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Carvone's Hypoglycemic and Hypolipidemic Potent Activity Via Regulation Insulin-Induced Genes in Diabetic Hyperlipidemic Rats

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Abstract: Carvone is a monoterpene and component of many essential oils from medicinal plants. Our research intended to evaluate the therapeutic efficacy of carvone in the handling of diabetes and hyperlipidemia in alloxan-induced type 2 diabetics. Alloxan (120 mg/kg) was administered intraperitoneally to male albino rats to induce diabetes as a single dose after diabetes, followed by two weeks of feeding the hypercholesterolemic atherogenic diet to develop hyperlipidemia. For one month, carvone (50 mg/kg) was administered orally to diabetic hyperlipidemic rats. Metformin, a common oral hypoglycemic medication, and atorvastatin, a standard oral hypolipidemic drug, were used to compare the findings. Our findings showed a reduction in blood glucose levels, low-density lipoprotein, cholesterol, and triglyceride. Upon administration of carvone, the encoded proteins (Insulin-induced gene-1and insulin-induced gene-2) were evaluated, which prevent Sterol regulatory-element binding proteins from being proteolytically activated. These transcription factors promote cholesterol and fatty acid production in the liver and other tissues. Histopathological alterations in pancreatic tissue were among the biomarkers chosen for hypoglycemic and hypolipidemic examinations. These findings suggested that carvone may be hypoglycemic and hypolipidemic potent activity by regulating insulin-induced genes.

Keywords: diabetes mellitus; alloxan; hyperlipidemia; Insig proteins; carvone; metformin.

1. Introduction

Diabetes mellitus has several risks, such as atherosclerosis, high blood pressure, and microcirculatory disorders. It also has long-term complications such as nephropathy and neuropathy [1]; it refers to metabolic diseases marked by hyperglycemia due to a defect in insulin secretion or insulin action [2, 3]. Persistent hyperglycemia is a symptom of this metabolic disease associated with inadequate insulin production or insulin resistance [4]. Hyperlipidemia has been linked to the development of coronary artery disease ,atherosclerosis, and other obesity-related complications [5]. Atherosclerosis is a condition caused by various factors, including the formation of atherosclerotic plaques and lipoprotein oxidation [6]; it is one of the most severe complications of diabetes. Most conditions linked to diabetes and atherosclerosis have vascular endothelium dysfunction as a defining feature [7, 8]. It also refers

to the fats, cholesterol, and other substances that build up in the walls of the arteries (plaque), which may reduce blood flow. This plaque causes complications in the body [9]. Cardiovascular diseases such as atherosclerosis and hypertension are promoted by higher cholesterol, triglycerides, and low-density lipoprotein [10]. Herbal medicine refers to medicinal plants in folk medicine to treat various diseases [11]. Natural products have received increased attention. Essential oils are natural products with various biological characteristics caused by monoterpenes, including anticonvulsant and anxiolytic properties. Therefore, essential oils have a high probability of producing new drugs [12]. Carvone is present in various essential oils, notably in caraway, spearmint, and dill seed oils also has a variety of biological properties, including antimicrobial, antitumor, and antioxidant [13, 14]. It also stops pro-inflammatory mediators like TNF- and IL-6 from being produced in lipopolysaccharide (LPS) -induced acute lung injury and obese mice fed a high-fat diet [15]. The study's goal was to compare the effects of carvone on diabetic hyperlipidemic rats to metformin and atorvastatin combination therapy in diabetic hyperlipidemic rats to treat diabetes and its complications with natural compounds rather than standard diabetes medications with known dire effects. Carvone's hypoglycemic and hypolipidemic effects were evaluated in diabetic hyperlipidemic rats using alloxan-induced hyperglycemia and cct diet-induced hyperlipidemia compared to metformin atorvastatin drugs. The intraperitoneal route of alloxan was used in this study because it has the same structure as glucose and causes a significant reduction in insulin by destroying B-cells of the islet of Langerhans [16]. Carvone's effect was investigated by measuring lipid profile, glucose, insulin, INSIG-1and INSIG-2 (encoded proteins that block proteolytic activation of sterol regulatory element-bound protein (SREBPs). This investigation found that carvone had an antidiabetic and antihyperlipidemic impact by looking at relevant biomarkers, verified by statistical analysis and histopathological study of the pancreas.

2 .Materials and Methods

2.1. Chemicals and kits.

Cholesterol was supplied by techno Pharma chem (Bahdurarh India). The alpha chemical company (Mumbai, India) provided cholic acid. Lobachemie supplied thiouracil in Mumbai (India). Alloxan (98 %) was obtained from (Sigma-Aldrich) and kept out of direct sunlight. Atorvastatin was provided by the medical unit of pharmaceuticals MUP, Egypt. Metformin was purchased from a pharmacy (Cadila, Healthcare Ltd, Ahmedabad, India). Across-Organics Chemical Co. (USA) provided the vitamin mixture, mineral mixture, and L-cystine .

2.2. Design of experiment.

2.2.1 Extraction of carvone.

Carvone was obtained from caraway seeds as described [17]. HNMR spectra from the Applied Nucleic Acid Research Center, Faculty of Sciences, Zagazig University, Egypt, have confirmed this .

2.3. Animals.

32 Male albino Wistar rats (150-190 g) were raised in the central animal house of medicine (Zagazig University, Egypt). The animals were subjected to the laboratory conditions

for one week before beginning the experiments, and then they were kept in the lab for one week before starting the trials, which were set at 25°C, 50–60% relative humidity, and a 12/12 h light-dark cycle, with normal chow and water. The experimental protocol was carried out according to the National Institutes of Health (NIH) guidelines for the care of laboratory animals. The study was certified by Zagazig University's Ethical Committee (ZU-IACUCL / 1/F/16/2019) .

2.4. Induction of experimental diabetes and hyperlipidemia.

The rats in the study were fasted for 16 h before receiving a single intraperitoneal administration of alloxan (120 mg/kg) which was dissolved in saline [18, 19]. To prevent hypoglycemia, the drinking water was fortified with a 5% glucose solution 24 hours after the injection [20]. After 3 days, a diagnostic ACC-check test strip (Roche. diagnostics, Monheim, Germany), and a blood sample from the cut tip of the tail were used to confirm the development of diabetes. For two weeks, Diabetic rats (blood glucose >150 mg/dl) were chosen and fed a CCT diet [21].

2.5. Treatment of rats.

32 rats were separated into four groups, each with eight rats. A regular chow diet was given to a single group identified as the normal control group (NC). The diabetic hyperlipidemic control group (DHC) received no treatment. For the other two groups, carvone (50 mg/kg) was administered intragastrical in 1 ml of corn oil daily for one month [22, 23]. The combination group received atorvastatin (10 mg/kg) which was dissolved in tween 80, and metformin (100 mg/kg) which was dissolved in saline daily for one-month intragastrically [24, 25].

2.6. Blood sampling and tissue collection.

At the end of the experiment, blood samples were obtained from the retro-orbital plexus of each fasted rat and anesthetized with urethane vapors. Part of the blood was collected at 37 ^{o} C, part of the blood was in vacuum tubes, and the other was in an EDTA tube. Separated serum and plasma were stored at -20^{o} C in Eppendorff [26, 27]. After blood was obtained, the rats were decapitated for the histopathological study. The pancreas was extracted and fixed in neutral buffered formalin at a concentration of 10%. For gene expression of Insig proteins (INSIG-1 and INSIG-2), the liver was immediately separated and stored in liquid nitrogen (-80^{o}C) [28,29].

2.7. Biochemical analysis.

Fasting Glucose, total cholesterol, and triglyceride levels in the blood were evaluated colorimetrical (Spin react Company, Girona, Spain) [30]. The colorimetric determination of HDL-c levels was done (Spin react Company, Girona, Spain) [29]. Friedewald's formula calculated LDL-c levels [31, 32]. Plasma insulin was determined colorimetrical using an ELISA kit (Ls. Bioscience, Inc, German) [33].

2.8. RNA extraction and (RT-PCR) assay for gene expressions INSIG proteins.

Total RNA was extracted from the frozen liver using Qiagen tissue extracted kits (Qiagen, USA). After extraction, total RNA (2 g) was converted to cDNA using a high-capacity cDNA reverse transcription kit (Fermentas, USA). The Applied Biosystem 3.1 software was used to perform real-time qPCR amplification and analysis (StepOneTM, USA). The relative quantitation (relative expression) was calculated according to[34], where (βeta actin) was utilized as a housekeeping gene, as shown in (Table 1).

Table 1. Primers used in gene expression of Insulin-induced gene -1 and Insulin-induced gene- 2 in liver

Gene expression	Primers
Insig-1	Forward primer :5'-TGCAGATCCAGCGGAATGT -3′ Reverse primer :5'- CCAGGCGGAGGAGAAGATG -3′
Insig-2	Forward primer :5'- GACGGATGTGTTGAAGGATTTCT-3′ Reverse primer :5'-TGGACTGAAGCAGACCAATGTC-3′
βeta Actin	Forward primer 5′-TGTTTGAGACCTTCAACACC-3′ Reverse primer 5′-CGCTCATTGCCGATAGTGAT-3′

2.9. Histopathological examination

The paraffin-embedded pancreas was sectioned at 4 μm using a semi-automated microtome (RM2155; Leica Microsystems). The tissue sections are then fixed with a hot plate on glass slides (HI1220; Leica Microsystems). Afterward, The tissue parts were subsequently parafined by a range of ethanol dilution and rehydrated (100 percent, 90 percent, and 70 percent). Hematoxylin and eosin were used to stain the sections (H&E). All slides have been studied by means of light microscopy with an x400 magnification digital camera.

2.10. Statistical analysis.

For data processing, SPSS Statistics 23.0 software was used to check, enter, and analyze data (SPSS Inc., Chicago, IL, USA). For the analysis of the current study's findings, the following statistical methods were used. Quantitative variables were expressed as mean $\pm$ standard deviation (SD), while qualitative variables were expressed as numbers and percentages. To calculate the difference between quantitative variables in more than two groups, a one-way ANOVA (F-test) test was used, followed by LSD (least significance difference) [35]. Significant results are indicated by a p-value of less than 0.05.

3. Results and Discussion

3.1. HNMR of carvone.

As shown in Figure 1, carvone was found to be the main component extracted using HNMR. 1H-NMR spectra were recorded on Bruker high-performance Digital FT-NMR spectrometer advance III 400 MHz using dimethyl sulfoxide (DMSO)-d6 as solvent. Chemical shifts are reported in δ (ppm) relative to the internal tetramethylsilane (TMS) standard.

3.2. Effect of carvone and combination drug on blood glucose, insulin, and lipid profile.

Glucose is the primary fuel source in animal cells. It comes from hepatic gluconeogenesis, glycogenolysis, and dietary carbohydrates. Glucose homeostasis is a balance between glucose supply and usage, which is assessed by the level of circulating insulin and tissue responsiveness to it [36].

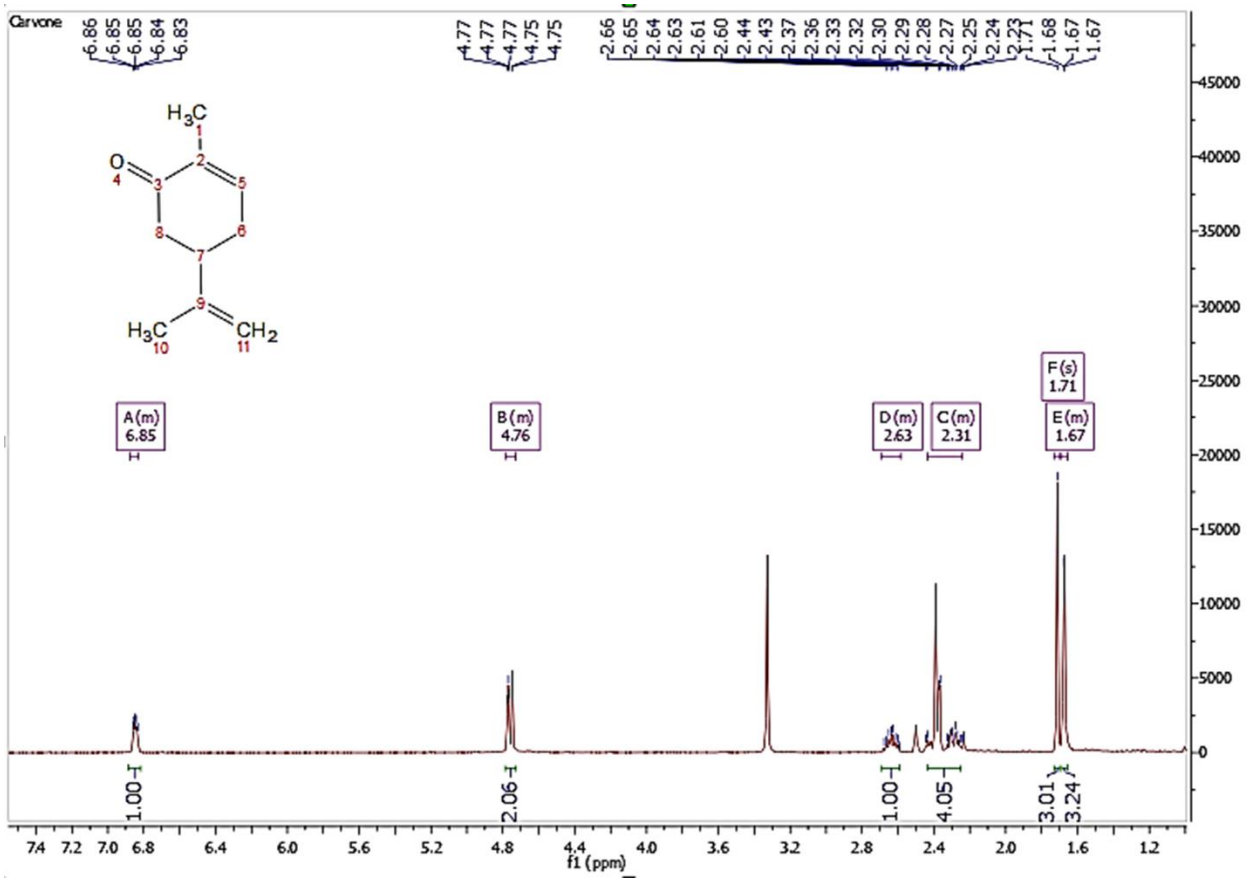

Figure 1. 1H NMR (400 MHz, DMSO-d6) δ ppm: 6.87 – 6.83 (m, 1H, H-5), 4.78 – 4.73 (m, 2H, H-11), 2.69 – 2.59 (m, 1H, H-7), 2.43 – 2.25 (m, 4H, H-6,8), 1.71 (s, 3H, H-10), 1.69 – 1.66 (m, 3H, H-1).

Insulin is the most important hormone for maintaining blood glucose homeostasis by encouraging the liver to use glucose. Insulin binds to the insulin receptor in normal conditions, triggering the insulin signal transduction cascade, regulating glucose metabolism and transport while enhancing glycogen synthesis [37]. We had diabetic hyperlipidemic control rats after inducing diabetes with alloxan and hyperlipidemia in the CCT diet (DHC). Thus, increasing glucose levels and decreasing insulin levels resulted in adipose tissue fat mobilization, leading to a high lipid profile. After the intervention of carvone, metformin, and atorvastatin for one month, we found that blood glucose levels decreased in combination drugs compared with the positive group and also decreased in the carvone group compared with the positive and this may be related to improvement in insulin resistance found that decrease in lipid profile. A study by researchers [23] reported that carvone alleviates hyperglycemia by modulating the activity of a major carbohydrate metabolism enzyme, indicating that an increase in the activity of glucose -6-phosphate dehydrogenase may be attributed to an increase in insulin production, lowering blood glucose levels in circulation, which come in the same line of our result**.** The lipid profiles were tested to examine hyperlipidemia. In this study, it has been found that combination groups had lower levels of cholesterol, triglycerides, and LDL than the positive group, which agreement with [38], which said that when we use this combined therapy, the lipid profile will decrease in diabetic hyperlipidemic rats and also carvone group decrease lipid profile. These findings match those observed in earlier studies indicating that carvone inhibited weight gain and fat accumulation in the liver caused by a high-fat diet [15] (as shown in Table 2, Figure 2, and Figure 3).

Table 2. Effect of Carvone and combined drugs on levels of Fasting Glucose, Cholesterol, Triglycerides, HDL, LDL, Insulin,

Groups / Parameters	1st group Negative control	2nd group positive Control(DHC)	3rd group Carvone treated group	4th group atorvastatin & metformin-treated group (combination group)
Fasting blood glucose level (mg/dl)	93.1±5.6**	208.7±20.3	98.5±12.2**	94.1±18.5**
Cholesterol level (mg /dl)	54.1±3.1**	95.3±6.3	67±8.5**	64.9±9.7**
Triglycerides level (mg /dl)	53.5±3.2**	165.7±18.6	70.3±11.8**	52.5±8.6**
HDL level (mg /dl)	60.4±3.8**	31.9±1.3	39.7±4.7**	42.0±4.4**
LDL level (mg /dl)	13.33±3.79**	60.07±11.46	34.73±5.46*	29±3.61**
Insulin level (ng/ml)	8.32±0.58**	4.12±0.66	6.97±0.49**	6.72±0.14**

In this table, there was a statistical difference between the Negative control group and the DHC group. Also, there was a statistically notable difference between the DHC group and treatment groups (carvone treated group and atorvastatin & metformin-treated group). Values are expressed in Mean ± SD, * Statistically significant difference ($p \leq 0.05$),** indicates a statistically significant difference ($P \leq 0.001$) compared to the positive control group.

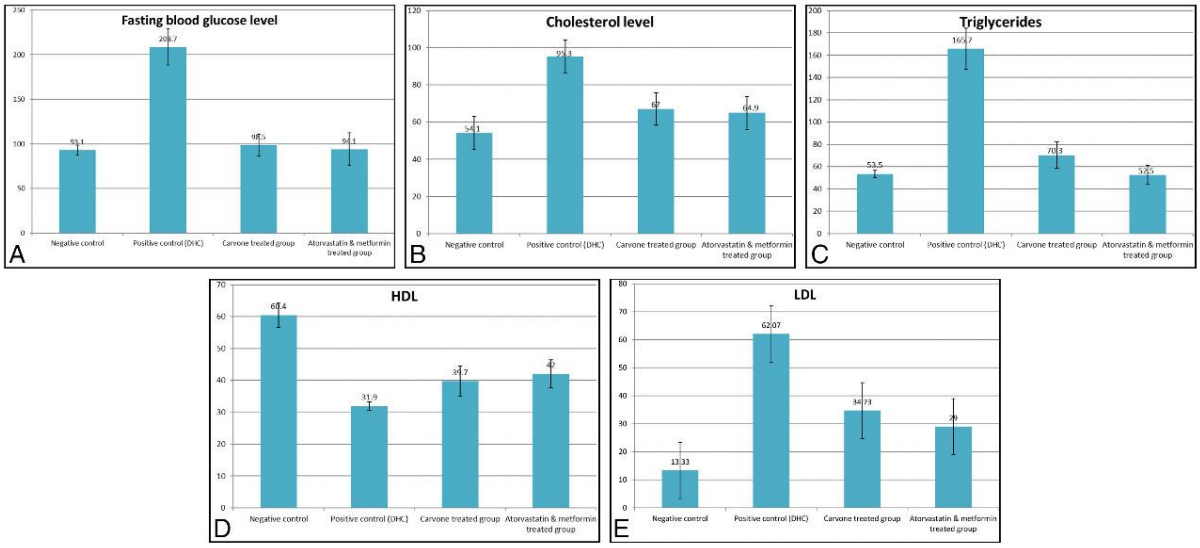

Figure 2. Bar chart for (**A**) Fasting blood glucose level; (**B**) Cholesterol level; (**C**) Triglyceride level; (**D**) HDL level; (**E**) LDL level, the positive control rats (DHC) reported a marked increase in (blood glucose, cholesterol, triglyceride, and LDL) levels and a decline in HDL. In carvone and combination, treated groups demonstrated a significant decrease in (blood glucose, cholesterol, triglyceride, and LDL) levels and an increase in HDL compared to the positive group.

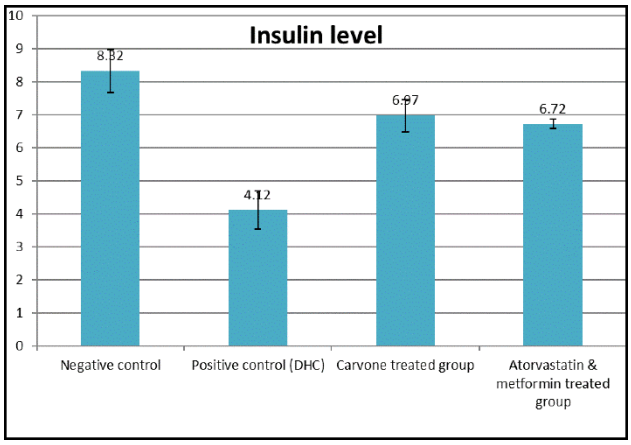

Figure 3. Bar chart for Insulin level. Compared to the negative control, DHC showed a decrease in serum insulin ($P \leq 0.001$). When compared to the DHC Group, serum levels of insulin were significantly increased ($P \leq 0.001$) in the carvone and combination-treated groups**.**

3.3. Effect of carvone and combined drugs on the expression of (INSIG-1) and (INSIG-2).

Because of changes in our lifestyles and practices, hyperlipidemia has become a standard deadlock in our society. A high-fat diet can lead to insulin resistance, as saturated fatty acids interfere with insulin action, increasing the risk of diabetes mellitus [39]. The sterol regulatory element-bound protein family of three membrane-bound transcription factors regulates lipid synthesis in the liver and other organs (SREBPs). In this study, the encoded proteins INSIG-1 and INSIG-2 were evaluated, which block the proteolytic activation of SREBPs. These transcription factors activate cholesterol synthesis and fatty acid synthesis in the liver and other organs [16]. Through binding to SCAP or HMG-COA reductase, Insig proteins play an essential role in cholesterol metabolism. When cellular cholesterol levels are high, Insig proteins bind to SCAP and inhibit the SCAP|SREBPs complex from being delivered to the Golgi, resulting in a decrease in a transcriptional gene required for cholesterol triglyceride, phospholipid, and fatty acid uptake and synthesis. In another mechanism, Insig proteins bind to HMG-COA reductase, causing the reductase to degrade and thus inhibit cholesterol synthesis [16].

It has been found that the expression of Insig protein decreased in diabetic hyperlipidemic rats but increased when they were treated with carvone or a combination of drugs. Hence, increasing the level of Insig proteins regulates the expression of SERBPs, which controls the expression of LDL receptors, allowing the hepatocyte to remove cholesterol from LDL particles [1, 16]. As shown in (Table 3, Figure 4)

Table 3. Effect of Carvone and combined drugs on Insig-1 and Insig-2.

Groups / Parameters	1st group Negative control	2nd group Positive Control (DHC)	3rd group Carvone treated group	4th group atorvastatin & metformin-treated group (combination group)
Insulin-induced gene_1	1.16±0.04**	0.42±0.11	1.01±0.01**	0.84±0.01**
Insulin-induced gene_2	1.08±0.01**	0.44±0.09	1.01±0.009**	0.93±0.04**

In this table, there was a statistical difference between the Negative Control Group and DHC group. Also, there was a statistically notable difference between the DHC group and treatment groups (carvone treated group and atorvastatin & metformin-treated group). Values are expressed in Mean ± SD, ** indicating a statistically significant difference ($P \leq 0.001$) compared to the positive control group.

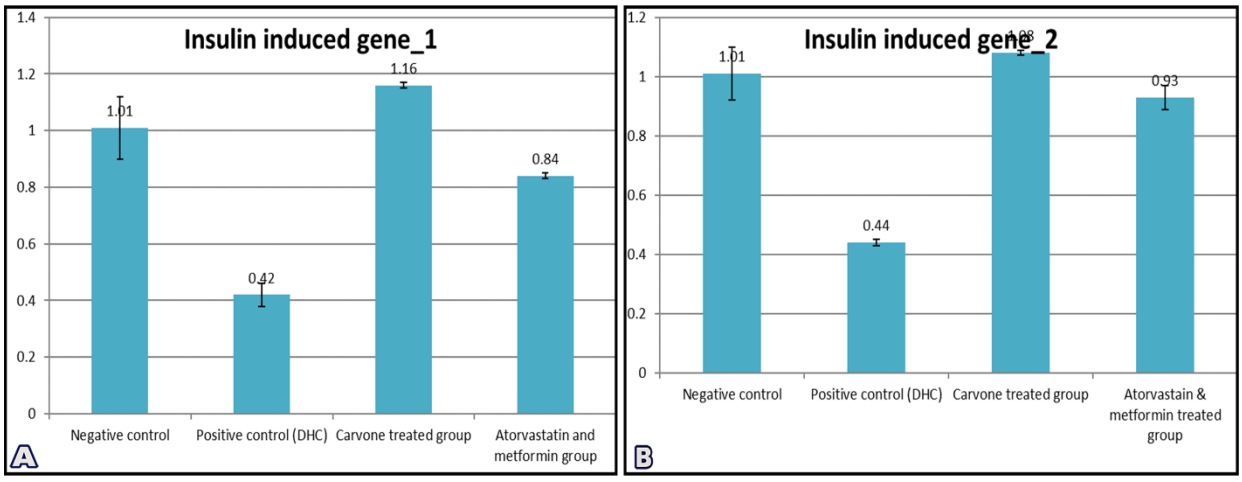

Figure 4. Bar chart for **(A)** Insulin-induced gene_1 level; **(B)** Insulin-induced gene_2 level among the studied. Positive control rats (DHC) marked a decrease in Hepatic Insig-1 and Insig-2 ($P \leq 0.001$) when compared to the negative control. Carvone and combination-treated groups revealed an increase in hepatic Insig-1 and Insig-2 ($P \leq 0.001$) compared to positive groups.

3.4. Histopathological examination.

It has been suggested that carvone may be renewable of the pancreas' beta cells or improve insulin secretion by increasing plasma insulin. The results revealed that the diabetic pancreas had fewer islets of Langerhans, which were lighter in color than the surrounding acinar cells(H&E)x400. However, Langerhans' appearance was improved in the carvone and combination, as evidenced by histopathological studies. These findings are consistent with earlier research which reported that carvone improved insulin secretion from pancreatic beta cells in diabetic rats [23].

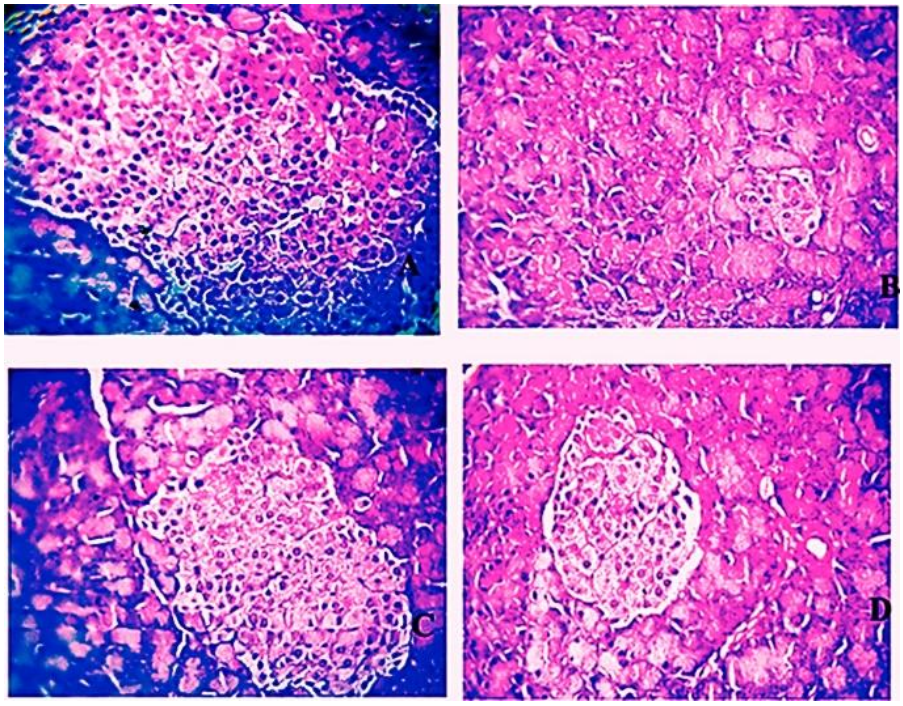

Figure 5. Histopathological examination in the islet tissues of the pancreas, where (**A**) control group; (**B**) diabetic hyperlipidemic group; (**C**) carvone group; (**D**) Combination drugs group. The islets of Langerhans appeared normal in the normal group. The islet was lighter in color than the surrounding acinar cells (Figure 5A). In contrast, alloxan diabetic hyperlipidemic control rats showed a decrease in islets of Langerhans (Figure 5B). The pathological examination in tissues of carvone and combination-treated diabetic rats showed improved the appearance of islets of Langerhans (H&E)x400 (Figures 5C&D).

4. Conclusions

Carvone plays an antidiabetic and antihyperlipidemic role in diabetes mellitus, which may be renewable of beta cells of the pancreas or improve the secretion of insulin by increasing plasma insulin and help maintain lipid and lipoprotein profile homeostasis by increasing the expression of Insig proteins that block SERPs. This transcription factor activates cholesterol synthesis. Overall, this breakthrough is critical for its use as an alternative medicine to treat diabetes and its complications.

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Conflicts of Interest

The authors declare no potential conflicts of interest concerning this article's research, authorship, and/or publication.

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