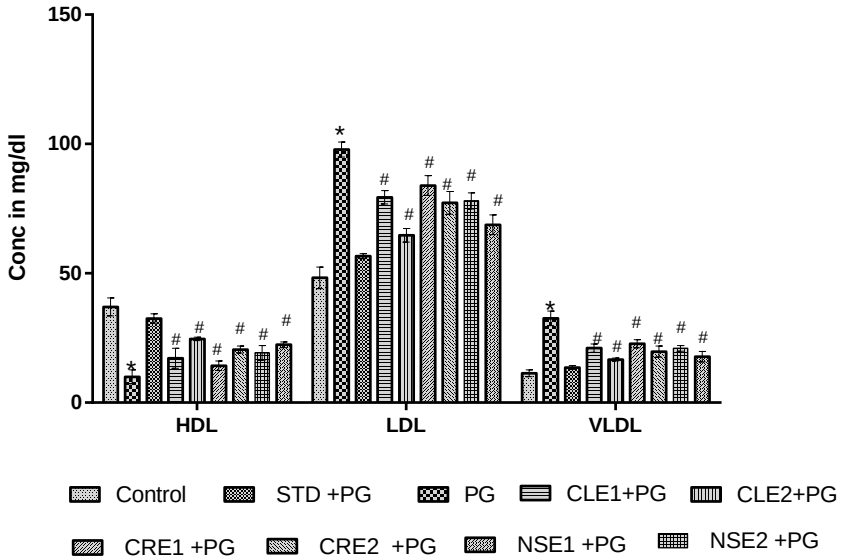
Effect of progesterone on lipid profile by plant extracts

Obesity is due to deregulation in lipid metabolism leading to dyslipdemia Progesterone treated group showed increases serum lipid profile; total cholesterol, triglycerides, LDL and VLDL with decreased in HDL levels.

A.



B.

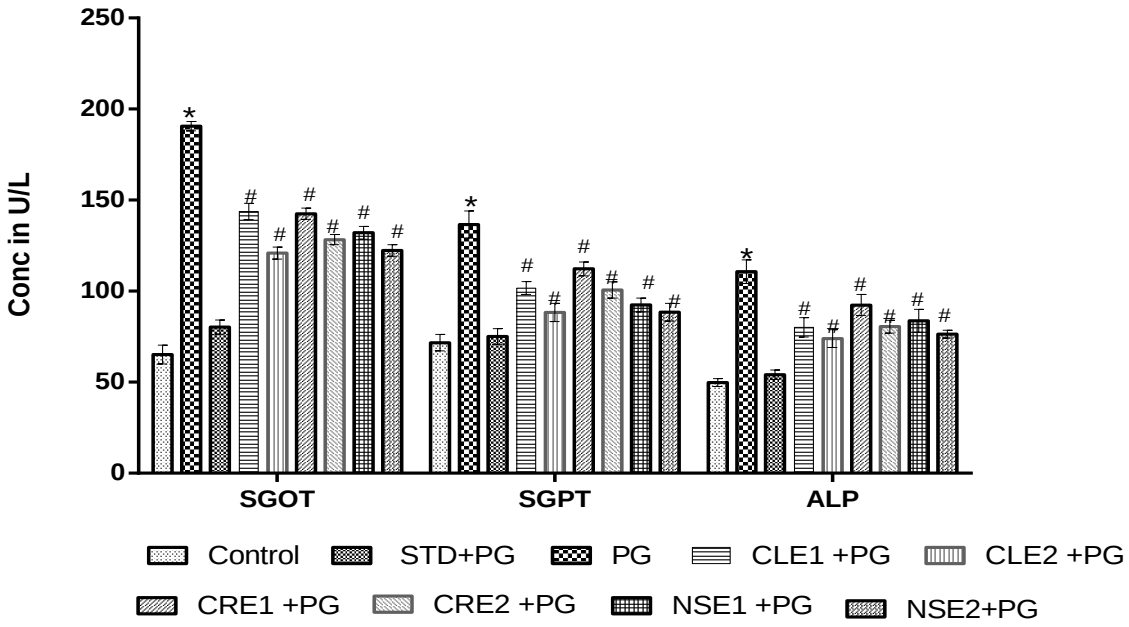


Lipid profile A and B: Data represented mean ± SD *p < 0.05 versus control group and # p< 0.05 verses progesterone group.

3.6. Liver function test

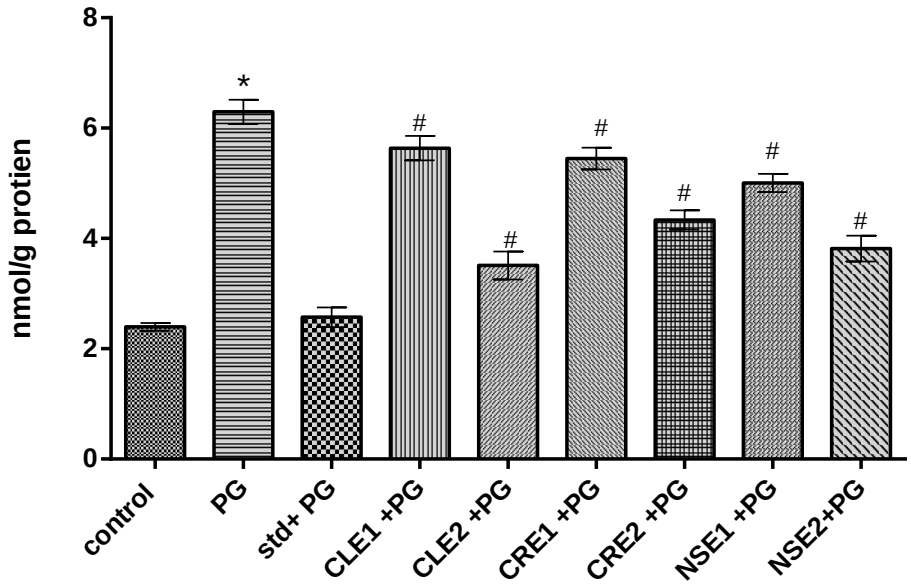
Effect of progesterone on liver function test by plant extracts

During obesity there is excess infiltration of lipids which causes liver tissue damage which in turn releases SGOT, SGPT and ALP.



3.7. Free Fatty Acid Estimation

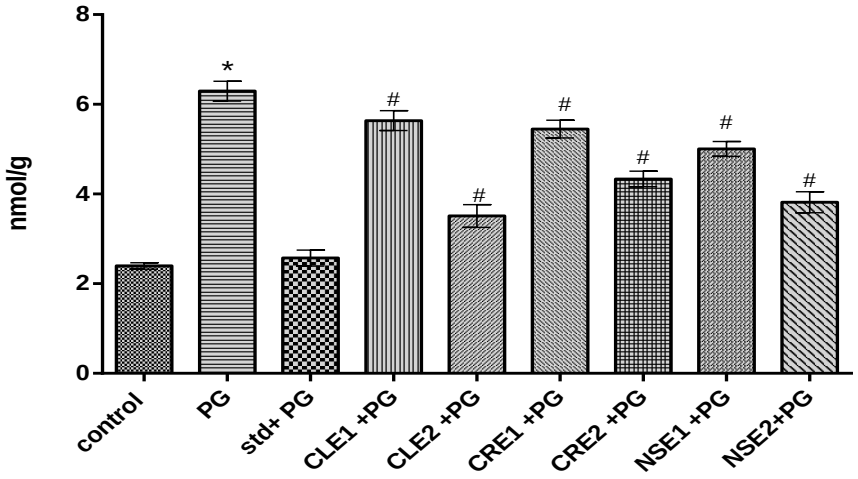
During obesity there is increase in free fatty acid in serum Progesterone treated group showed increases free fatty acid in serum concentration Co – administration of plant extracts at different concentration significantly reduced.



Data represented ± SD *p < 0.05 versus control group and # p< 0.05 verses progesterone group.

3.8. Lipid Peroxidation

Excess of lipid in liver causes lipid peroxidation which generate free radical that is measured by malanoldehyde release.



Data represented ± SD *p < 0.05 versus control group and # p< 0.05 verses progesterone group

3.9. Fasting blood glucose

Fasting blood glucose

Fasting blood glucose was measured before sacrificing mice. Progesterone treated group showed increased glucose concentration. Co – administration of plant extracts at different concentration significantly reduced glucose level compared with progesterone treated group. 400mg/kg b.w of *Citrus limon* and *Nigela sativa* showed better result compared to other treatment group.

Sl.no.	Groups	Glucose mg/dl
1	Control	96 ± 1.2
2	Progesterone	134 ± 3.5*
3	Standard 10mg/Kg + Prog	78 ± 2.2#
4	*Citrus limon*200mg/Kg b.w	89 ± 1.6#
5	*Citrus limon* 400mg/Kg b.w	86 ± 2.4#
6	*Nigella sativa* 200mg/kg b.w	92 ± 2.7#
7	*Nigella sativa* 400mg/kg b.w	87 ± 1.1#
8	*Cyperous rotundus* 200mg/kg b.w	94 ± 2.3#
9	*Cyperous rotundus* 400mg/kg b.w	90 ± 1.5#

Table 1: FBS Data represented ± SD *p < 0.05 versus control group and # p< 0.05 verses progesterone group

3.10. Nuerotransmitters

There is strong neurondocrine regulation in food intake serotonin and dopamine play important role in appetite regulation

Group	Serotonin (ng/g wet tissue)	Dopamine (pg/g wet tissue)
Control	17.77 ± 0.4	43.3 ± 0.3
Progesterone	10.35 ± 0.1*	37.7 ± 0.3*
Citrus limon 200 mg/kg b.w + progesterone	19.2 ± 0.2#	51.6 ± 0.2#
Citrus limon 400 mg/kg b.w + progesterone	24.6 ± 0.1#	54.6 ± 0.3#
Cyperous Rotundus 200 mg/kg b.w + progesterone	17.4 ± 0.21#	38.3 ± 0.1#
Cyperous Rotundus 400 mg/kg b.w + progesterone	20.3 ± 0.3#	41.2 ± 0.5#
Nigella Sativa 200 mg/kg b.w + progesterone	18.6 ± 0.2#	47.1 ± 0.2#
Nigella Sativa 400 mg/kg b.w + progesterone	23.7 ± 0.3#	51.7 ± 0.3#
Standard drug+ progesterone	26.4 ± 1.3#	57.5 ± 0.4#

Table 2: Neurotransmitters. Data represented ± SD *p < 0.05 versus control group and # p< 0.05 verses progesterone group

3.11. Histology of liver

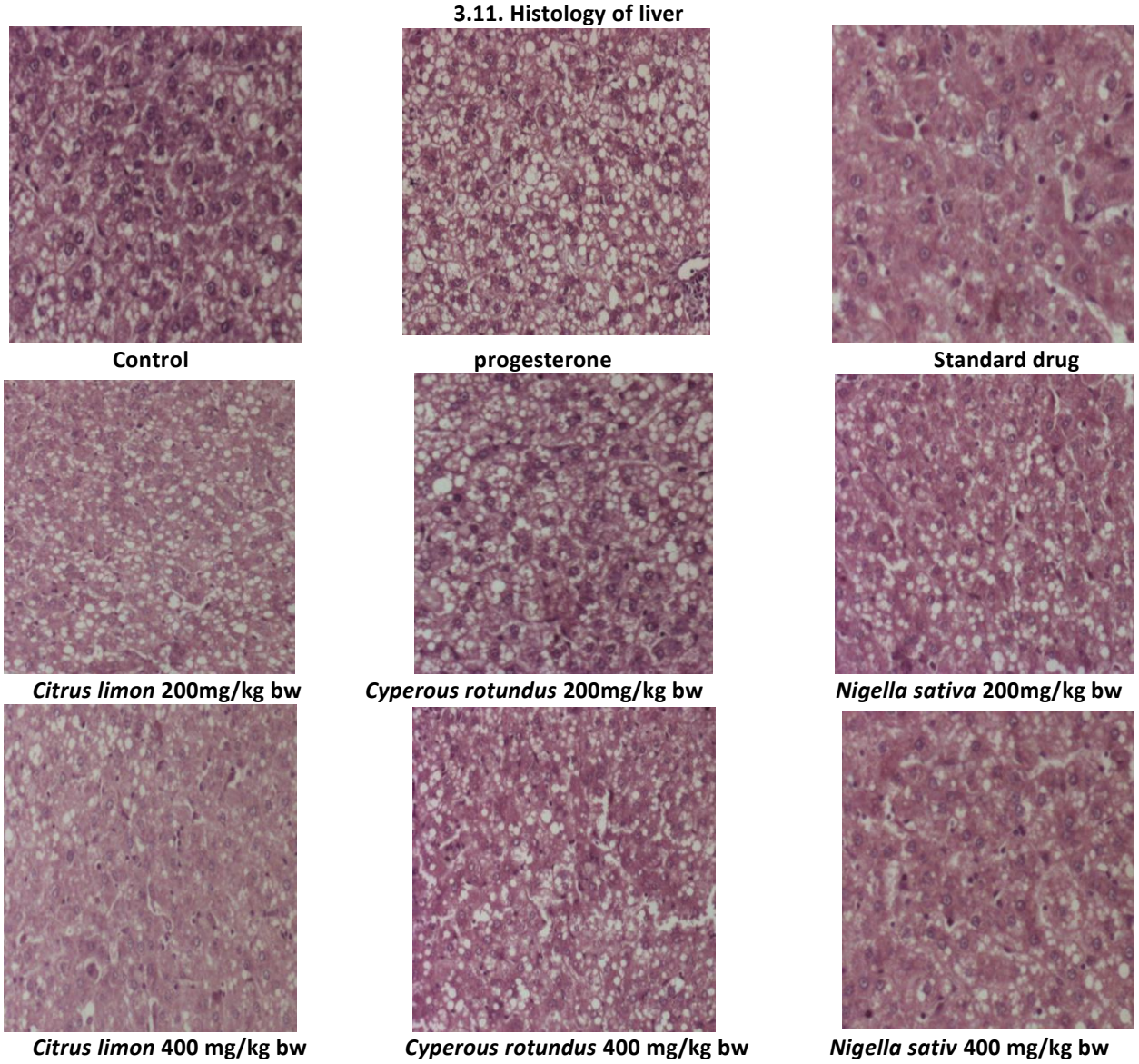


Figure 11: Histological section of liver stained with H and E - 40X

4. DISCUSSION

Obesity is a disorder caused due to imbalance in calorie inatake and expenditure. It is multifactorial in origin. There is strong interaction between horomone imbalance and development of obesity [21]. Progesterone, a neurosteriod impairs fat metabolism and induces lipid accumulation leading to obesity [22]. Fundamental cause of obesity is increase in fat mass. Progesterone treatment led to increase in body weight however co–administration of a plant extracts along with progesterone significantly reduce body weight in a dose dependent manner with respect to plant extracts. *Citrus limon* and *Nigella sativa* at 400 mg/ kg bw showed comparatively better results than *Cyperous rotundus* group. Body mass index (BMI), a measure which compares weight and height, defines people as over weight (pre-obese), if their BMI is between 25 to 30 kg/m^2, and obese when it is greater than 30 kg/m^2. Lee's Index is also a factor considered for measurement of obesity [23]. Progesterone treatment increases BMI and LEE index compared to control, standard drug and plant extracts treatment groups. Progesterone administration was associated with increase in food intake leading to increase in body weight. In the present study, subcutaneous administration of progesterone produced significant increase in food intake and thereby body weight gain in agreement with previous reports [24]. There was significant decrease in food intake comparatively with plant extracts and orlistat standard drug as measured on 1st ,14th and 28th day (Figure 1).

Progesterone has been reported as the steroid hormone that promotes synthesis and storage of fats. There was increase in mass of adipose tissue in progesterone treated mice. As liver is a highly metabolically active organ, prone to deposition of fat, the present investigation showed significantly increased liver weight with progesterone administration. Plant extracts treated mice showed significant decrease in mass of adipose tissue and liver weight which is represented in (Figure 6). An increase in liver weight and adipose tissue suggests hypertrophy or hyperplasia [25].

The impairment in fat and carbohydrate metabolism is reflected in the biochemical parameters like blood glucose and lipid profile associated with progesterone administration. It is reported that increased progesterone level in pregnant women may lead to gestational diabetes due to hyperphagia [26].In our study progesterone treated group showed significant rise in the blood glucose level which was completely reversed by the effect of *Citrus limon* and *Nigella sativa* (400 mg/kg bw) treatment.

Progesterone modulated various biochemical parameters in female mice by stimulation of lipoprotein lipase which is responsible for hydrolysis of dietary fat leading to enhancement of fat storage in body, and a significant increase in serum lipid profile [27].Progesterone produced an increase in blood glucose levels, and other parameters of lipid profile viz. TG, TC, LDL and VLDL while decrease in levels of HDL. Herbal extracts treated mice showed a significant decrease in TG, TC, LDL and VLDL. Oral administration of herbal extracts produced a significant increase in levels of HDL which is shown in (Figure 7).

The present study indicated an increase level of marker enzymes of liver damage viz. SGOT, SGPT and ALP with progesterone administration indicating hepatic injury or abnormality. 28 days treatment with 3 different plant extracts (200 mg/kg and 400 mg/kg bw) significantly reduced the levels of SGOT, SGPT and ALP when compared to progesterone treated group. All the results suggesting maximum anti-obesity action at higher dose which is represented in (Figure 8).

As seen from graph 9, plant extracts stimulated a significantly lower free fatty acid response when compared to progesterone treated group. The concentration of plasma fatty acids may be regulated by the utilization or release of fatty acids by a number of tissues such as adipose tissue, muscle and liver. The slow release of glucose contributes to the suppression of free fatty acids in blood, thereby lowering the serum triglycerides [28].Since free fatty acids have been shown to impair insulin mediated glucose disposal and enhance hepatic glucose output, prolonged free fatty acid suppression results in improved glucose and triglyceride concentrations, both of which directly influence the factors leading to obesity (Figure 9).

Malonaldehyde (MDA) is a degradation product that is generated by oxidative degradation of polyunsaturated fatty acids in the cell membranes which is an important cause for cell damage and cell membrane destruction [29]. lipid peroxidation (MDA) has been used as a marker for the oxidative stress. In this study, we found that treatment with plant extracts significantly inhibits the peroxidation of lipids (Figure10).

Progesterone producing hyperphagia via progestin receptors, which has been reported to be expressed on the serotonergic neurons [30].Progesterone-induced hyperphagia by inhibiting reuptake of 5-HT (serotonin) at the hypothalamic site which regulate the food intake, which suggests the possible interaction exists between the neurosteroid and serotonin receptor system in regulating food intake and body weight. Further, these data implicate that disturbances in the ovarian hormone levels may predispose females to eating disorders. The reduction in the food intake by alteration in dopamine and serotonin levels by administration of plant extracts [31,32].From this study, we are predicting that after absorption of plant extracts from gastro intestinal track it cross the blood brain barrier (BBB) and enter the brain and amplify signaling in the basal hypothamus energy sensing function, which is the master regulator of food intake and energy expenditure or it may also possibly inhibits the re-uptake of 5-HT in the hypothalamus to activate β-adrenergic receptors which are involved in the burning of fats [33]. (Table 2)

Leptin and adiponectin are the key hormones in treatment of obesity. Leptin, a satiety hormone regulates energy balance by inhibiting hunger regulating food intake and body weight to regulate adipose tissue mass by decreasing food intake and modulating glucose and fat metabolism. Circulating adiponectin is capable of targeting multiple tissues and regulating insulin sensitivity as well as energy homeostasis in our study *Citrus limon* and Nigella *sativa* at 400mg / kg bw followed by *Cyperus rotundus* also at 400mg / kg showed decreased leptin concentration and increased adiponectin levels [34]. (Table 3).

The histological examination reveals the probable toxic effect of the drug on vital organs. The sections

of liver from progesterone treated mice indicated distorted structures of functional unit as mild congestion and focal necrosis in hepatocytes. However, the hepatic damage was reversed with the co–administration of 3 different plants extracts in a dose dependent manner visualized in (Figure 11).
Thus, our results suggest that plant extracts *Nigella sativa* and *Citrus limon* of 400mg / kg bw can be used for the treatment of obesity.

5. CONCLUSION

In general, the culmination with the present results we suggest that 3 different plants extract posses anti obesity efficacy against progesterone induced obesity model. From this study *Citrus limon* at 400mg / kg bw, *Nigella sativa* 400mg / kg bw followed by *Cyperus rotundus* 400mg / kg bw exhibited a significant anti-obesity activity. Oral administration of these extracts reduced the level of circulating lipids resulting in the decrease of food intake and body weights in female BALB/C mice, which bearing close resemblance to human obesity. Thus, it can be considered as an effective treatment in management of obesity owing to its potential anti-obesity effect.

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