

Andrographolide Cause Retardation of Insulin Resistance Changes in Pancreatic and Adipose Tissues of Male Mice: Immunological and Biochemical Study.

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ABSTRACT

The continual increase in the incidence of insulin resistance and the associated metabolic syndrome has necessitated the thrust for the development of therapeutic agent that could ameliorate this condition. Even though the use of andrographolide for therapeutic purposes has gained wide acceptability, it's used in the treatment of insulin resistance that associated with immunological, and pathophysiological derangements have not been evaluated. This study was undertaken to investigate the ameliorative effect of andrographolide on high fat diet induced insulin resistance in male mice immunologically. Forty-eight apparently healthy ICR male mice were divided into 4 groups, CN, CP, HFA25 and HFA50 (12 mice per group). CP, HFA25 and HFA50 were fed with high fat diet for 24 weeks than HFA25 and HFA50 groups were treated for 15 days with 25 mg/kg and 50 mg/kg body weight respectively. The CN group was fed a standard diet, while the CP group was fed with high-fat diet containing 45% of energy derived from fat, 20% from protein and 35% from carbohydrate. The mice were sacrificed at the end of the treatment. Blood leptin, Glucose, total cholesterol, high-density lipoprotein (HDL), triglycerides, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT), and IgM, IgG, GGT, TNF α , IL-1 β .IL-6 MD, adiponectin were determined. High fat diet was observed to induce increase in levels of leptin, AST, ALT, Glucose, insulin, ALP, Cholesterol, TG, VLDL, GGT, TNF α , IL-1 β .IL-6 MDA and decline in adiponectin, IgM, IgG, and HDL, while 15 days treatment with andrographolide ameliorated the pathophysiological changes of insulin resistance in mice by use anti- insulin beta cell receptor to detection the number of beta cell of pancreas in treated groups according to immunoperoxidase protocol. It was concluded that Andrographolide has potential ameliorative in insulin resistant mice.

Keywords: Andrographolide, insulin resistance, immunoglobulin, immunohistochemistry, inflammatory markers

INTRODUCTION

Andrographolide is the major diterpenoid in *A. paniculata*, making up about 4%, 0.8%~1.2% and 0.5%~6% in dried whole plant, stem and leaf extracts respectively (Burgos et al., 1997; Pholphana et al., 2004, Song et al., 2013). It is used as a wonder drug in traditional Sidha and Ayurveda system of medicine as well as tribal medicine in India and China for multiple clinical applications, since ancient and also been shown to be effective against certain cancers and is an effective purgative. The plant extracts exhibit anti-typhoid and antifungal, anti-hepatotoxic, anti-biotic, anti-malarial, anti-hepatitis, anti-thrombogenic, anti-inflammatory, anti-snake venom and antipyretic properties to mention a few, besides its general use as an immunostimulating (De Silva et al., 2010; Puri et al., 1993) Andrographolide has been tested in different experimental studies on human and animals which proved andrographolide was a safe

drug with no harmful side effects (Bothiraja et al., 2013). Insulin resistance which plays a major role in the pathogenesis of type 2 diabetes could be detected 10 to 20 years even before the onset of hyperglycemia. The inability of the peripheral target tissues to respond effectively to insulin stimulation gives rise to IR (National Diabetes Information Clearing house, Insulin Resistance and Prediabetes, 2014). It has been demonstrated that extracts of *Andrographis paniculata* and its constituents, namely the diterpene lactone andrographolide, have anti-inflammatory (Tang et al., 2007; Chang et al., 1986a), anti-Allergic (Cáceres et al., 1997), immuno-stimulatory (Puri et al., 1993) and anti-viral activity (Chang et al., 1991; Wiart et al., 2005). Little is known about the mode of action of andrographolide as anti-prediabetes activity. It was demonstrated that anti-inflammatory effects of *Andrographis paniculata* is likely associated with inhibition of PAF-mediated

inflammatory response (Puri et al., 1993; Ernst et al., 2001) and inhibition of expression of nitric oxide (NO) synthesis in macrophages (Chao et al., 2011). However, anti-inflammatory mechanism was not connected with inhibition of the biosynthesis of eicosanoids, as in conventional non-steroidal anti-inflammatory drugs (NSAID) (Wiert et al., 2005). Other data indicates that andrographolide has an immunostimulatory activity. For example it has been shown that administration of an ethanol extract of *A. paniculata* (25 mg/kg), or purified andrographolide (1 mg/kg) to mice by gastric lavage stimulated antibody production and the delayed-type hypersensitivity response to foreign antigen (sheep red blood cells) (Puri et al., 1993). This extract also stimulated migration of macrophages, phagocytosis and proliferation of splenocytes. (Puri et al., 1993). Since the crude extract was more effective than purified andrographolide or neoandrographolide alone, it was suggested that other constituents might be involved in the immunostimulant response (Puri et al., 1993). In other words the mechanism of the immunostimulating action of Andrographis and its active constituents was not fully clarified. Immunomodulation effects were also shown by a methanolic extract from *A. paniculata* and isolates andrographolide, 14-deoxyandrographolide and 14-deoxy-11, 12-didehydroandrographolide that enhanced the proliferation and interleukin-2 (IL-2) induction in human peripheral blood lymphocytes (Kumar et al., 2004; Zhang et al., 2001). More than 300 herbal extracts have been used to reduce blood glucose, but only a small number of these have received scientific and medical evaluation to assess their efficiency especially as anti-insulin resistant, anti-inflammation, and immunostimulating effects (Sykietis, 2001; Shoelson et al., 2006; Chao et al., 2011a) in ICR male mice. Among them, Anti-diabetic property of aqueous extract of Andrographolide was further confirmed (Dandu et al., 2009; Chao 2011b). Even though the insulin – sensitizing property of Andrographolide has been noted, the molecular mechanisms by which Andrographolide reduce insulin resistance and immunosuppression, and inflammation mechanisms are not clear. The current study is part of PhD program and the aim from it is to investigate the role of andrographolide as immunostimulating properties by measure IgM, IgG, and some anti – inflammatory markers that included TNF α , IL-1 β , IL-6, leptin and adiponectin, adding to that, detection the effect of andrographolide on β -cell in pancreatic tissue that affected by insulin resistance by using immunoperoxidase protocol. We are demonstrated this the first time that andrographolide effected and caused retardation of insulin resistance histopathological changes of pancreatic tissue and elevated immunostimulating.

MATERIALS AND METHODS

Animal study

Forty-eight (48) male mice aged 6 weeks 20-22 weighted were purchased from 45 Jalan Indah 1, 22. Taman University Indah 43300 Sei Kembangan Selangor. Darul Ehsan, Malaysia and housed in Animal house (FPV) under a 12-hour light-dark cycle at 21 \pm 2°C and 60 \pm 10 % humidity. After 2 week adaptation period, the body weight and fasting blood glucose level of the 8-week-old mice were measured. Then, the mice were equally divided into four groups (n = 12): (1) control negative (CN), (2) Control positive (CP), (3) High fat diet + andrographolide 25 mg/kg and (4) High fat diet + andrographolide 50 mg/kg. After feeding high fat diet (commercial butter mixed with fresh chew pellets) to (CP, HFA25, and HFA50) for 24 week accordance with diet –induced obesity (DIO) (Warden, 2008) and Fresh chow was provided to control group daily. The mice fasted for 8 hours, and then sacrificed by cervical dislocation blood samples were collected from the heart puncture for the measurement of the blood and serum parameters such as glucose, and insulin, lipid profile, immunoglobulin, and anti-inflammatory biomarkers.

Biochemical analysis

At the end of 26th week, after the estimation of blood glucose level, the animals were sacrificed by cervical dislocation. Serum separated for determination of parameters the level of serum triglyceride (TG) (GP0-PAP method mmol/l, high density lipid cholesterol (HDL), total cholesterol (TC), insulin and using commercial available kits. (SPAN Diagnostics) Furthermore, the serum lipids such as total cholesterol and triglyceride were determined with commercial kits (Sigma-Aldrich, USA). Mouse Insulin Kit was estimated by (Dia Metra (Italy)-DKO 078 ELSA specific to mice), Total cholesterol and triglycerides (TG) were assayed by the colorimetric enzymatic method. The high-density lipoprotein -cholesterol (HDL-c) was measured using the Homogenous enzymatic colorimetric assay (Roche /Hitachi 912/917/MODULAR analyzers: ACN059).

The enzymes alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase A (ALK) were also measured by indicates Roche/Hitachi analyzers on which kits (MODULAR P/D analyzers: CAN 15. While adipocytokines were assessed by Mouse /Rat Leptin ELSA Kit (A05176SPI bio), (TNF α -Kit (ARIG 80206-Mouse TNF α ELISA). Interleukin-6 (CSB-E04639m Mouse IL-6 ELSA Kit). Adiponectin Kit (CSB E046738 Mouse adiponectin kit). Moreover, IgM, IgG was measured (Mice immunoglobulin M, G (IgM, IgG) ELISA Kits (CSB-E07991r), (CSB-E07981r).

Immunoperoxidase protocol

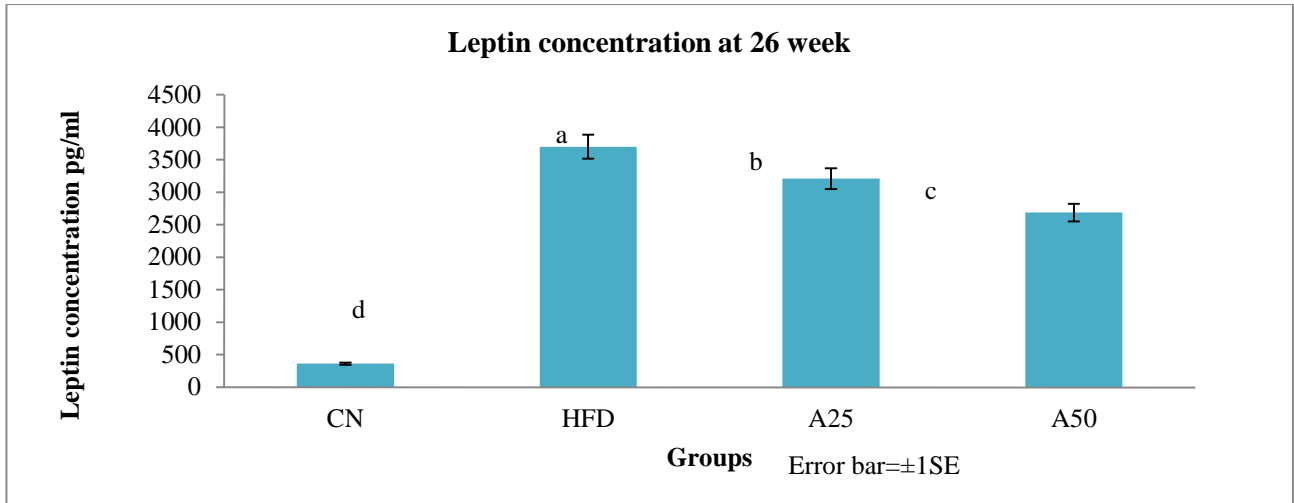


Figure 1: Serum Leptin concentration after andrographolide treatment. Different superscripts (^{a,b,c,d}) showing significant difference ($P<0.05$) between (CN, HFA25, HFA50) ($n=12$ mice per group) among the treatment groups. Error bar = ± 1 SE. Data is expressed as means ± 1 standard error.

CN=control negative group

CP=control positive group

HFA25=high fat diet +andrographolide 25mg/kg

HFA50=high fat diet+ andrographolide 50 mg/kg

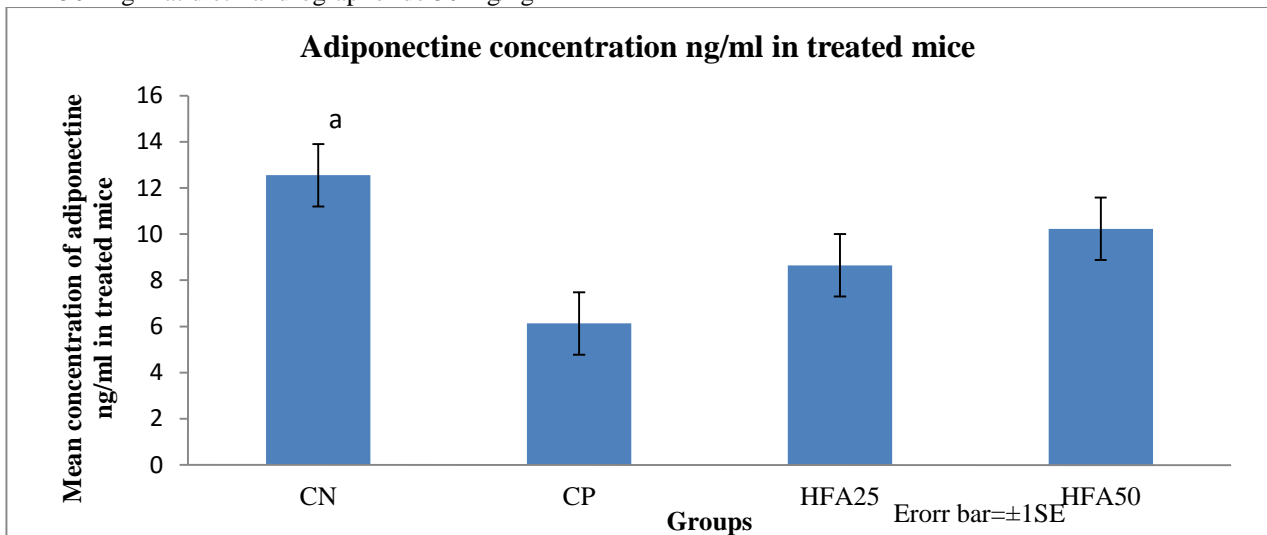


Figure 2: Mean concentration of adiponectin after andrographolide treatment. Different superscripts (^{a,b,c,d}) showing significant difference ($P<0.05$) between (CN,CP, HFA25, HFA50) ($n=12$ mice per group) among the treatment groups. Error bar = ± 1 SE. Data is expressed as means ± 1 standard error.

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HFA25=high fat diet +andrographolide 25mg/kg

HFA50=high fat diet+ andrographolide 50 mg/kg

For the immunohistological staining of insulin, the pancreas, liver, adipose tissues were removed from the animal immediately after sacrificing. Immunohistochemical examination and interpretation all specimens were previously fixed in 10% formalin solution and prepared for the immunohistochemical procedure using the Dako En Vision +System-HRP(DAB) Immune peroxidase staining protocol. The principal steps are as follows; selected blocks were cut into 5 micron sections, deparaffinised in xylene, rehydrated in different

grads of alcohol and rinsed in Tris-buffered saline Tween 20 (TBST). Antigen-retrieval (pH=9) was done using microwave. The sections were incubated slides with peroxidase Blocking Reagent (H2O2) for 5 min. Firtely incubated with primary antibody (anti-insulin mouse monoclonal beta cell receptor) (dilution 1: 600; Dako 076 Company) for 40 min. Then incubated with Dako real En Vision HRP polymer for 40 min and rinse 2x3 in TBST. The slides were visualized using 3, 3'-diaminobenzidine (DAB. Meyer's Hematoxylin, methylene blue, was

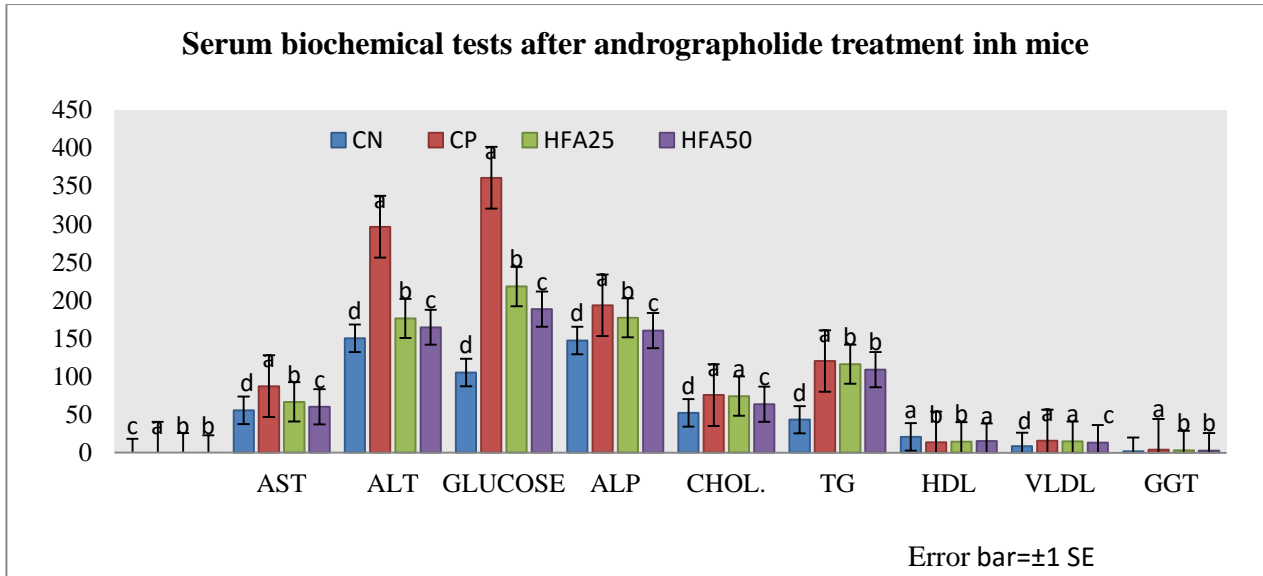


Figure 3: Mean serum biochemical levels of AST, ALT, Glucose, ALP, Cholesterol, TG, HDL, VLDL, and GGT. Different superscripts (a,b,c,d) showing significant difference ($P<0.05$) between (CN,CP, HFA25, HFA50) (n=12 mice per group) among the treatment groups. Error bar = ± 1 SE. Data is expressed as means ± 1 standard error.

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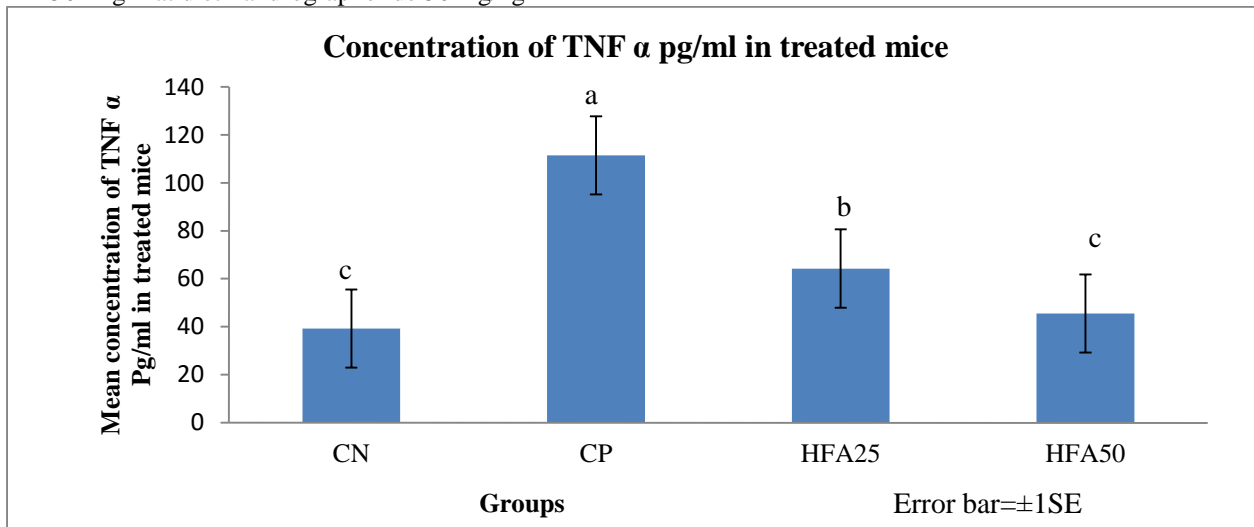


Figure 4: Mean concentration of TNF α at week 26 following 15 days treatment with Andrographolide. Different superscripts (a,b,c,d) showing significant difference ($P<0.05$) between (CN,CP, HFA25, HFA50) (n=12 mice per group) among the treatment groups. Error bar = ± 1 SE. Data is expressed as means ± 1 standard error.

CN=control negative group

CP=control positive group

HFA25=high fat diet +andrographolide 25mg/kg

HFA50=high fat diet+ andrographolide 50 mg/kg

used as counter stain. Positive control of normal pancreas, liver, adipose tissue and were used with each run of immunoassay. After completion of the immunohistochemical staining, the cases were examined microscopically for the localization of the antibody. The beta cells were positive by brown staining for the used antibody and the degree of

immunoreactivity in the targeted cells was evaluated image j software.

Statistical analysis

All data were expressed as the mean \pm standard error of mean (SEM). Statistical analysis was performed using the SPSS software package

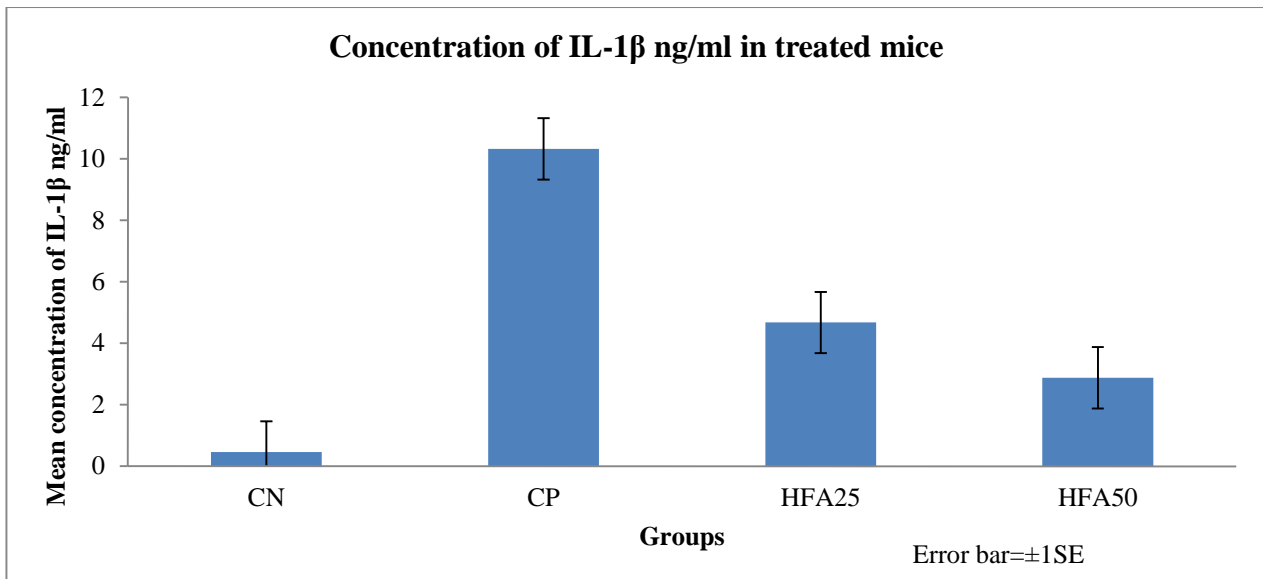


Figure 5. Mean concentration of IL-1 β at week 26 following 15 days treatment. Different superscripts (a,b,c,d) showing significant difference ($P<0.05$) between (CN,CP, HFA25, HFA50) (n=12 mice per group) among the treatment groups. Error bar = ± 1 SE. Data is expressed as means ± 1 standard error.

CN=control negative group

CP=control positive group

HFA25=high fat diet +andrographolide 25mg/kg

HFA50=high fat diet+ andrographolide 50 mg/kg

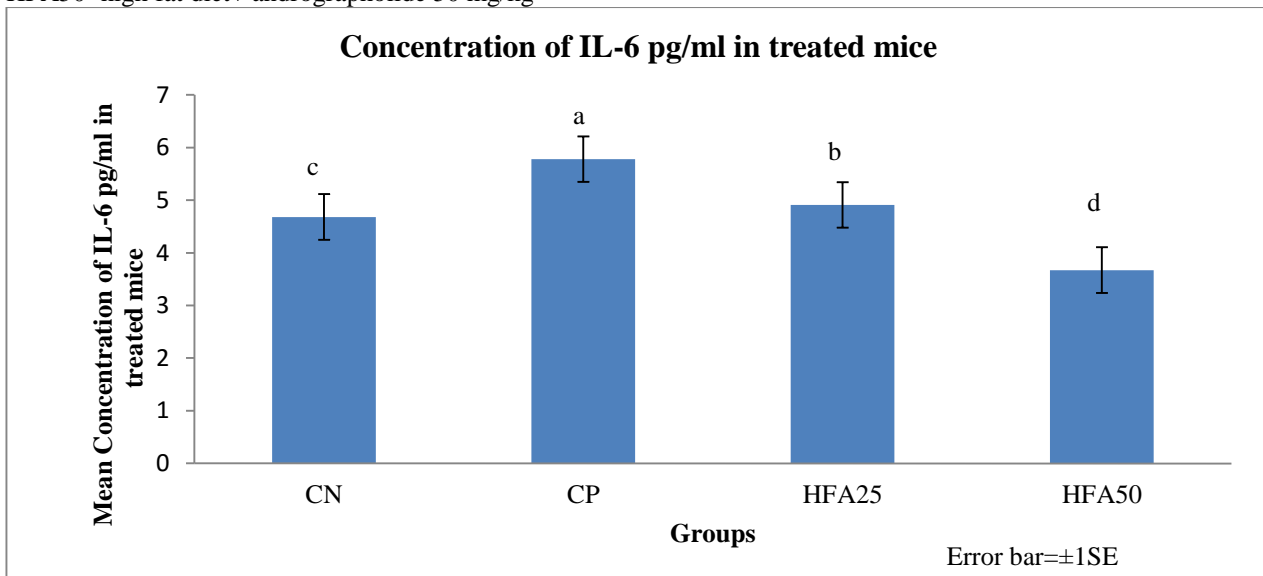


Figure 6. Mean concentration of IL-6 at week 26 following 15 days treatment with andrographolide. Different superscripts (a,b,c,d) showing significant difference ($P<0.05$) between (CN,CP, HFA25, HFA50) (n=12 mice per group) among the treatment groups. Error bar = ± 1 SE. Data is expressed as means ± 1 standard error.

CN=control negative group

CP=control positive group

HFA25=high fat diet +andrographolide 25mg/kg

HFA50=high fat diet+ andrographolide 50 mg/kg

(Version.20). One-way ANOVA (analysis of variance) with post-hoc test by Duncan's multiple-range test was used to examine differences among groups. The level of significance was set at 0.05

RESULTS

Leptin concentration Serum leptin concentration was assayed and the result is presented in figure 1. The control negative (CN) group showed the least serum leptin concentration while the (CP) recorded the highest serum leptin concentration. Unlike the positive control group, the serum leptin concentration for the treated

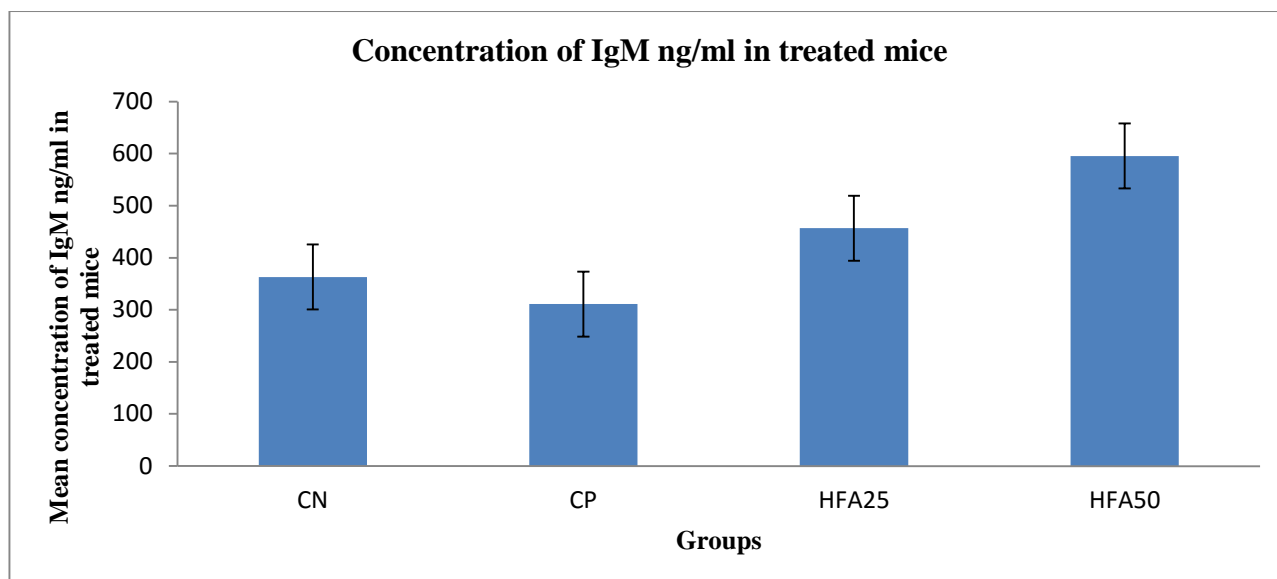


Figure 7. Mean serum levels of immunoglobulin M (IgM) in treated mice. Different superscripts (^{a,b,c,d}) showing significant difference ($P < 0.05$) between (CN, CP, HFA25, HFA50) ($n = 12$ mice per group) among the treatment groups. Error bar = ± 1 SE. Data is expressed as means ± 1 standard error.

CN=control negative group

CP=control positive group

HFA25=high fat diet +andrographolide 25mg/kg

HFA50=high fat diet+ andrographolide 50 mg/kg

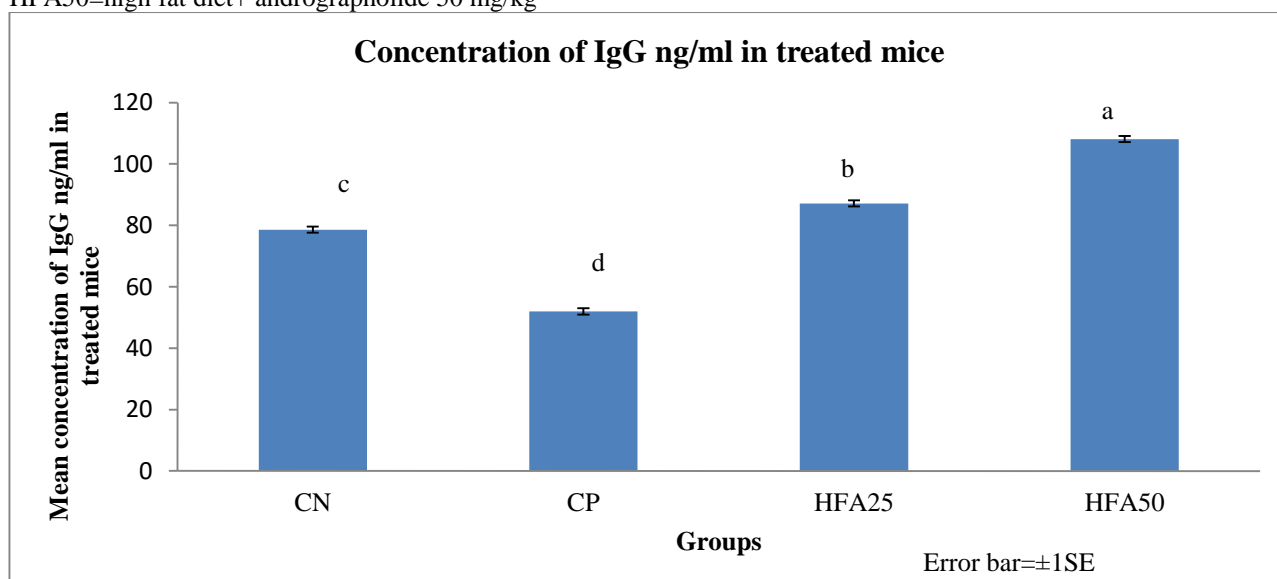


Figure 8. Mean serum levels of immunoglobulin G (IgG). Different superscripts (^{a,b,c,d}) showing significant difference ($P < 0.05$) between (CN, CP, HFA25, HFA50) ($n = 12$ mice per group) among the treatment groups. Error bar = ± 1 SE. Data is expressed as means ± 1 standard error.

CN=control negative group

CP=control positive group

HFA25=high fat diet +andrographolide 25mg/kg

HFA50=high fat diet+ andrographolide 50 mg/kg

groups (HFA25 and HFA50) showed relatively reduced leptin concentration with the HFA50 group showing the most significant reduction and these decreases were due to the 15 days treatment with Andrographolide

Serum level of adiponectin

The mean concentration of adiponectin after andrographolide treatment is shown in figure 2.

Twenty four weeks feeding with HFD was observed to induce significant decrease ($P < 0.05$) in adiponectin level (CP). 15 days treatment with Andrographolide at the doses of 25 mg/kg and 50 mg/kg body weight (A25 and A50 respectively) was observed to induce increase in adiponectin levels significantly ($P < 0.05$).

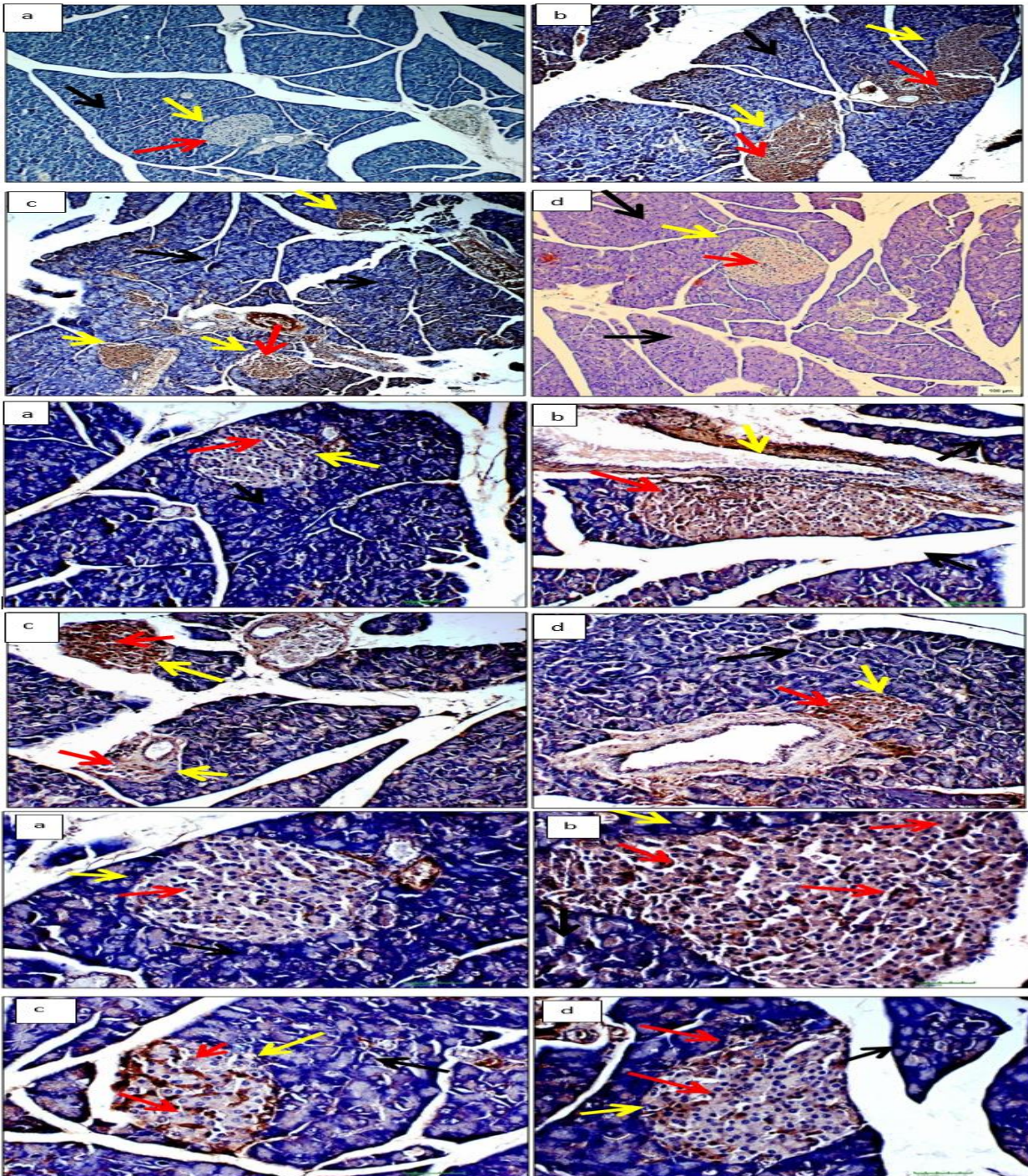


Figure 9: Photomicrograph of pancreas tissue showing (a) (x100, x200, x400). CN group showed pancreas Acinar (black arrow), islands of Langerhans normal (yellow arrow), beta cell (red arrow) (. (b) (x100, x200, x400) CP mice pancreas showing there were brown activity in pancreatic tissue (black arrow) special in beta cell (black spot) (red arrow) due to reactive with anti-insulin beta cell receptor, the number of beta cell inside of islets of Langerhans increased as a compensatory mechanism for pancreas to increase production of insulin at the first stage of in insulin resistance condition (hyperinsulinemia), the islands of Langerhans cells larger compare to (yellow arrow) CN, HFA25, and HFA50. (c) (x100, x200, x400) the reactive of beta cell less intensive (black spot) (red arrow), islets of Langerhans (yellow arrow). (d) (x100, x200, x400). light brown beta cell reaction (red arrow), islets of Langerhans (yellow arrow). pancreatic acini (black arrow).

Biochemical analysis

The mean serum biochemical levels of AST, ALT, Glucose, ALP, Cholesterol, TG, HDL, VLDL and GGT at week 26 after 15 day treatment with

andrographolide is presented in (Figure 3). All the biochemical tests performed recorded increase with HFD, suggesting that high fat diet is associated with elevation of these biochemical tests. However, 15

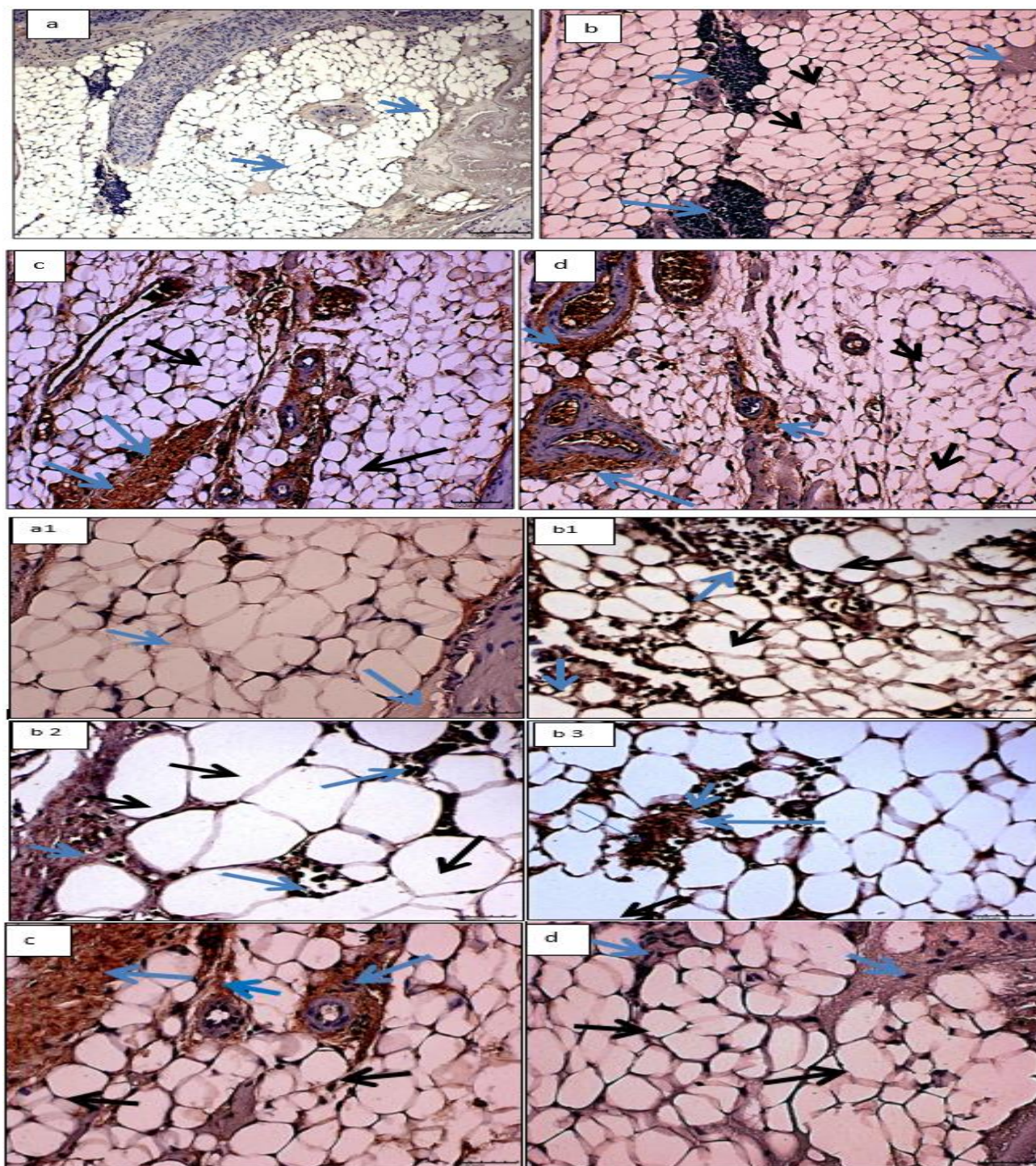


Figure 10: showing immunohistochemistry micrographs of abdominal adipose tissues of treated mice. (a) (x100, x400) CN group shows normal organized adipocyte surrounded (blue arrow). (b1, b2, b3) (x100, x400) the micrograph of the adipocyte CP of mice feeding high fat diet showed enlargement of adipose cell (black arrow), infiltrations of inflammatory cell between adipose cell (blue arrow). (c) Micrograph of adipose cell of mice HFA 25 group noticed number of cell filtrations between clusters of adipocyte (black arrow). Different adipocyte structure (blue arrow). (d) (x100, x400) Micrograph of adipocyte of mice HFA50 noticed clearly reduced filtrations of inflammatory cell between (black arrow) multilocular clusters of semi-small adipocyte (blue arrow) surrounded with vascular capillaries.

days treatment with Andrographolide at the doses of 25 mg and 50 mg/kg body weight (HFA25 and HFA50 respectively) was observed to induce a decline in all the biochemical tests performed,

implying that Andrographolide has the potential of ameliorating biochemical alterations associated with HFD induced insulin resistance. The HFA50

mg/kg body weight appeared to be a better treatment option.

Concentration of tumour necrosis factor Alfa (TNF α)

Tumour necrosis factor Alfa (TNF α) was determined at week 26 after andrographolide treatment and the result is presented in figure 4. The CN group had normal concentration of TNF α compared with the CP group which had significantly elevated level of TNF α , suggesting high fat diet induces increased concentration. However, 15 days treatment with Andrographolide the doses of 25 mg/kg and 50 mg/kg body weight (HFA25 and HFA50 respectively) were observed to significantly ($P < 0.05$) reduced TNF α .

Effect of Andrographolide concentration of IL-1 β at week 26

The mean concentration of IL-1 β at week 26 following 15 days treatment with Andrographolide is shown in figure 10. Twenty four weeks feeding with HFD was observed to induce significant increase ($P < 0.05$) in IL-1 β (CP). However, 15 days treatment with 25 mg/kg and 50 mg/kg body weight Andrographolide (HFA25 and HFA50 respectively) was observed to reduced IL-1 β levels significantly ($P < 0.05$) at week 26, with HFA50 having the lowest value.

Serum Interleukin 6 (IL-6) level

The mean serum concentration of IL-6 at week 26 is shown in figure 11. Feeding with HFD for 26 weeks was observed to induce increase in serum IL-6 level. However, treatment with Andrographolide at the dose of 25 mg/kg body weight and 50 mg/kg body weight (A25 and A50 respectively) significantly reduced IL-6 level; with A50 group have the lowest IL-6 level.

Serum levels of Immunoglobulin M (IgM)

The mean serum levels of immunoglobulin M (IgM) as recorded in this study at week 26 is presented in figure 7. Feeding with HFD for 24 weeks was observed to reduce IgM levels as seen in the (CP) after 15 days treatment with Andrographolide at doses of 25 mg/kg body weight and 50 mg/kg body weight (HFA25 and HFA50 respectively) were observed to significantly enhanced IgM production, with the A50 group having the highest IgM level $P < 0.05$.

Effect of Andrographolide on mean immunoglobulin G (IgG)

The mean serum levels of immunoglobulin G (IgG) at week 26 is presented in figure 9. Twenty four weeks feeding with HFD was seen to induce decrease in the IgG serum level (CP). However, the 15 days treatment with Andrographolide at the doses of 25mg and 50 mg/kg body weight (HFA25 and HFA50 respectively) was observed to induce significant IgG production, with the H FA50 group having the highest IgG level

Immunohistochemical results

1-Pancreatic tissue

Effect on insulin expression in this study, the pancreatic insulin was determined using Immunohistochemical method. The insulin expression represents the content of insulin in the Langerhans islets (Gupta et al., 1981; Nugroho et al., 2013) Brown area in the islets indicates

insulin staining. β -cells of the insulin resistance group (CP) stained with strong positive reactive for anti-insulin β -cell receptor antibodies as deep brown granules occupying the cytoplasm, the great numbers of the β -cells with black spot as a compensatory mechanism in insulin resistance (hyperglycemia) (Figure 9b) (x100,x200,x400) compare with control negative group (CN) (Figure 9a)(x100,x200,x400) .(Figure 9c) (x100,x200,x400) treatment with andrographolide for 15 days (HFA25) ,positive immunoreactions of β -cell with anti-insulin β -cell receptor antibodies were clearly less brown intensive staining compare to (CP) group this indicated to andrographolide enhance the β -cell activity by reduce in minimal way abnormal level antigen and antibody β -cell reactive mechanism to normal level (red arrow) . (Figure 9d) (x100,x200,x400) administration of andrographolide at (HFA50) for 15 days showing succeed restoration the β -cell activity by reduce in a major way abnormal level antigen and antibody β -cell reactive mechanism to normal level the pancreatic insulin content in insulin resistance male mice comparison to (CP, CN) groups, the area of β -cell reaction noticed light brown semi-like to normal one

Immunoperoxidase staining of the pancreas (Islets of Langerhans "across "treatment groups (Immunostaining for anti-insulin beta cell antibodies x400, diluent % (1:600 (Dako EnVision +System HRP (DAB)

2-Adipose tissue

Immunohistochemical analysis of visceral adipose tissue in mice is shown in (Figure 10) (x100, x400 respectively). Adipocytes CN group were presented clusters of small adipocytes (black arrow) surrounded with stoma cells and vascular structures (a) (x100, x400). Most of them adipocytes in CP group large in size(black arrow) and often close association with capillaries, which may facilitate the exchange of endocrine cytokines, cell infiltrations (blue arrow) or free fatty acids between capillary lumen and adipocyte. (b1), (b2), (b3) (x100, x400). Multilocular adipocyte in had abundance of brown. Micrograph of in (HFA25) group noticed adipocyte close to gather (black arrow) surrounded by stoma can be distinguish, few number of cell filtrations (blue arrow) (c) (x100, x400). In macrograph (HFA50) noticed clearly reduced filtrations of inflammatory cell between (black arrow) multilocular clusters of semi-small adipocyte (blue arrow) surrounded with vascular capillaries (d) (x100, x400).

DISCUSSION

The continual increase in the number of patients suffering from diabetic cases emerging from insulin resistance has necessitated the thrust to find new therapeutic agents for the management of the disease. Several herbs and extracts have increasingly been given attention especially those with less or no side effects (Gurib-Fakim, 2006). A newer anti-insulin resistance agent should have a strong hypoglycemic effect. However, the most optimal therapeutic agent should be capable of protecting and preserving pancreatic beta cell function (Zhang et al., 2009), in addition to

hypoglycemic effect. Andrographolide has been reported to have potent hypoglycemic effects in both type 1 and type 2 diabetes mellitus (DM) (Nugroho et al., 2011; Zhang 2000a,b) thereby making it a credible therapeutic candidate for managing insulin resistance. The evidence on the link between dietary fat and insulin resistance has been increasingly shown in previous studies (Alsaif et al., 2004; Haag et al., 2005). In this study, we successfully developed an insulin resistant model in young male mice based on feeding high fat diet for 24 week. In the present study, we have demonstrated that high fat diet-induced insulin resistance mice treated with andrographolide experienced normalization of blood glucose level, augment blood insulin level and also protect and preserve pancreatic beta cell mass and function. This perhaps suggests andrographolide is a potential therapeutic agent for the management of type 2 diabetes as similarly indicated in previous studies (Zhang et al., 2009; Nugroho et al., 2011), that alloxan-induced diabetic female BALB/CN mice treated with andrographolide – lipoic acid (20,50,80mg/kg) (AL-1). High fat diets were reported to induce insulin resistance in a rat model, the insulin resistance is said to be as result of protracted increase in insulin level (hyperinsulinemia) which consequently leads to decrease in insulin sensitivity (Pooranaperundevi et al., 2010). Although leptin has been known to moderate appetite as a systemic signal, high systemic level of leptin has been reported in obese subjects due to elevated percentages of body fat (Ahren et al., 1997; Considine et al., 1996). In this study, the increase in plasma leptin following 24 weeks of feeding with HFD could be associated with the HFD feeding. This finding is in accord with earlier findings (Ahren et al., 1997) where it was reported that HFD induced increase in plasma leptin concentration. The plasma level of leptin was observed to decline following 15 days treatment with andrographolide. Since elevated levels of leptin is associated with high body fat (Zhang et al., 2000b), the decline in plasma leptin level observed in this study could be due to the decrease in body fat due to treatment with andrographolide. Adiponectin on the other hand, is a protein hormone that regulates several metabolic processes including fatty acid oxidation and regulation of blood glucose and its level is said to be reduced in diabetic subjects (Díez et al., 2003; Coppola et al., 2009). Adiponectin is partly responsible for the suppression of metabolic dysregulations that could lead to obesity and type 2 diabetes. Adiponectin level has been observed to be inversely correlated with percentage of body fat (Ukkola et al., 2002). In this study, feeding with high fat diet for 24 weeks induced decline in adiponectin level. However, 15 days treatment with andrographolide resulted in elevated levels of the hormone. Previous study has

shown that adiponectin in combination with leptin could completely reverse insulin resistance in mice (Yamauchi et al., 2001) and it is on this background that opine the anti-insulin resistance effect of andrographolide could be at least partly due to its ability to induce increase in adiponectin production. In this study, the biochemical analysis performed revealed elevated levels of AST, ALT, ALP, blood glucose, cholesterol, TG, and VLDL with HDL showing no significant alteration following 24 weeks feeding with high fat diet. The decrease in the levels of AST, ALT, and ALP observed following 15 days treatment with andrographolide in this study is suggestive of being induced by andrographolide as similarly reported in earlier studies (Trivedi et al., 2007; Tripathi et al., 2007). In a similar vein, the observed decrease in the levels of blood glucose, VLDL, LDL, cholesterol, TG, GGT and the increase in HDL levels are in agreement with the results observed in earlier studies (Nugroho et al., 2013) where fructose-high fat diet were observed to induce increase in levels of blood glucose, VLDL, cholesterol, TG, LDL and a decrease in HDL in rat model. However, fifteen days treatment with andrographolide was observed to significantly reduce blood glucose level, cholesterol, TG, LDL and VLDL levels, increase HDL and this observation is in accord with the findings of a previous study (Nugroho et al., 2011; Nugroho et al., 2013; Rammohan, et al., 2009; Subrananian et al., 2008). The development of obesity is associated with an increase in visceral adipose tissue, especially the size of the white adipocytes and this change precede the development of insulin resistance. Insulin resistance induced via increase in visceral adipose tissue are usually marked on cellular levels by hepatic fat infiltration and TNF- α expression (Akagiri et al., 2008). The observed decrease in TNF- α activity following 15 days treatment with andrographolide could be as a result of andrographolide induced inhibition of the TNF- α as similarly observed in a previous study (Abu-Ghefreh et al., 2009) where andrographolide was observed to inhibit ovalbumin induced increase in TNF- α . Interleukin 6 is a pleiotropic cytokine that plays a key role in host defense system by regulating immune responses in addition to its ability to induce either differentiation or growth of several types of cells (Stanton et al., 2011). Diet-induced increase in IL-6 and IL-1 β levels have been previously reported (Stemmer et al., 2012; Stanton et al., 2011) and elevated levels of IL-6 and IL-1 β have been observed to play some roles in the development of type 2 diabetes which is associated with insulin resistance via its actions on skeletal muscle cells, hepatocytes, adipocytes and pancreatic β -cells (Kristiansen et al., 2005; Stanton et al., 2011). In this study, both IL-6 and IL-1 β levels were found to be elevated following 24 weeks feeding with high fat diet. However,

following 15 days treatment with andrographolide, the levels of IL-6 and IL-1 β were found to decrease. These findings are in accord with the report of a previous study (Yu et al., 2003) where it was observed that andrographolide decreased the increased serum levels of IL-6 in a C57BL/6 mice model and (Chun et al., 2010) where andrographolide was reported to decrease the expression of IL-6 in a DU145 cell line. Pathophysiologically, both insulin resistance (IR) and beta-cell dysfunction are partly responsible for the development of IgM (Pallayova, 2012). The decrease in the level of IgM and IgG as observed in this study following 24 weeks feeding with HFD were in agreement with a previous study (Crevel et al., 1992) where high fat diet was observed to induce significant decrease in the levels of IgM and IgG in C57BL mice model. The increase in the IgM and IgG levels following 15 days treatment with andrographolide could be due to immune enhancing and immunostimulatory activity of andrographolide via antibody dependent cellular cytotoxicity as reported recently (Sareer et al., 2014). When an anti-insulin antibody was applied to the beta cells, we found that the beta cells of the andrographolide treated animals have significant amounts of insulin (Figure 9), suggesting that these cells can secrete insulin and this study agreement with (Zhang et al., 2009; Nugroho et al., 2012a,b). In contrast to the AL-1-treated animals, suggesting that the ability of these beta cells to secrete insulin has been retardation. The reason behind the discrepancy between these results needs to be further investigated. These results indicate that even relatively short-term obesity can indeed induce small defects in rapid pulsatile insulin secretion that could be a sign of early compensatory insulin secretion during insulin resistance. Furthermore, extensive fat deposition, both extracellular and within the beta-cells was seen, which could, in the longer term, be detrimental to their function. Beta-cell volume was increased in response to obesity, as has also been reported in humans (Ogilvie, 1933; Klöppel et al., 1985; Butler et al., 2003; Yoon et al., 2003) as further sign of compensation for obesity. The mechanism behind this increase in beta-cell volume has, in another study in obese minipigs, been shown to be via increased islet number indicating neogenesis of islets in response to obesity (Larsen et al., 2007b). Other mechanism behind the loss of B-cell mass in prediabetes condition has been proposed to be increased apoptosis rate (rather than a loss of the ability to produce new beta-cells) (Butler et al., 2003) and, as is also seen for insulin resistance and reduction in BCF, both gluco- and lipotoxicity are probably involved (Del Prato, 2004; Donath et al., 2004). Our study agreement with several studies have reported increased beta-cell volume (Ogilvie, 1933; Klöppel et al., 1985), and dis agreement with other studies

whereas islet size is reported to be unchanged (Butler et al., 2003; Yoon et al., 2003). Previous studies have shown that Andrographolide and LA are both potent antioxidants (Kaneto et al., 1993; Packer et al., 1995; Shen et al., 2000; Suzkudelski et al., 2001). Other study show that AL-1 had protective effects toward H₂O₂-induced oxidative damage in RIN-m cells at concentrations from 0.01–1 μ M, which are achievable in animals (Shen et al., 2000). Thus, it is likely that, in diabetic animals, AL-1 functions as an antioxidant to quench ROS and protect beta cells. These data and those reported by others (Iwakura et al., 2000; Tsubouchi et al., 2005). Dietary excess and obesity are associated with the accumulation of lipid into adipocytes and the expansion of the adipose tissue (Steven et al., 2007). Hypertrophic adipocytes may produce proinflammatory cytokines such as TNF-, IL-6, resistin, MCP-1, and PAI-1 (Figure 10). The production of these substances undoubtedly has local effects, including on the endothelium leading to up-regulated adhesion molecule synthesis (intracellular adhesion molecule and vascular cell adhesion molecule) and increased vascular permeability, and on circulating monocytes, which are recruited chemo way. These pathways are activated by many of the same proinflammatory stimuli including cytokines such as TNF-, which in addition to being activators of NF-B are also NF-B-regulated products. Both pathways are also activated by pattern recognition receptors, such as the receptor for advanced glycation end products and the toll-like receptors, which are gatekeepers of the innate immune system. In addition to being activated by bacterial, viral, and fungal products, toll-like receptors can be activated by fatty acids, 40, 41 which suggests a potential link between elevated circulating or tissue lipid concentrations and the immune system. Reactive oxygen species, endoplasmic reticulum stress, and ceramides are increased by adiposity, and all have also been shown cause adipocyte pathophysiological changes included large adipocyte and infiltrations immune cell such as macrophage in insulin resistant mice. This result agreement with (Steven et al. 2007).

CONCLUSIONS

A study was undertaken to evaluate the effect of relatively short-term (6-7 month) obesity and mild insulin resistance in male mice, to investigate the hypothesis that disturbed rapid pulsatile insulin secretion is a consequence of obesity, and thus an early sign of beta cell failure. Therefore, use of andrographolide in the treatment of diabetes and numerous other conditions is increasingly gaining wider acceptance. The anti-insulin resistance property of andrographolide appeared to be multifaceted. In this study, andrographolide was shown enhance biochemical and immunological

insulin resistant mice state and successful restoration of pancreatic and adipose tissues dysregulations associated with insulin resistance. Moreover, enhanced adiponectin, IgM and IgG. The was also observed to reduce oxidative damage linked to obesity, insulin resistance (prediabetes). Based on the findings of this study, it was concluded that treatment with andrographolide has the potential of significantly ameliorating insulin resistance condition modulating biochemical alterations, TNF- α , IL-6, IL-1 β and ameliorate oxidative cellular damage. The exact mechanism through which andrographolide induced the desired changes and the subsequent validation of this extract for human treatment is still the thrust for ethnopharmacologists.

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