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Research Article

PTEROCARPUS MARSUPIUM EXTRACT EXAGGERATES ANTI DIABETIC ACTIVITY OF METFORMIN

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ABSTRACT

Diabetes mellitus refers to a group of chronic metabolic disorders affecting human societies worldwide which are generally characterized by hyperglycemia. The single drug therapy does not provide adequate clinical results for the treatment of diabetes mellitus and its complications. The patients use herbal drugs and conventional drugs at the same time during their treatment which can lead to both benefit and risk to the health of the patient. Co-treatment of Metformin and *Pterocarpus marsupium* (PM) on pancreatic α-amylase activity in vitro has been studied to find out the drug-herb interaction in diabetes mellitus. Combination index for the combination was calculated to find out the synergistic, additive and antagonistic effects. Significant synergistic effect was seen at lowest herb-drug combination (10µg/ml Metformin + 10µg/ml hydroalcoholic extract of PM) treated group. The additive inhibition of the combination of Metformin with PM against pancreatic α-amylase was also found at other concentrations. The present study also investigated the in vivo antidiabetic and antilipidemic effect of alone herb and in combination with oral Metformin. Parameters like blood glucose, serum insulin, lipid parameters, liver glycogen and reduced glutathione were determined. Combination showed better effect than Metformin alone, reduced dose of Metformin may help to reduce the long term side effects associated with it. These results provide the first evidence for the effect of combination of Metformin and PM in vitro against pancreatic α-amylase as a potential inhibitor to control and prevent diabetes mellitus and reduce the dose of Metformin in the treatment. Results showed that combination treatment group showed significant normalization of all parameters compared to disease and metformin treated group in vivo. But no significant difference was observed in combination treatment with increased dose of metformin.

Keywords: Alpha amylase, Synergism, Antagonism, Herb-drug interaction.

1. INTRODUCTION

Diabetes mellitus has been defined by American Diabetes Association Expert Committee in their 1997 recommendations as a group of metabolic diseases characterized by hyperglycemia, altered metabolism of lipids, carbohydrates & proteins resulting from defects in insulin secretion, insulin action or both. The chronic hyperglycemia is associated with long damage, dysfunction & failure of various organs especially the eyes, kidneys, nerves, heart & blood vessels thus covering a wide range of heterogeneous disease [1]. There are two types of diabetes: Type I diabetes (β-cell destruction, leading to insulin deficiency) and Type II diabetes (Insulin resistance with relative insulin deficiency to a predominantly insulin secretory defect with insulin resistance) [2]. Assuming that age-specific prevalence remains constant, the number of people with diabetes in the world is expected to approximately double between 2000 and 2030, based solely upon demographic changes [3]. Many antidiabetic drugs are available, but adverse effects are associated with long term use of these drugs.

The use of complementary and alternative medicine (CAM) for the managing diabetes has hastily increased over the last decade. Most people use CAM therapies in addition to, rather than in place of, conventional medicine. A large number of antidiabetic plants are present in the nature and have been reported to be used in the disease for better management. However the parallel use of antidiabetic herbs and antidiabetic medications has greatly raised safety concerns. Herbal medicine contains numerous bioactive components and limited data are available of how these components interact with conventional antidiabetic medicines when

taken in combination [4]. It is generally believed that the use of herbs with medicine enhances the effect of the latter and reduces its adverse effects. So in such studies a perfect combination of phytochemicals and commercial oral antidiabetic drugs should be used. Positive interactions (additive or synergistic) between herbs and drugs may lead to improved effectiveness of the antidiabetic agents. An overview of the interaction between Pterocarpus marsupium and metformin has been provided.

Two or more drugs when administered together have the possibility to cause interactions-chemical or pharmacological. Herb-drug interaction can take place at pharmacodynamic or pharmacokinetic level [5]. Plant extracts and their ingredients could be a more effective strategy for the management of diabetes mellitus because of the likelihood of high compliance. They are largely free from side effects, have better effectiveness, act on multiple target sites and are of relatively low cost [6]. When using a combination therapy, the dosing of both the compounds is very important. These combinations can show one type of behavior at one concentration mixture and other type at another concentration. Herbs, vitamins, and other dietary supplements may modify, magnify or oppose the action of drugs when taken together [7]. Insulin resistance is a common problem with the Type-II diabetic patients. Pterocarpus marsupium is a widely used plant in many herbal formulations available in the market for the treatment of diabetes. Antidiabetic activity of alcoholic extract of *Pterocarpus marsupium* has been proven against STZ induced diabetic rat. The phytochemical analysis has shown the presence of flavonoids, terpenoids, tannins, glycosides, sterols, phenols and saponins which are known to be bioactive antidiabetic principles. In STZ diabetic rats flavonoids are known to regenerate the damaged beta cells as β cells are damaged in STZ induced rats [8]. (-)-Epicatechin was found to possess potent anti-diabetic activity on rat and effectively helped in β-cell regeneration in STZ induced diabetes in rats and reduced the blood sugar level. The insulinogenic activity of (-) epicatechin and its beneficial effect on islet insulin was found to be protective and restorative, by preventing metabolic alteration as a consequence of diabetes [9]. There are many synthetic drugs that are available in the market for treatment but have side effects and long term complications and patient noncompliance too. Metformin is a primary line and widely used drug for the treatment of diabetes and it acts via suppression of hepatic gluconeogenesis and glucose output from liver. Also enhances GLUT 1 transport from intracellular to site of plasma. It interferes with mitochondrial complex and promotes peripheral glucose utilization by anaerobic glycolysis and retards intestinal absorption of glucose [10].

2. MATERIAL AND METHODS

2.1. Preparation of extract

The fresh bark of the plant was collected from the local market of Rajkot. The fine bark powder (30g) was used for the extraction purpose. Powdered material was evenly packed, thimble was prepared and extraction was carried out with 95% ethanol and water in ratio of 1:1 at 40-50°C for 72 h using a Soxhlet apparatus. The above extract was dried on a water bath maintained at 40 °C to get dark brownish mass. The dry crude extracts were weighed and stored in air-tight container [11].

2.2. Chemical characterization of the extract

The extract was tested for the presence of flavonoids, alkaloids, tannins, phenols, saponins, terpenoids, glycosides etc. Flavonoids in the extract were determined using Shinoda test. The extract solution was made to react with, few fragments of magnesium ribbon and concentrated hydrochloric acid drop wise. Yellowish-orange color showed the presence of flavonoids. Total phenols were determined by ferric chloride test, a few drops of 10% aqueous ferric chloride were added to the extract, and appearance of blue color was the positive indicator of phenols [12].

2.3. *In Vitro* studies

The alcoholic extract of bark of *Pterocarpus marsupium* and metformin was tested for its alpha amylase inhibitory activity. 600 µl of (10,20,40,60,80,100µg/ml) test sample, 1.2 ml of starch in phosphate buffer (pH 6.9) containing 6.7mM of sodium chloride were added. The reaction was initiated by adding 600 µl of pancreatic amylase and incubated at 37 °C. From the above mixture 600 µl was taken and 300 µl of DNSA (1g of DNSA, 30g of sodium potassium tartarate and 20 ml of 2N NaOH was added and made up to a final volume of 100 ml with distilled water) and was kept in a boiling water bath for 15 min. The reaction mixture was diluted with 2.7 ml of water and absorbance was read at 540 nm. For each concentration, blank tubes were prepared by replacing the enzyme solution with 600 µl in distilled water. Control, representing 100% enzyme activities were prepared in a similar manner, without test sample. The experiments were repeated thrice using the same

protocol. The α-amylase inhibitory activity was calculated using the formula [13]:

Inhibition % = {Abs 540 nm (Control) - Abs540 nm (drug sample)/ Abs540nm (Control)} × 100

The following treatment schedule was framed [14]:
Test Tube 1: Negative control
Test Tube 2: Positive control
Test Tube 3: 10µg/ml of Metformin
Test Tube 4: 20µg/ml of Metformin
Test Tube 5: 40µg/ml of Metformin
Test Tube 6: 60µg/ml of Metformin
Test Tube 7: 80µg/ml of Metformin
Test Tube 8: 100µg/ml of Metformin
Test Tube 9: 10µg/ml of *Pterocarpus marsupium* extract
Test Tube 10: 20µg/ml of *Pterocarpus marsupium* extract
Test Tube 11: 40µg/ml of *Pterocarpus marsupium* extract
Test Tube 12: 60µg/ml of *Pterocarpus marsupium* extract
Test Tube 13: 80µg/ml of *Pterocarpus marsupium* extract
Test Tube 14: 100µg/ml of *Pterocarpus marsupium* extract
Test Tube 15: 10 µg/ml of Metformin + 10µg/ml of *Pterocarpus marsupium* extract
Test Tube 16: 10 µg/ml of Metformin + 100µg/ml of *Pterocarpus marsupium* extract
Test Tube 17: 100 µg/ml of Metformin + 10µg/ml of *Pterocarpus marsupium* extract

2.4. *In Vivo* studies

2.4.1. Experimental animals

All experiments were approved by Institutional Animal Ethics Committee (IAEC) procedure guidelines of CPCSEA. Male and female (10 weeks old) Sprague Dawley rats (body weight 200±25 g each) were housed in 7 groups of 6 animal in cages and maintained under standardized condition (12-h light/dark cycle, 24 ºC, 35 to 70% humidity) and provided free access to pellet diet and purified drinking water *ad libitum*, unless specified.

2.4.2. Chemical preparation and induction of diabetes

Fructose (21% solution) was dissolved in drinking water (prepared every 2 days) and given orally. The experimental rats developed diabetes after 8 weeks and then the Sprague Dawley rats were divided into groups as stated below for treatment with Metformin and *Pterocarpus marsupium* extract. Metformin and *Pterocarpus marsupium* were given orally as diabetic standard [7, 15].
Group I (control): free access to water
Group II-VII: diabetes-induced animals were divided into 6 groups. The experimental Sprague Dawley rats were sacrificed at 14th day.
The following treatment groups were used in the experiment:
Group II: diabetic control (21% fructose; p.o.)
Group III: diabetic standard (Metformin 50mg/kg; p.o.)
Group IV: diabetic standard (*Pterocarpus marsupium* extract 300mg/kg; p.o.)
Group V: treatment with combination- Metformin (12.5mg/kg; p.o.) + *Pterocarpus marsupium* extract (300mg/kg; p.o)
Group VI: treatment with combination- Metformin (25mg/kg; p.o) + *Pterocarpus marsupium* extract (300mg/kg; p.o)
Group VII: treatment with combination- Metformin (50mg/kg; p.o) + *Pterocarpus marsupium* extract (300mg/kg; p.o) [15]
Combination index: The interaction of two compounds can be calculated by a constant known as combination index (CI) [16].
CI = Ac/Ae + Bc/Be
Where, Ac and Bc correspond to the concentrations of A and B when used in combination, and Ae and Be correspond to those concentrations able to produce an effect of the same magnitude if used alone.
If D = 1, additive
D > 1, antagonism
D < 1, synergistic

2.5. Statistical analysis

All the values were expressed as mean±S.E.M. Statistical significance between more than two groups were tested using one-way ANOVA followed by the Bonferroni test as appropriate using computer based fitting program (Prism, Graphpad 5.01). Differences were considered to be statistically significant when $p < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Qualitative phytochemical analysis

Pterocarpus marsupium hydroalcoholic extract was found to contain flavonoids, tannins, sterols, triterpenoids and phenols. Proteins, alkaloids, carbohydrates were found to be absent. Flavonoids are reported to have anti α-amylase activity. It stimulates insulin secretion by islet cells. It also controls blood glucose levels. The phenol in the extract is responsible for the anti amylase activity. Analysis of the phytochemical constituents revealed the strong evidence for the anti diabetic activity of *Pterocarpus marsupium*.

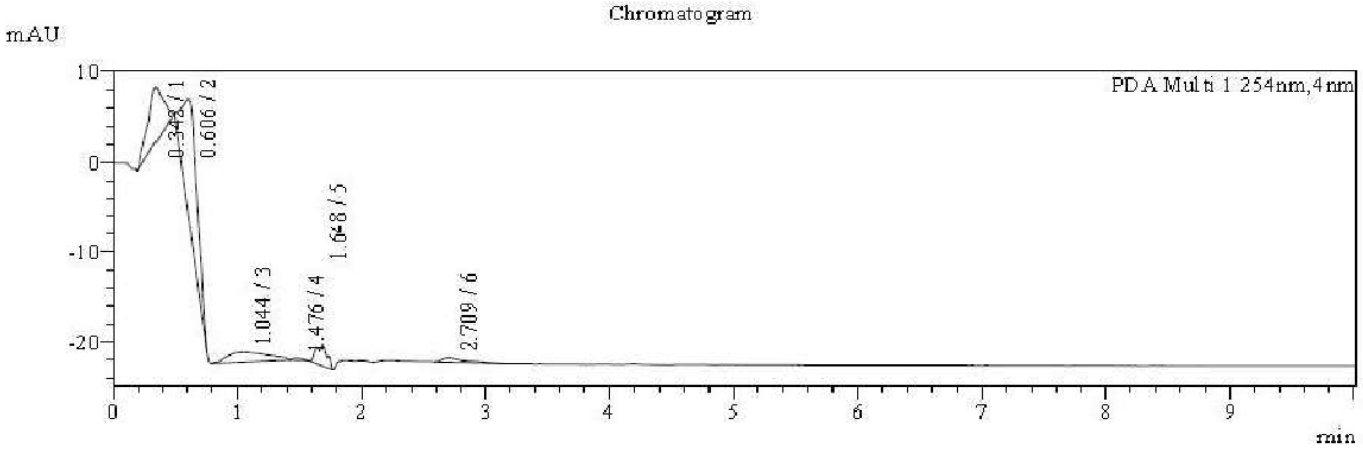

Fig. 1: LC-MS Chromatogram of *Pterocarpus marsupium* extract

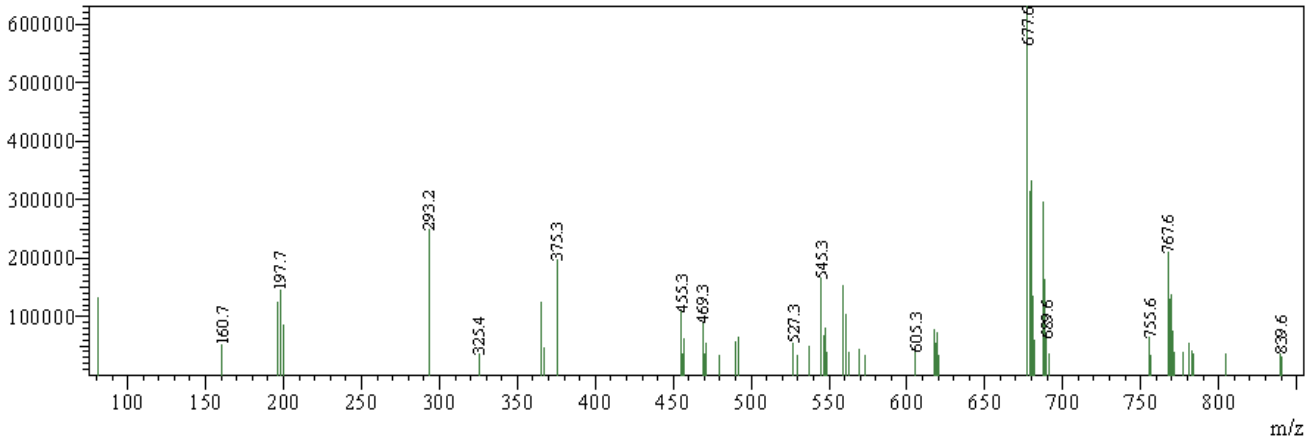

Fig.2: Identification of compound by mass value

Table 1: Phytochemical analysis of *Pterocarpus marsupium*

Phytochemicals	Result
Carbohydrates	A
Proteins	A
Sterols and triterpenoids	P
Glycosides	A
Alkaloids	A
Phenolic compounds	P
Flavonoids	P
Tannins	A

P- Present A- Absent

Table 2: Identification of compound by mass value

Name of compound	Mass of compound
Epicatechin	293.2
Pterostillbin	262.1
Marsupsin	306.1
Pteropsin	455.3

3.2. Inhibitory activity of Metformin and *Pterocarpus marsupium in vitro*

3.2.1. Effect of Metformin on α amylase

Metformin at different concentrations was able to produce inhibition of alpha-amylase. Increase in concentration leads to increase in % inhibition of alpha-amylase activity.

3.2.2. Effect of *Pterocarpus marsupium* extract on α amylase

Extract of *Pterocarpus marsupium* extract at different concentrations was able to produce inhibition of alpha-amylase. Increase in concentration leads to increase in % inhibition of amylase.

The combination showed better alpha amylase inhibition as compared to individual Metformin and *Pterocarpus marsupium* extract treatment. Synergism and additive effect was shown at different concentrations. The lowest dose combination of Metformin and extract of

Pterocarpus marsupium shows strong synergistic activity (CI = 0.24)

10 + 100 dose combination shows nearly additive activity (CI = 0.99)

100+10 dose combination shows additive activity (CI=1)

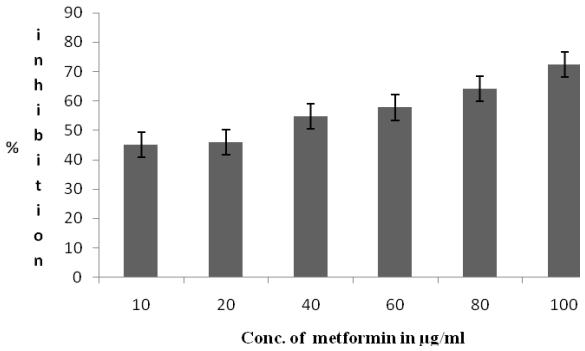

Fig. 3: Alpha amylase inhibitory activity of Metformin *in vitro*

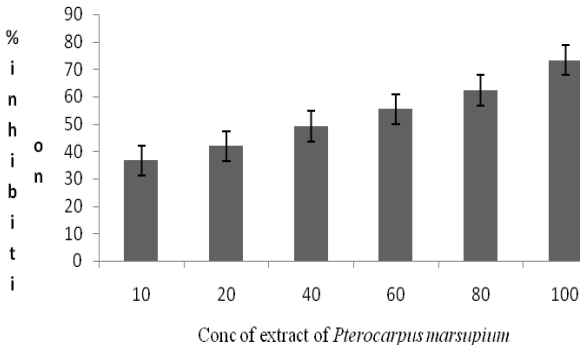

Fig. 4: Alpha amylase inhibitory activity of *Pterocarpus marsupium* extract *in vitro*3) Effect of Metformin + *Pterocarpus marsupium* extract on α amylase

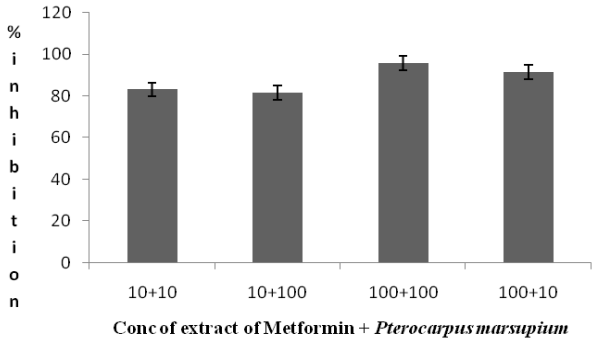

Fig. 5: Alpha amylase inhibitory activity of combination of Metformin + *Pterocarpus marsupium* extract in vitro

3.3. Inhibitory activity of Metformin and *Pterocarpus marsupium* in vivo

3.3.1. Effect on fasting blood glucose

The fasting glucose level was significantly increased ($p<0.05$) with administration of 21% fructose for 8 weeks in all the groups as compared to normal control group. A significant decrease in blood glucose level was observed in all the treatment groups in comparison with the disease control group. The decrease in combination treatment groups were significant ($p<0.05$) as compared to metformin treated group. But there was no significant change observed in blood glucose lowering activity when dose of metformin was increased in combination treatment. Determination of glucose was done using Dr. Morepen Advantage Blood Gluco meter.

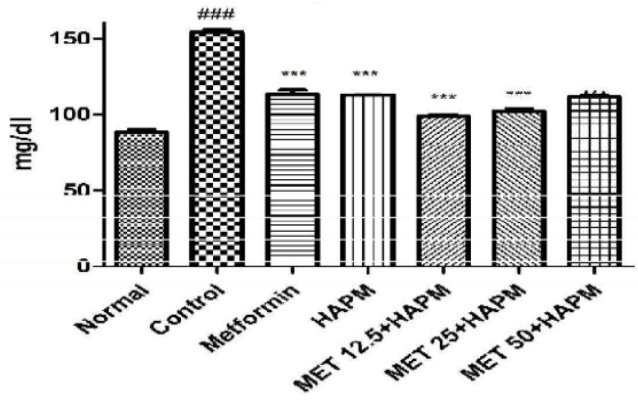

Fig. 6: Effect of combination treatment on serum fasting glucose in fructose induced insulin resistance in rats

3.3.2. Effect on fasting insulin

The fasting insulin level was significantly increased ($p<0.05$) with administration of 21% fructose in all the groups as compared to normal control group. A significant decrease in serum insulin level was observed in all the treatment groups in comparison to the disease control group The decrease in combination treatment groups were significant ($p<0.05$) compared to metformin treated group. But there was no significance change observed in insulin activity when dose of metformin was increased in combination treatment. Serum fasting insulin was estimated using CLIA kit.

3.3.3. Effect on fasting insulin resistance index

The fasting insulin resistance index shows significant ($P<0.05$) increased in all the groups as compared to normal control group. A significant decrease in FIRI was

observed in all the treatment groups in comparison to the disease control group. The decrease in combination treatment groups were significant ($P<0.05$) compared to metformin treated group. But there was no significance change observed in FIRI index when dose of metformin was increased in combination treatment.

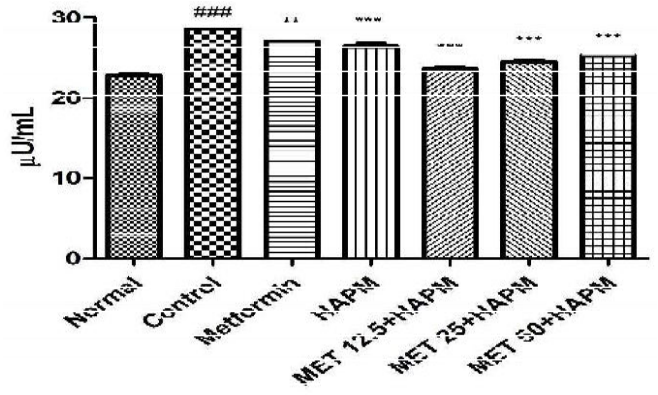

Fig. 7: Effect of combination treatment on serum fasting insulin in fructose induced

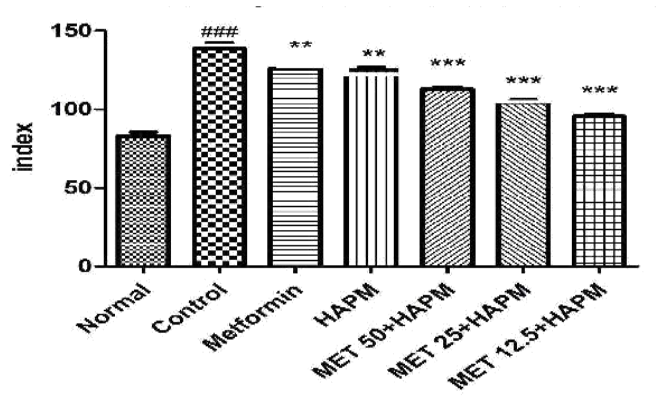

Fig. 8: Effect of combination treatment on fasting insulin resistance index in fructose induced insulin resistance in rats

3.3.4. Serum Triglyceride and Total Cholesterol

The fasting serum triglycerides and total cholesterol level were significantly increased ($p<0.05$) with administration of fructose in all the groups as compared to normal control group. Serum triglyceride and total cholesterol levels were decreased in all the treatment groups as compared to the disease control group. The triglycerides and total cholesterol level found to be restored in combination treatment are significant ($p<0.05$) as compared to metformin treated group. But with increase in dose of metformin in the combination treatment group showed no significance difference observed in serum triglyceride and total cholesterol level. The quantitative determination of the activity of cholesterol and triglyceride in serum was done using enzymatic kit.

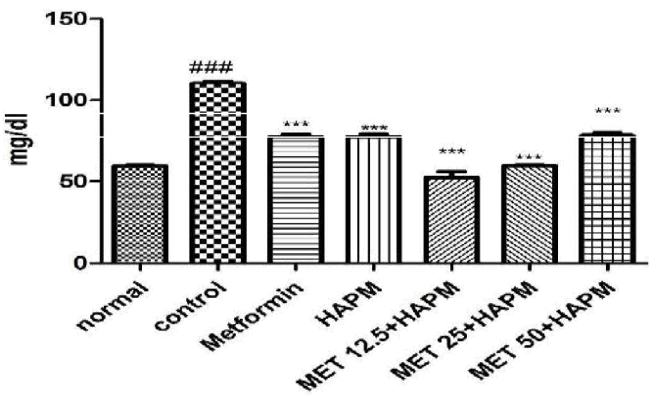

Fig. 9: Effect of combination treatment on serum triglyceride in fructose induced insulin resistance in rats

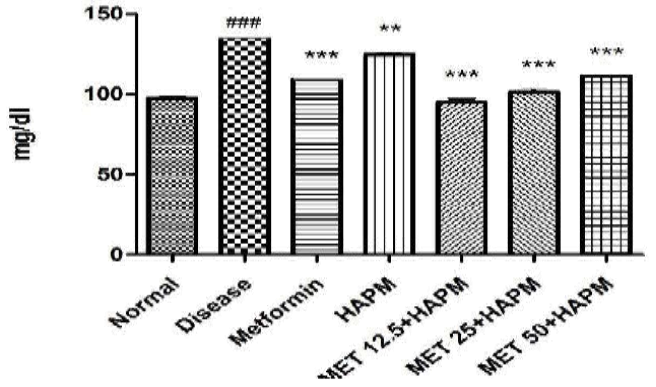

Fig. 10: Effect of combination treatment on total cholesterol in fructose induced insulin resistance in rats

3.3.5. Serum HDL, LDL, VLDL parameters

The fasting serum HDL level were significantly decreased ($p<0.05$) with administration of fructose in all the groups as compared to normal control group, and serum LDL, VLDL levels were significantly ($p< 0.05$) increased in all groups as compared to normal control. Serum HDL levels were normalized in all the treatment groups in comparison to the disease control group. The HDL level were found to be normalized in combination treatment and are significant ($p<0.05$) as compared to metformin treated group. With increase in dose of metformin in the combination treatment groups the reduced levels there was no statistically significant

change observed. Serum LDL and VLDL levels were found to be significantly ($p<0.05$) increased in all treatment group as compared to disease control group. Increase in serum LDL, VLDL was significant in treatment as compared to metformin alone. But with increase in dose of metformin in the combination treatment groups the reduced levels become statistically insignificant. Quantitative determination of the activity of cholesterol in serum was done using enzymatic kit-Phosphotungstate method. Estimation of LDL and VLDL cholesterol was done using the Friedewald formula [17].

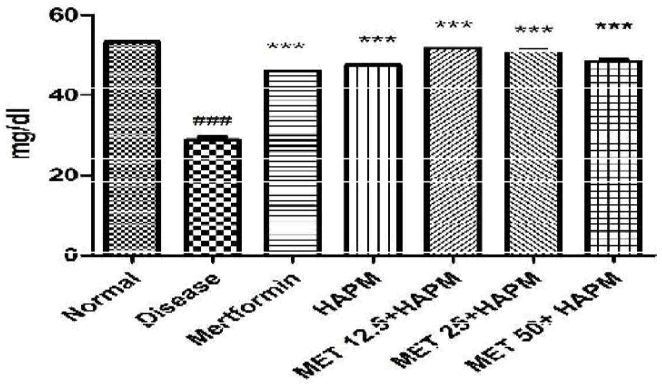

Fig. 11: Effect of combination treatment on serum HDL in fructose induced insulin resistance in rats

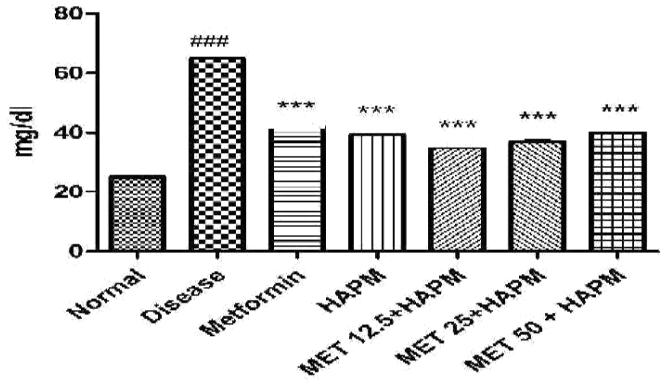

Fig. 12: Effect of combination treatment on serum LDL in fructose induced insulin resistance in rats

3.3.6. Liver glycogen

Liver glycogen stores were found to be decreasing ($p<0.05$) significantly with fructose supplement, as compared to normal control group, indicating development of insulin resistance. A significant increase in liver glycogen levels were observed in all the treatment groups in comparison to the disease control group which showed the increase in insulin sensitivity. The decrease in combination treatment groups were significant ($p<0.05$) compared to metformin treated group. No significant difference was observed with increase in dose of metformin in the combination treatment groups.

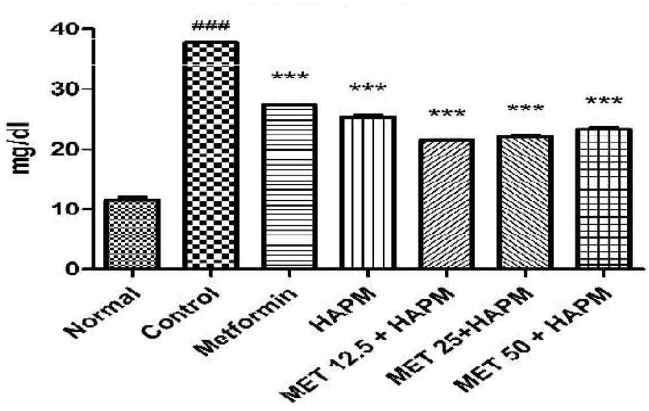

Fig. 13: Effect of combination treatment on serum VLDL in fructose induced insulin resistance in rats

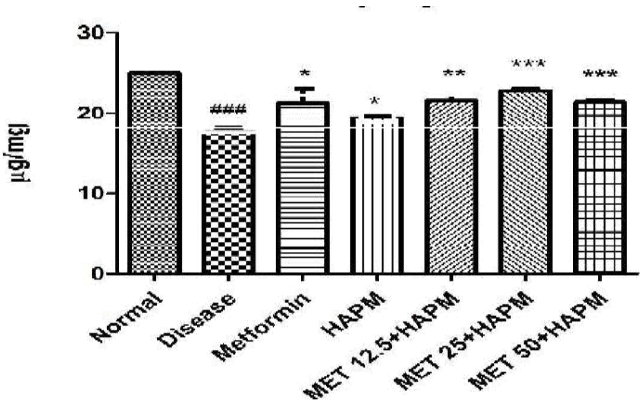

Fig. 14: Effect of combination treatment on liver glycogen parameters in fructose induced insulin resistance in rats

3.3.7. Reduce Glutathione

Reduced Glutathione were measured in terms of anti-oxidant activity, was found to be decreasing ($p<0.05$) with fructose supplement, as compared to normal group. A significant increase in antioxidant activity was observed in all the treatment groups in comparison to the disease control group. The increased in activity in combination therapy groups were significant ($p<0.05$) compared to metformin treated group. But with

increase in dose of metformin in the combination therapy groups the antioxidant activity become statistically insignificant. Anti-oxidant activity may be due to prevention of oxidation of free fatty acid by increasing insulin sensitivity. Reduced glutathione was determined according to the method of Ellman [18].

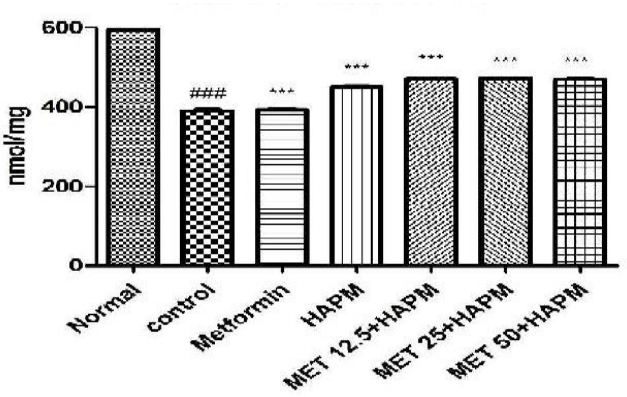

Fig. 15: Effect of combination treatment on serum reduce glutathione in fructose induced insulin resistance in rats

3.3.8. Effect on Body Weight

Body weight were found to be increasing ($p<0.05$) respectively with fructose supplement, as compared to normal group. A significant normalization in body weight was observed in all the treatment groups in comparison to the disease control group the normalized in combination treatment groups were significant ($p<0.05$) compared to metformin treated group. But with increase in dose of metformin in the combination treatment groups the decrease in body weight become statistically insignificant.

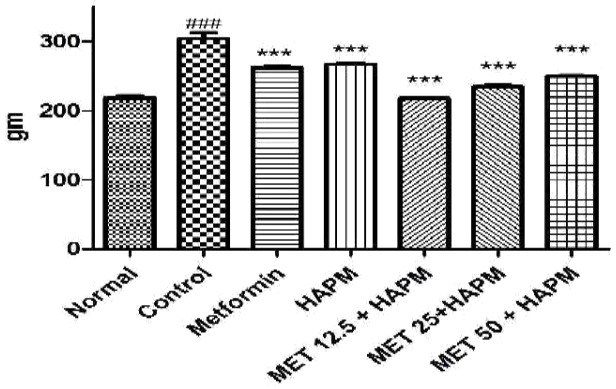

Fig.16: Effect of combination treatment on body weight in fructose induced insulin resistance in rats

In recent years, the search for new chemical compounds as possible pancreatic α-amylase inhibitors with a high specific affinity has increased. In this study, in vitro inhibitory effect of Metformin and *Pterocarpus marsupium* extract on pancreatic amylase activity was evaluated. Flavonoid is reported to have inhibitory activity against α-amylase and epicatechin is the flavonoid present in *Pterocarpus marsupium*. The lowest dose concentration showed the best synergistic activity from which we can conclude that the dose of metformin can be reduced if given in combination with *Pterocarpus marsupium*. Results suggested that individual drug or extract shows concentration dependent inhibition. As we go on increasing the concentration of drug, significant inhibition was observed. Fructose feeding for 60 days resulted in hyperglycaemia, hyperinsulinaemia, dyslipidemia and decreased peripheral uptake of glucose [19]. The resultant increase in fasting insulin resistance index (FIRI) is also indicative of aggravation of insulin resistance. Earlier fructose was considered as a glucose alternative in diabetic patients. But later on it was revealed that chronic use of fructose culminates in development of metabolic syndrome, including induction of insulin resistance. Research in metabolism of fructose has unmasked difference between short-term positive effects and the negative effects of chronic use of fructose. Long term derogatory effects include changes in digestion, absorption, plasma hormone levels, appetite, and hepatic metabolism, leading to precipitation of insulin resistance, diabetes, obesity, and inevitably cardiovascular disease. Fructose is a potent regulator of glycogen synthesis and liver glucose uptake. Therefore any catalytic improvements are due to hepatic glucokinase and glucose uptake facilitation. However, as mentioned, the beneficial effects do not continue with chronic fructose utilization. Because of its lipogenic properties, excess fructose in the diet can cause glucose and fructose mal absorption, and greater elevations in triglycerides and cholesterol compared to other carbohydrates. Of the key importance is the ability of fructose to bypass the main regulatory step of glycolysis, the conversion of glucose-6-phosphate to fructose 1, 6-bisphosphate, controlled by phosphofructokinase. Thus, while glucose metabolism is negatively regulated by phosphofructokinase [20], fructose can continuously enter the glycolytic pathway. Therefore, fructose can uncontrollably produce glucose, glycogen, lactate and pyruvate, providing both the glycerol and acyl portions of acyl-glycerol molecules. These particular substrates, and resultant excess energy flux due to unregulated

fructose metabolism, will promote the overproduction of TG [21]. It has been further reported that fructose causes metabolic syndrome mediated through leptin, adiponectin, and free fatty acids. Metformin decrease hepatic glucose output by inhibiting hepatic gluconeogenesis, enhance insulin mediated glucose disposal in muscle and fat. It also retards intestinal absorption of glucose other hexose and amino acids. Thus blood glucose level is normalized. These benefits of *Pterocarpus marsupium* are presumed to be the single or synergistic effects of several antidiabetic ingredients like (-) epicatechin, pterosupin, marsupin and pterostiblene present in it. The antidiabetic effect of pterostilbene may be due to increased release of insulin from the existing β-cells of pancreas. Flavonoids are known to regenerate beta cells [22]. In normal metabolism insulin activates the enzyme lipoprotein lipase hydrolyse. Insulin resistance causes inactivation of this enzyme thus causing hypertriglyceridemia. Fructose feeding causes increased production of VLDL particles through the elevated synthesis of cholesterol, fatty acid and triglycerides in the liver, causing insulin resistance accompanied by hyperlipidemia and hypertension. It was reported that continuation of fructose feeding caused down regulation of PPAR-α, which in turn leads to a reduction in triglycerides catabolism which is involved with LDL, Acyl Co-An oxidase and β-oxidation [23]. The hypolipidemic effect may be due to inhibitory of fatty acid synthesis. Treatment may be directly attributed to improvement in insulin levels and decrease in sensitivity of insulin in tissue.

4. ACKNOWLEDGEMENT

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