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Research Article

Low-Dose 17β-Estradiol Supplemented with *Andrographis Paniculata* Improved Glucose and Lipid Homeostasis in a Type-2 Diabesity Mice Model

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ABSTRACT

Estrogens play an important role in metabolic homeostasis. However, its risk of oncogenecity and cardiovascular adverse effects underscores its therapeutic benefits. This study investigated the metabolic effect of low dose estrogen supplemented with *Andrographis paniculata* on type-2 diabesity mice model. The experimental animals maintained on high fat diet were induced diabetes with streptozotocin (100 mg/kg) after intraperitoneal injection of 50 mg/kg nicotinamide. Low dose estrogen (0.02 mg/kg) was administered alone as well as in combination with 50, 150 and 500 mg/kg of the ethanol extract of *A. paniculata*. These doses of the extract, vehicle (5 ml/kg distilled water) and two reference standards pioglitazone (30 mg/kg) and metformine (100 mg/kg) were used as controls. Oral glucose tolerance test was used to determine the effect of treatment on pancreatic β-cell function and insulin sensitivity following oral glucose load of 2 g/kg. Lipid profile tests and blood glucose measurements were used to evaluate effect of treatment on lipid homeostasis and chronic diabetes respectively. Combination of low dose estradiol with 150 and 500 mg/kg of the extract showed significant ($p<0.05$) reduction in blood glucose when compared to their individual monotherapeutic effects. Co-administration of the extract with estradiol at all doses of the extract produced significant ($p<0.05$) improvement in oral glucose tolerance as depicted by smaller AUC when compared to either the extract or estradiol alone. Low dose estradiol was unable to significantly improve diabesity associated lipid profile abnormalities. However, combination of both low doses of the extract (50 mg/kg) and estradiol showed significant ($p<0.05$) reduction in serum triglyceride (TG) and LDL -cholesterol as well as significant ($p<0.05$) increase in HDL compared to vehicle control group. These findings established that augmentation of low-dose estrogen with *A. paniculata* resulted in the improvement of glucose and lipid homeostasis in a type-2 diabesity mice model compared to their individual effects.

Keywords: Estrogen; Metabolism; *Angrographis paniculata*; Diabesity

BACKGROUND

Diabesity is a term describing diabetes in the context of obesity and sometimes referred to as obesity-dependent diabetes [1]. It is the continuum of progressive abnormal biology, which ranges from mild insulin resistance to full-blown type-2 diabetes [2]. Obesity-dependent diabetes has been recognized as a major public health challenge that is evolving to become an epidemic [3]. According to the report by Zambard et al [4], diabesity and cardiovascular disease share many common risk factors including central obesity, hyperinsulinaemia, hyperglycaemia, elevated blood pressure and dyslipidaemia.

Beyond the well-recognised role of estrogen in the reproductive system, estrogens are important participants in metabolic regulation [5]. A strong correlation between estrogen deficiency and metabolic dysfunction has also been established [6]. This is consistent with studies demonstrating accelerated development of insulin resistance and type-2 diabetes in postmenopausal women with reduced estrogen production [7]. Estrogen therapy due to its

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risk of oncogenecity undermines its therapeutic benefits in the maintenance of glucose and lipid homeostasis [8]. This potential risk can be averted by using a low-dose estrogen treatment which at the same time may possibly augment the therapeutic benefits of other bioactive compounds when combined together. This approach may provide superior benefits in glucose and lipid metabolism while at the same time keeping the risk of estrogen therapy in check.

The plant *Andrographis paniculata* (Family Acanthaceae) is one of the most popular medicinal plants used traditionally for the treatment of array of diseases including diabetes [9]. In more recent studies, compounds isolated from the alcoholic extract of the plant showed great potential to ameliorate diabetic nephropathy in MES-13 cells [10], while the ethanol extract significantly reduced blood glucose level in streptozotocin-induced hyperglycaemic rats [11]. Given the acclaimed blood glucose-lowering potentials of this plant, little or nothing has been documented about its effectiveness in diabesity presenting classical features of insulin resistance with consequent hyperglycaemia and hyperlipidaemia. Also the metabolic potential of low-dose 17β-estradiol (E2) suggested to reduce hepatic glucose output compromised in insulin resistance has not been fully exploited especially when combined with medicinal plants.

It is to this end that this study was set to investigate the contributions of low-dose 17β-estradiol (E2) augmentation on glucose and lipid homeostasis in male type-2 diabesity mice model treated with *Andrographis paniculata*.

MATERIALS AND METHOD

Plant collection and extraction

The aerial part (leaves, seeds and stem) of *A. paniculata* was collected from the botanical garden of the Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Agulu. The plant was air dried at room temperature and pulverized into coarse powder. The powdered plant (200 g) was macerated in 2 L of ethanol for 72 h with intermittent shaking, filtered and concentrated using rotary evaporator at 50°C. The resulting extract was stored at 0-4°C in the refrigerator till further use.

The percentage yield of the extract was calculated using the following formula

Yield (%)=(Weight of the concentrated extract (g))/(weight of pulverized plant material (g))

Animals

Swiss male Albino mice (25-30 g) were used for this study. The animals were obtained from the Animal House of the Department of Pharmacology/Toxicology, Nnamdi Azikiwe University, Awka. The animals were housed in standard laboratory condition. All animal studies were performed in accordance with NIH guidelines outlined in the Guide for the Care and Use of Laboratory Animals, as described in protocols reviewed and approved by the NnamdiAzikiwe University institutional Animal Care and Use Committee.

Phytochemical analysis

The extract was subjected to qualitative determination of alkaloids, saponins, tannins, flavonoids, terpenoids and cardiac glycosides as well as quantitative determination of terpenoids, saponins, flavonoids and tannins were using standard procedures described by Odoh et al., [12].

Acute toxicity study

Acute toxic effect of the extract was determined using Lorke's method as described by Agyigra et al., [13]. The first phase comprised of nine mice randomized into three groups of three mice each. Each group of animals was administered different doses (10, 100 and 1000 mg/kg) of the extracts. The mice were observed thereafter for 24 hours for signs of toxicity as well as mortality. The second phase was made up of four groups of one mouse each. Based on result of the first phase, they were administered 2000, 3000, 4000 and 5000 mg/kg of the extract respectively. Observations for toxicity and death were also done for 24 h post administration.

Formulation of high fat feed

High fat feed was formulated as described by Mbagwu et al., [14]. The diet was composed of 45% fat, 35% carbohydrate and 20% protein having total caloric energy value of 4057 Kcal/kg (Animal Care Feeds, Asaba, Nigeria) against normal mice diet that was found to composed of 10% fat, 70% carbohydrate and 20% crude protein with the same total caloric energy value of 4057 Kcal/kg(Animal Care feeds, Asaba, Nigeria).

Effect of the extract on high-fat diet streptozotocin-nicotinamide-induced type 2 diabetic mice

A total of 100 mice were used for this study. The animals were maintained on high fat diet with free access to water *ad libitum* for 4 weeks. Prior to induction of diabetes, 50 mg/kg of nicotinamide was injected intraperotoneally to provide partial protection of the beta cells from complete pancreatectomy. Thereafter, streptozotocin (100 mg/kg) was administered intraperitoneally within an interval of 15 min as described by Tahara et al., [15]. After 5 days, animals were assessed for successful induction of diabetes (fasting blood glucose > 160 mg/dl). The diabetic animals were divided into 10 groups of 10 animals with mean blood glucose of 232 ± 2 mg/dl per group. The grouping was as described below:

Group 1 - 5 ml/kg distilled water

Group 2 - 0.02 mg/kg of estradiol

Group 3 - 50 mg/kg extract +0.02 mg/kg estradiol

Group 4 - 150 mg/kg extract +0.02 mg/kg estradiol

Group 5 - 500 mg/kg extract +0.02 mg/kg estradiol

Group 6 - 50 mg/kg extract

Group 7 - 150 mg/kg extract

Group 8 - 500 mg/kg extract

Group 9 - 30 mg/kg pioglitazone

Group 10 - 100 mg/kg metformin

In each group, 5 animals were used to monitor effect of treatment on lipid metabolism while the other half was used to monitor effect of treatment on glycermic control. Treatments were done orally for 4 weeks while the animals were still maintained on high fat diet.

Effect of treatment on chronic diabetes

Blood samples were drawn from tail vain of the diabetes animals in all the groups for the determination of pre-treatment fasting blood glucose concentration using One Touch Glucometer (Lifeshield, Johnson and Johnson, California). After 4 weeks treatment, blood samples were obtained again from the animals for the determination of post-treatment fasting blood glucose concentration.

Effect of treatment on oral glucose tolerance test (OGTT)

Prior to the test, the animals were fasted overnight and fasting blood glucose determined. The mice were given 2 g/kg oral glucose solution. At 15, 30, 45, 60, and 120 min after the administration of glucose, blood samples were collected by tail milking and the glucose concentration estimated. The Area under the curve (AUC) of the plot of blood glucose against time was used to determine the oral glucose tolerance.

Effect on lipid parameters

Lipid parameters (total cholesterol, triglyceride, LDL-Cholesterol, and HDL-Cholesterol) were assayed using standard serum lipid assay kits (Randox). The procedure was followed as prescribed by the manufacturer.

Statistical analyses

Statistical analyses were done using SPSS software (version 18). The data obtained was expressed as mean + SEM, analysed by Kruskal-Wallis ANOVA test. The differences between various groups were determined by multiple comparisons of mean ranks for all groups. In all cases, a probability error of less than 0.05 was selected as the criterion for statistical significance.

RESULT

Yield and phytochemical content

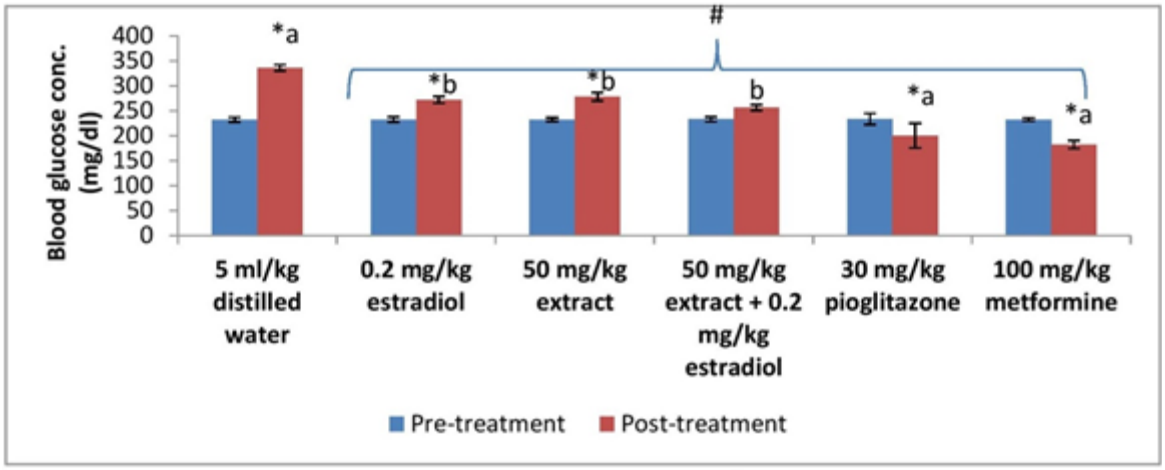
The concentration extract weighed 10.4 g and the yield was calculated to be 5.2%. Qualitative phytochemical analysis showed positive test for all the phytocompounds tested. Further quantitative analysis showed that terpenoids, saponins, flavonoids and tannins were 30.8, 11.8, 8.6 and 6.9% respectively.

Acute toxicity study

Administration of the extract at 10 - 5000 mg/kg did not produce mortality or obvious signs of toxicity throughout the period of observation. Reduction in physical activities and eating were however observed after drug administration but normalized 30 minutes post administration.

Effect of supplementation of low dose estradiol with *A. paniculata* on chronic diabetes

Result of the pre-treatment blood glucose concentration showed no significant (P>0.05) differences across groups. However, after 4 weeks treatment, significant (P<0.05) reductions in blood glucose were recorded across the treatment groups when compared with vehicle control post-treatment value (Figure 1). Compared with individual group pre-treatment values, low doses of the extract (50 mg/kg) and estradiol (0.02 mg/kg) as monotherapy showed significantly (P<0.05) increased blood glucose concentration just like the vehicle control group. However, this significant increase was offset when these low doses were given as combination therapy. Combination of low dose estradiol with 500 mg/kg of the extract produced significant (P<0.05) reduction in blood glucose just like the reference standards pioglitazone (30 mg/kg) and metformine (100 mg/kg) when compared with their pre-treatment diabetic values. Also combination of low dose estradiol with 150 and 500 mg/kg of the extract showed significant (P<0.05) reduction in blood glucose when compared to their individual monotherapeutic effects.



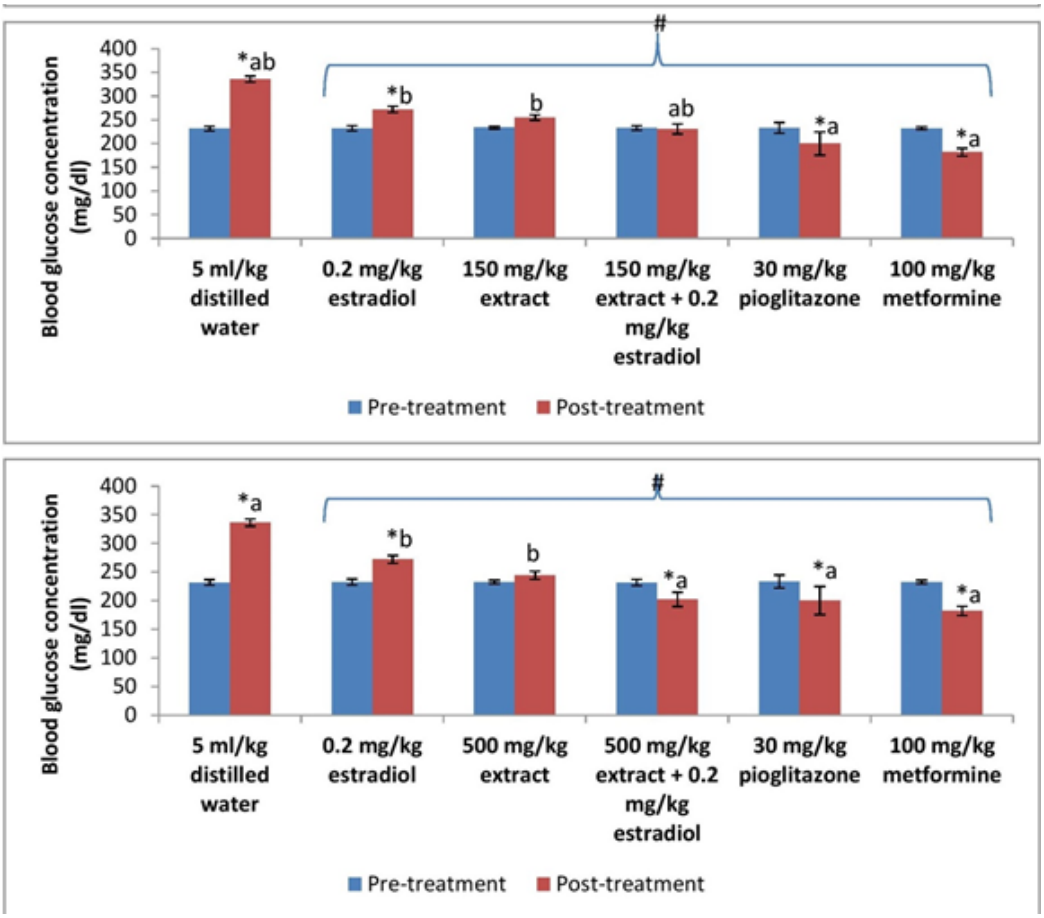


Figure 1: Pre-treatment and post-treatment blood glucose concentration. *=P<0.05 compared to pre-treatment; #=P<0.05 compared to post-treatment 5 ml/kg distilled water (vehicle control); a=P<0.05 compared to extract/estradiol alone post-treatment; b=P<0.05 compared to pilocarpine/metformine post-treatment.

Effect of the supplementation of low dose estrogen with *A. paniculata* on oral glucose tolerance

The plasma glucose levels of the diabetic animals in each group peaked at 15 minutes post oral glucose load (Figure 2). However, animals treated with the extract and estradiol either alone or in combination produced lower blood glucose peak level in comparison to the vehicle control group (5 ml/kg distilled water). Oral glucose tolerance of the treated animals showed significant (P<0.05) improvement when compared to the vehicle control group (Figure 3). Co-administration of the extract with estradiol at all doses of the extract produced significant (P<0.05) improvement in oral glucose tolerance as depicted by smaller AUC when compared to either the extract or estradiol alone. The combination effect of the extract was dose dependent and at 150 and 500 mg/kg produced better oral glucose tolerant effect than low dose estradiol (0.02 mg/kg). However, the combination of the least dose (50 mg/kg) of the extract with estradiol produced similar effect as the highest dose of the extract (500 mg/kg). Combination effect of 150 mg/kg extract and estradiol was similar to the reference standard pioglitazone (30 mg/kg) as depicted by non-significant difference (P>0.05) in their AUC while at 500 mg/kg of the extract, the combination effect was significantly (P<0.05) better than pioglitazone.

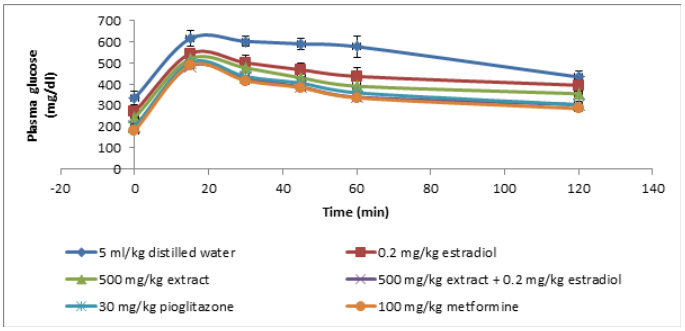


Figure 2: Plasma glucose concentration curve for 2 h oral glucose tolerance test

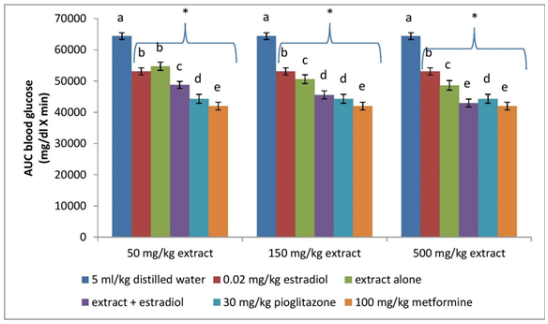


Figure 3: Area under the curve of oral glucose tolerant test.

*P<0.05 compared to 5 ml/kg distilled water (vehicle control); the alphabets a–e represents improved glucose tolerance in increasing order. Bars with different alphabets in each category indicates significant (P<0.05) difference.

Effect of the supplementation of low dose estrogen with *A. paniculata* on lipid profile

From figure 4, it was evident that low dose estradiol was unable to significantly improve diabesity associated lipid profile abnormalities. Similarly, low dose of *A. paniculata* extract (50 mg/kg) among other lipid parameters showed significant (P<0.05) reduction only in serum triglyceride (TG). Combination of both low doses of the extract and estradiol showed significant (P<0.05) reduction in serum TG and LDL-cholesterol as well as significant (P<0.05) increase in HDL compared to vehicle control group. Compared with low dose estradiol, combinations with the extract at 150 and 500 mg/kg produced significant (P<0.05) reduction in serum TG, LDL and increased HDL while combination with 50 mg/kg of the extract only showed significant (P<0.05) difference on serum TG and LDL. Compared with the extract monotherapy, combination of estradiol with the extract at all the tested doses showed improvement in lipid profile with significant (P<0.05) reduction and increase recorded for LDL and HDL respectively. Combination of estradiol with the extract at 50 mg/kg produced similar effect on TG, LDL and HDL when compared to the reference standard pioglitazone (30 mg/kg). The monotherapeutic effects of low doses of the extract (50 mg/kg) and estradiol (0.02 mg/kg) on TG, LDL and HDL were significantly (P<0.05) lower than the reference standard pioglitazone. However, similar effects of pioglitazone were recorded on these lipid parameters when both treatments were given as combination therapy. Combination of low dose estradiol with 500 mg/kg of the extract produced significant (P<0.05) reduction in LDL and increase in HDL when compared to the reference standard metformine (100 mg/kg).

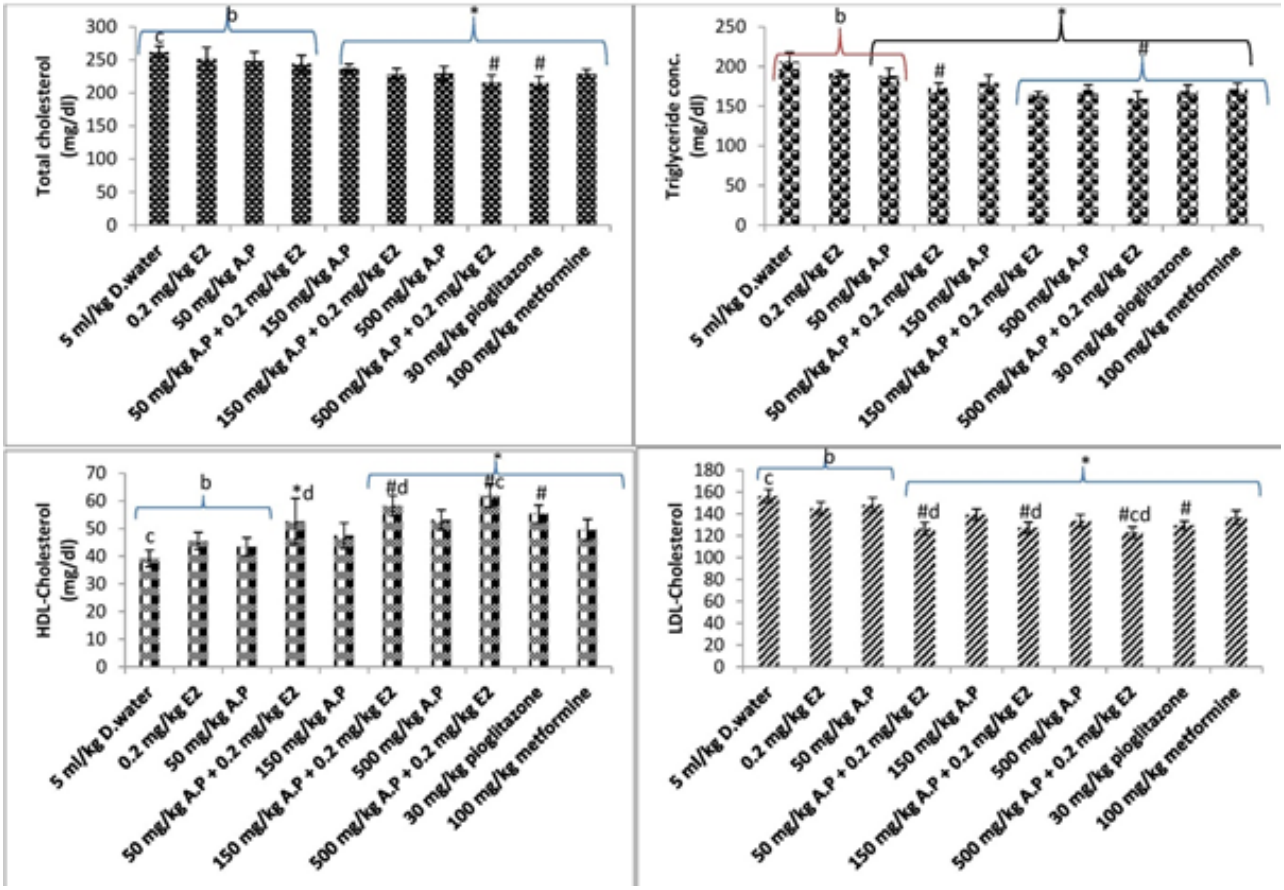


Figure 4: Effect of treatment on lipid profile. D.water=distilled water, E2=extradiol, A.P=*A. paniculata* extract, HDL=high density lipoprotein; *=P<0.05 compared to 5 ml/kg distilled water (vehicle control); #=P<0.05 compared to estradiol (0.2 mg/kg); b=P<0.05 compared to 30 mg/kg pioglitazone; c=P<0.05 compared to 100 mg/kg metformine; d=P<0.05 compared to extract alone.

DISCUSSION

One promising but yet poorly explored aspects of the regulation of glucose and lipid homeostasis is the use of estrogen. There is increasing evidence both in humans and rodents linking estrogen to the maintenance of glucose and lipid homeostasis [16]. Estrogen deficiency clearly predisposes males to increased adiposity and metabolic dysregulation [17]. In apparent contrast, however, obesity in men has been associated with hyperestrogenemia, and further excessive estradiol exposure has been postulated to play an exacerbating role in the progression of obesity and attendant metabolic dysregulation [18]. This work is set to investigate the

effect of estrogen supplementation on blood glucose and lipid homeostasis produced by Andrographis paniculata using a type 2 diabesity mice model

High-fat diet-fed/STZ-NAD induced type 2 diabetes is a well-documented model of obesity-induced diabetes used for screening antidiabetic agents. STZ preferentially accumulates in the β-cells via GLUT2 glucose transporter and induces the DNA strand breakage in β-cells causing a decrease in endogenous insulin release [19]. Many studies have reported that a long-term high-fat diet leads to insulin resistance and hyperinsulinaemia [20-21]. Intraperitonial administration of nicotinamide provides partial protection of the beta cells from complete STZ induced chemical pancreatectomy [14]. In other words, the high-fat diet combined with STZ-NAD induced diabetic rats have the characteristics of later-stage T2DM including hyperglycaemia, moderate impairment of insulin secretion, abnormalities in lipid metabolism, destruction of islet cells and reduced glycogen synthesis [22].

Dyslipidemia is a common abnormality associated with HFD consumption. Accumulation of excess fatty acid from lipid metabolism in non-adipose tissues (liver, pancreas and muscle) is a predominat feature of metabolic diseases like obesity and diabetes [23]. Subsequent metabolism of these fatty acids leads to decreased insulin-stimulated glucose uptake in skeletal muscle, unsuppressed hepatic glucose production and altered glucose-stimulated insulin release from β-cells [24]. Hyperglycermia resulting from these dysregulations in addition to FFA combine to generate major oxidative stress in tissues, further aggravating insulin resistance and deficiency. Estrogen modulates lipid concentration in plasma by regulating lipogenesis in adepocytes and hepatocytes [25]. The reduction in LDL and cholesterol level by low dose estrogen administration was probably as a result of estrogen induced accelerated conversion of hepatic cholesterol to bile acids and increased expression of LDL receptors on cell surfaces, resulting in augmented clearance of cholesterol and LDL from the plasma [26]. Other documented beneficial roles of estrogen on lipid metabolism include increase in lipoprotein lipase expression, increased fat oxidation and the regulation of acetyl-CoA oxidase as well as uncoupling proteins (UCP2-UCP3), which enhances fatty acid uptake without lipid accumulation [6].

A. paniculata also has hyperlipidemia-lowering effect profile. One of its active compounds-Andrographolide has been reported to reduce serum cholesterol, triglycerides and LDL-cholesterol in hypercholesterolaemic patients and high-fat diet animals [27-28]. The combined effects of estrogen and *A. paniculata* on the same lipid homeostatic targets and separately on different regulatory targets may account for the improved lipid lowering effect of low estrogen supplemented with *A. paniculata* compared to effects recorded when they were administered separately.

Among the series of indices for testing β-cell function and insulin sensitivity, oral glucose tolerance test (OGTT) has emerged as a simple method that provides a reasonable approximation of whole-body insulin sensitivity [29]. The index of insulin sensitivity obtained from the oral glucose tolerance test has also been documented to be applicable to advanced type-2 diabetes [30]. Based on these documented evidences, we chose OGTT as our index for estimating insulin secretion and insulin sensitivity.

E2 regulates insulin action directly via actions on insulin-sensitive tissues or indirectly by regulating factors like oxidative stress which contributes to insulin resistance. In skeletal muscles, E2 via ERα have positive effect on insulin signalling and GLUT4 expression [31]. E2 also suppresses oxidative stress via both non genomic and genomic actions, by activating pathways that prevent generation of reactive oxygen species and increasing efficient scavenging of ROS [32]. The enhanced tolerance to oral glucose load by low-dose estrogen administration may have resulted from estrogen-mediated increase in sensitivity of skeletal muscle to insulin-stimulated glucose uptake. This enhanced response can account for improvement of the diabetic state in the partially pancreatectomized animals since small amount of endogenously insulin are likely to be secreted by the pancreatic remnant. Although estrogen is not an insulin secretagogue, it has however been reported to induces pancreatic beta-cell proliferation which may represent additional mechanism of improved glucose tolerance in this diabesity model [33].

Phytocompounds of *A. paniculata* has been found to induce the mRNA and protein levels of GLUT4-increasing glucose uptake in a time- and dose-dependent manner [34]. They also increase insulin secretion, acting as insulin secretagogue, as well as preventing loss of β-cells and/or their dysfunction through inhibition of ROS production and cytokine-stimulated NF-KB activation which are part of the mechanisms through which HFD and STZ damage the β-cells [35]. Supplementing estradiol with *A. paniculata* may have contributed to increase in insulin secretion with complementary stimulation of more insulin-mediated glucose uptake.

CONCLUSION

Augmentation of low-dose estrogen with *A. paniculata* resulted in the improvement of glucose and lipid homeostasis in a type-2 diabesity mice model compared to their individual effects. The low-dose estrogen augmentation is expected to reduce the side effects of estrogen monotherapy while at the same time exploiting its metabolic potentials in glucose and lipid homeostasis. *A. paniculata* augmentation with low-dose estrogen elicited a better control of glucose and lipid parameters associated with diabesity.

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