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BLOOD BIOCHEMICAL CHANGES ASSOCIATED WITH ACUTE TREATMENT OF *ORCHIS ANATOLICA* PLANT ROOTS ETHANOL EXTRACT IN ADULT ALBINO RATS.

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ABSTRACT: We explore through this study blood parameters changes after 7 days treatment with *Orchis anatolica* (*O. anatolica*) plant roots ethanol extract in Albino rats. An intra-peritoneal glucose tolerance test (IPGTT) was carried on to evaluate the hypoglycemic effect of the extract on blood glucose and insulin titer using Albino rats fasting for 18 hours. Blood glucose and serum insulin levels where determined throughout one hour period prior to a single treatment dose of 400mg/kg/BW *O. anatolica* extract. Oral treatment with *O. anatolica* was then performed using another group of 10 Albin rats treated with 400mg/kg/BW/day for 7 days and where compared with a control group. Using UV/Visible spectrophotometer, glucose, cardiac and lever serum blood biochemical markers were investigated in addition to blood total protein urea. Significant reduction in cardiac markers namely serum cholesterol (LDL) and Creatinin Kinase (KC) titers were detected after the treatment. Alanine AminoTransferase (ALT), Aspartate Amino Transferase (AST and) ad, triglycerides representing hepatic markers were normal concomitant with normalized values of bilirubin, total protein and blood urea when compared with the control. Blood glucose levels were reduced to significant levels after 7 days treatment with the plant extract an observation that was further demonstrated by IPGTT. Concomitant, an increase in serum insulin level was observed also when IPGTT was performed. We can conclude that *O. anatolica* root extract induces hypoglycemia, insulin-releasing and cholesterol lowering effects in rats. Together, a normal biochemical marker indicates an organ protective ability of the plant.

Key words: *Orchis anatolica*, Biochemical serum blood markers, Diabetes, Hypoglycemia, Organ protective.

Abbreviation

O. anatolica – Orchis anatolica.
BW - Body Weight
IPGTT - Intra-Peritoneal Glucose Tolerance Test.
UV – Ultra Violet.
LDL – Low Density Lipoprotein "Cholesterol).
KC - Creatinin Kinase.
ALT - Alanine AminoTransferase.
TnI - Trponin I.
AST - Aspartate Amino Transferase.
LD_{50} – Lethal Dose 50 (half time)

INTRODUCTION

Hypoglycemic properties of several medicinal plants had been studied and reported (McWhorter, 2005, Leduc, et al., 2006, N'guessan, et al., 2009, Ayyanar et al., 2008). Accordingly, the chemical components of more than 1200 plants have been reported to contain components with hypoglycemic activity and considered a natural anti-diabetic agent (Marles, et al., 1995, Bailey, et al., 1989). Majority of modern drags originally derived from ancient herbal remedies (Gragg et al., 1997). Medicinal plants, howevere, have been used as remedies to cure many disorders ranging from inflammation to cancer as they contain components of therapeutic value (Blumenthal, 2003, Yu, et al, 2006, Lahans, 2007, Shoeb, 2006, Ramoutsaki, et al., 2002, Leduc, et al., 2006). Through time, physicians have attempted the treatment various medical conditions with indigenous plant.

Many herbs and plants exhibit hypo-glycemic and hypo-lipidymic activity have been described when taken orally (Jasmine et al**.,** 2007). Even after the discovery and use of insulin and modern oral hypoglycaemic agents, the search for safer and more effective drugs of plant origin for the treatment of diabetes has continued.

O anatolica is a delicate-looking plant that grows mostly in light shaded pine woods. It belongs to the *Orchidaceae* family which is known to be the largest plant family (Kong, et al., 2003, Dafni, et al., 1997). The use of this plant family in traditional medical has been considered one of the most popular widespread of all plant families (Gutiérrez, 2010, Hew, et al., 1997, Shanker, et al., 2007). Many Orchids species has been used in old traditional medicine for their therapeutic value as anti-inflammatory, diuretic, anti-diabetic, and also to treat some liver disorders contain chemical substances of pharmacological use (Bulpitt, et al., 2007, Bythrew 2005, Tovar-Gijon et al., 2006). Recently, this plant had been used and was considered to have therapeutic value when explored for their effects on male reproductive system, enhancing spermatogenesis and aggression in male rodents (Allouh, et al., 2009, Khouri et al.2006).

This study aims at finding new affordable therapies, inexpensive and able to normalize and stabilize the elevated blood glycemic values. Our main aim is to investigate this plant effects on glucose levels and whether the key biochemical marker of the lever, heart, and lipid profile are altered in this treatment. This is done in order to provide scientific evidence of the effectiveness of this plant as anti-diabetic and hypo-lipidemic agent.

MATERIALS AND METHODS

Plant collection*:*

O.anatolica plant roots were collected from Zoubia area (Northern region of Jordan) during spring period**.** The large potato shaped bulb forming the roots of this plant was used in this experiment. Roots were air dried and grinded into powder and prepared for ethanol extraction.

Ethanol extraction and dosage determination:

Ethanol extract was done using 500g dried and grinded root powder and was refluxed into (2 L) 70 % ethanol at 50° C for 36 hours in a continuous extraction (soxhlet) apparatus. The extract was then filtered and concentrated under reduced pressure at 50° C using a rotary evaporator where the net yield of this extract was 30g. The concentrate was dissolved in distilled water and administered orally in a dose of 400mg/kg/BW (as 1ml volume) as a single daily dose for 7 days. An animal feeding intubation needles (Popper and Sons, New York) was used for this procedure to.

Animals:

Male and female Albino rats weighing approximately 300g used in this experiment were raised in the Animal House Unit at Jordan University of Science and Technology, School of Medicine (JUST). Rats were maintained under controlled temperature of 21±1°C and a 12-hr-light: 12-hr-dark schedule where food and water were supplied *ad libitum*. A total number of 70 Albino rats were used to explore different aspects of this treatment.

Determination of LD_{50} in mice:

Acute toxicity of the plant was determined by the calculation of LD50 which represents the dose that can be fetal to 50% of any rats group (Hruskova, et al., 1961). Determination of LD_{50} in rats was conducted to determine the proper treatment dose that should be used in this experiment. For this procedure, 24 Albino rats were needed and used distributed into 4 different groups each containing 6 albino rats (300g). Different dosage *O. anatolica* root ethanol extract namely 200, 400, 600, and 1000mg each dissolved in 0.2 ml distilled water was administered intra-peritoneal to each corresponding rat group. Rats were then housed in transparent plastic cages under controlled temperature of 24°C and monitored for 24 hours representing the time length of this experiment for any toxic symptoms. The number of deceased mice was counted in each group after 24 hours and the percentage of mortality was calculated. Treated rats were compared with 6 controlled rats that had received an intra-peritoneal infusion of 1ml distilled water alone, in the same manor with the experimental rats.

Biochemical Parameters investigated:

In this part of the experiment, 20 male and female rats weighting 300g each were used and divided into two groups each containing 10 rats. One group was used as control receiving 1ml distilled water orally through a gastric intubations needle.

The other group was receives a daily oral dose (400mg/kg/BW) of *O. anatolica* roots ethanol extract through the same procedure for 7 days. During the experimental period, all rats were allowed to feed a regular rat chaw pallets and free access to water. Twenty four hours prior to the last treatment dose, rats were weighed and autopsied under light ether anesthesia. Blood serum samples were collected from rats trough cardiac puncture using a sterile syringe with needle attached. Biochemical parameters investigated in this study were carried on using commercially available diagnostic kits. Blood glucose serum level was determined using commercial laboratory kit obtained from Promega, USA. The determination of total protein, total cholesterol (LDL-cholesterol) and triglyceride were done using required laboratory kit obtained from Sternile, USA. Serum AST, serum AST, and serum CK were determined using TC kit Tecodiagnostic, USA. Finally, laboratory kit obtained from Sigayatia, Roma, was used to determine the total bilirubin and blood urea serum titers. Concentrations of each parameter investigated were determined using UV/Visible spectrophotometer.

Hhypoglycemic effect of O. anatolica ethanol extract (IPGTT):

To test this variant, twenty more Albino rats (male and females) were randomly, equally divided and assigned to either control or experimental groups. The control group received 1 ml distilled water where the experimental rats group received an oral dose of 400 mg/kg/BW of *O. anatolica* roots ethanol extract dissolved in 1 ml distilled water through a stainless steel intra-gastric intubation. One hour after both treatment and water administration, the IPGTT was performed in all rats by an intra-peritoneal dose of glucose (1.5g/kg/BW). Blood glucose and insulin levels were then measured using commercial kits from Cis-BIO International (Gif Sur Yvette, France) after 0, 15, 30, 45 and 60 minutes timing period.

Statistical analysis

Student T test was used to determine the significance of the differences between the groups. ± S.E. Data were expressed as mean ± SD (Ipstein, et al., 1970) (statistical package for social sciences [SPSS, version 11.5]). Differences between the control and the *O. anatolica* treated groups of rats were analyzed using Chi-square test, student *t* test and nonparametric (Kruskal-Wallis) tests when applicable. The P-value of ≤0.05 was considered to be significant.

RESULTS

The effect of *Orchis anatolica* on the cardiac biochemical markers

Blood serum LDL, TnI, CK and Triglycerides levels:

Administration of 400mg/kg/BW/day of *O. anatolica* roots ethanol extract caused a significant decrease in total blood serum cholesterol (LDL) levels ($p<0.01$) when compared with the control group. Blood serum titer of CK was decreased to significant levels ($p<0.05$) when treatment with *O. anatolica* roots ethanol extract is applied. TnI blood serum values were found to be slightly elevated to insignificant level when whereas, no changes in serum triglyceride levels were detected in treated rats when compared with controls (figure I).

Figure 1: The effect of O.anatolica root extract on cardiac markers

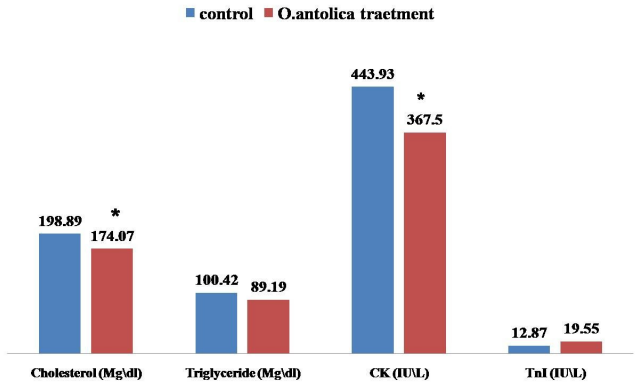


Figure 1 legend: The effect of *Orchis anatolica* on the cardiac biochemical markers

Figure 1 indicates various biochemical cardiac parameters that were investigated after 7 days treatment with 400mg/kg/BW/day *O.anatolica* roots ethanol extract. Down regulations of both blood serums Cholesterol (LDL) and Creatinin Kinase (CK) to a significant level were observed. Insignificant decrease in Triglyceride blood serum level was observed. Tropinin I (TnI) serum titer was elevated slightly but insignificantly. Results are expressed as mean ±S.D. *p < 0.05, **p < 0.01, ***p < 0.001. Significant difference from the control group (Student's "t" test).

The effect of the *O. anatolica* on key hepatic enzyme in rats:

Blood serum AST, ALT, and total bilirubin levels:

As Indicated in figure 2, treatment with of *O. anatolica* roots ethanol extract induces no changes in the serum hepatic enzyme AST concentrations when compared with the control group. In contrast to this, a slight reduction in the serum hepatic enzyme ALT concentration was observed after 7 days treatment when compared with the control rats. Treatment with *O. anatolica* ethanol extract did not affect the total bilirubin serum levels.

Figure 2: The effect of O. anatolic roots ethanol extract on hepatic markers in rats

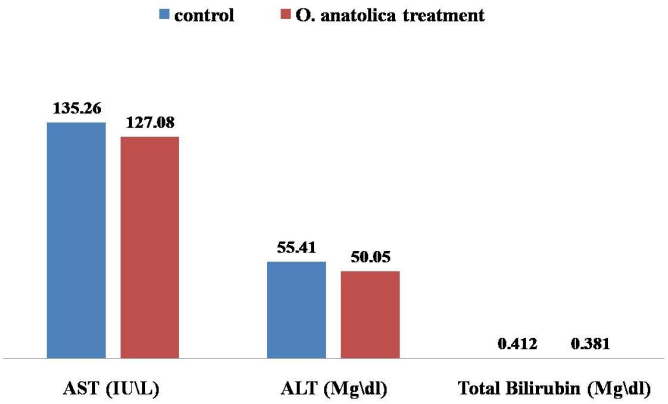


Figure 2 legends: The effect of *Orchis anatolica* on hepatic biochemical markers

Figure 2 showed the treatment effects of *O.anatolica* roots ethanol extract on lever parameters after treatment a dose of 400mg/kg/BW/day for 7 days. Down-regulation of ALT to significant levels was observed after daily oral treatment for 7 days with 400mg/kg/BW *O.anatolica*. The AST and total bilirubine values were unaffected by this treatment. Results are expressed as mean ±S.D. *p< 0.05, **p< 0.01, ***p< 0.001. Significant difference from the control group (Student's "t" test).

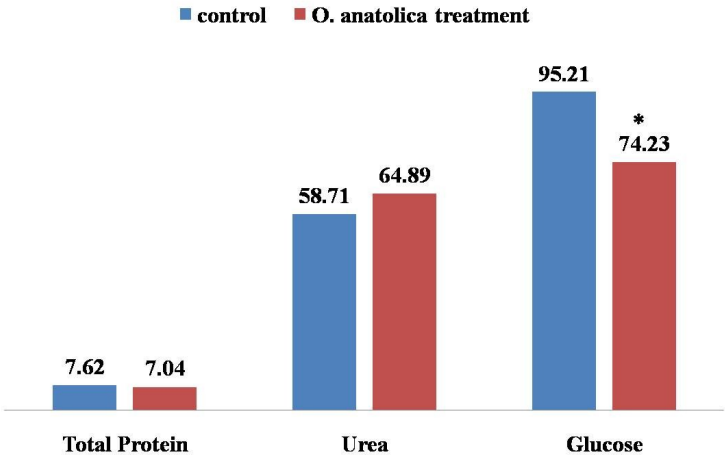
The effect of the *O. anatolica* on total blood serum proteins, urea and blood glucose levels in rats:

Blood serum glucose levels were evaluated and found to be significantly decreased after 7 days treatment with *O. anatolica* ethanol extract. Similar treatment did no affect the serum total protein concentration nor altered the serum concentration of blood urea (figure 3).

Figure 3 legend: The effect of the *O. anatolica* on total blood serum proteins, urea and blood glucose levels in rats:

As indicated by figure 3, the total blood serum protein and urea were not affected by the treatment with *O.anatolica* roots ethanol extract. However, 7 days treatment with 400mg/kg/BW/day *O.anatolica* roots ethanol extract induces down-regulations of serum blood glucose to significant levels throughout the treatment period. Results are expressed as mean ±S.D. *p< 0.05, **p< 0.01, ***p< 0.001. Significant difference from the control group (Student's "t" test).

Figure 3: The effect of O. anatolica on total protien, Urea and blood glucose levels in rats



The effect of *O. anatolica* on serum blood glucose levels and insulin titer after IPGTT

Data from the IPGTT experiment (Table II) indicated that administration of *O. anatolica* roots ethanol extract induces slight decrease in blood glucose levels that was observed at 5 minutes after IPGTT is performed. Treated rats exhibit a significant reduction in glucose titer to significant levels through out the remaining period, an effect that lasted throughout the one hour when compared with the control group of rats after intra-peritoneal glucose induction. In addition, significant increase in the insulin level was observed but only after 15, 30, and 45 minutes measuring period in treated rats group whereas the its levels were observed to return to almost normal levels after 60 minute similar to the controls. Readings of both gulose and insulin levels after IPGTT was performed are shown to go concomitant and with linear values (Table 1). On the other hand, and as indicated in figure 3, it was noticed that in rats group treated 7 days treatment with *O. anatolica* roots ethanol extract, the effect of this extract induces a reduction in glucose levels to significant levels ($p<0.01$) which signify the treatment effect enforcing the data obtained by IPGTT (Table 1).

Table 1: The effect of *O. anatolica* on serum blood glucose levels and insulin titer after IPGTT

	0.0 minute		5 minute		15 minute		30 minute		45 minute		60 minute	
	Glucose Mg/dl	Insulin µU	Glucose Mg/dl	Insulin µU	Glucose Mg/dl	Insulin µU	Glucose Mg/dl	Insulin µU	Glucose Mg/dl	Insulin µU	Glucose Mg/dl	Insulin µU
Control group (n=10)	116.39 ±5.84	18.54 ±2.93	119.48 ±4.57	20.6 ±2.50	236.9 ± 4.79	71.07 ±3.10	195.7 ±3.94	60.25 ±3.66	190.55 ±3.55	38.44 ±2.55	154.5 ±5.27	21.63 ±2.77
Treated group (n=10)	115.36 ±5.02	15.54 ±3.19	117.42 ±4.45	20.6 ±2.66	141.11** ±4.65	88.58* ±2.52	128.75** ±3.47	75.06* ±3.34	130.29** ±3.47	40.03 ±3.06	120.51** ±5.58	22.66 ±2.20

Table 1 legend:

Table 1 represents results obtained after IPGTT was performed in rats prior to treatment with a single dose of *O.anatolica* roots ethanol extract to 18 hours fasting and compared with the control. Blood obtained from rats tails were tested fro both glucose and insulin titers at different periods of time namely at 0.0, 15, 30. 45, and 60 minuts after intra-peritoneal glucose administration. Glocuse values were markedly reduced to significant values after 15, 30 and 45 minutes and remaining at constant levels at 60 minutes recording period when compared with control group. Elevation of the insulin levels where observed only after 0.0, 15 and 30 minute when compared with control group, returning to their constant values through out the remaining detection period. Results are expressed as mean ±S.D. *$p< 0.05$, **$p< 0.01$, ***$p< 0.001$. Significant difference from the control group (Student's "t" test).

DISCUSSION

Traditional medicinal plants are widely used in folk medicine to treat many diseases. Study of such plants might offer a key to unlock a dialectologist's pharmacy for the future (Blumenthal, 2003, Ayyanar 2008). Dabetes and its subsequent complications are possibly the word fastest growing metabolic disorder that requires more appropriate therapies (Dieye, et al., 2007). Treatment with *O. anatolica* induces no change in body weight in rat indicating that plants generally have no toxicity upon usage.

Hypoglycemic effect of *O. anatolica* roots ethanol extracts:

Oral administration of one single dose of *O. anatolica* root ethanol extract induces significant reduction in blood glucose together with an elevation in the insulin levels when IPGTT was performed. Concomitant hypoglycemic effect was also confirmed in the second part of this study prior to treatment with the plant extract to rats for 7 days. These evidences could suggest that the plant used in this study could have direct effect on glucose level either directly or indirectly stimulating insulin secreting cells in similar manor that observed with the sulphamides (Paris, et al., 2004). This mechanism could be explained by the presence of bioactive compounds for this metabolic transformation process acting without indirectly through enhancing insulin secretion ((Paris et al., 2004, Holz, et al., 1993). Others had suggested that many plants my contain substances that would exert an effect on the insulin-secretion through either a stimulation of pancreatic insulin secretion or by up-regulation in proliferation of Langerhance β-cells (Holz, 2004). Like many other plant research, the mechanisms by which this plants constituents act to induce hypoglycemia was not elucidated. Whoever, the significance of this plant extracts to induce hypoglycemic in rats suggested that this plant contains ingredients with anti-diabetic properties (El-Demerdash et al., 2005; Adolfo, et al., 2005, Emeka, et al., 2011). This action mechanism of the plant has been only hypothetically proposed where further studies needed to elucidate the action mechanisms at the molecular level.

Lipid profile after *O. anatolica* roots ethanol extracts treatment:

O. anatolica roots ethanol extracts reduces no changes in the serum blood total protein titer when compared with the controls. The fact that proteins present several features as potentially interesting biomarkers of toxicity they might serve as peripheral indicators of toxic events in relatively inaccessible target organs **(**Bernard et al., 1995). Protein titers stability after *O. anatolic* extract administration demonstrate the fact that this plant did not exhibited any protein degradation leading to propose a non toxic effect at the levels primary organ, in this case the liver. Blood serum cholesterol level was found to be downregulated in this study. It is known that high blood cholesterol levels and hyper-lipidemia can be the consequence and frequently associated with diabetes (Ravi et al., 2005, Rameshkumar et al 2004, Emeka, et al., 2011). The concomitant protein stabilization and the elevation in the serum cholesterol levels are considered an added value of this plant protective mechanisem. These events and together with the hypoglycemic properties of this plant indicated can be considered a preventive factor for long-term complications of diabetes. These findings are in agreement with other cholesterol modulating effects of several other plants (Rajagopal, et al., 2008, Momo et al 2006). The reduction of this lipid profile in rats after treatment can be attributed to their promotion in utilization glucose reflected by a decrease in blood glucose and elevated insulin levels and hence depressed mobilization of fat (Momo et al., 2006). Therefore we can speculate that this plant extracts may be helpful in reducing the complications of hyperlipidemia and hypercholesterolemia which coexist quite often in diabetics (Farnier, 2002).

Hepoatoprotective effect of *O. anatolica* roots ethanol extracts:

Normally, both AST and ALT assay indicates suspected liver damage that might be caused by the drug toxicity or any harmful chemicals compounds (Nelson, et al., 2005, Emeka, et al., 2011). Presently, normal or even slight decrease in both AST and ALT levels in rats were observed after plant extract treatment when compared with controls. In a study by Rosa et al., (2009) indicated some hepatic-protective of an orchid plant. It was demonstrated that ALT and AST pretreatment were significantly reduced in rats treated with different of methanol extract this plant and caused a significant reduction in parcetamol treated rats in a dose-related manner. These findings are similar to our finding which enforces the idea of the safe treatment usage of our extract leading to confirm that treatment with *O. anatolica* extracts did not altar the liver function by not elevating both ALT and AST levels suggesting no plant hepatic-toxicity (Pari 2004).

These normal values further indicate that liver functions should be also unaltered a fact that could enhance the transport of glucose to ameliorate diabetes (Bhandarkar, et al., 2003). This is in accordance with other research results suggesting some plants that are employed in the management of diabetes can be safely used due to the fact that they do not possesses liver toxicity (Hossain, 2011, Rosa et al., 2009).

Cardio-protective properties of *O. anatolica* roots ethanol extracts:

Administration of this *O. anatolica* ethanol extract decreases the total serum cholesterol with no evident changes in triglycerides levels. The mechanism behind the cholesterol lowering effect of this plant is unclear whoever a cardio-protective effect of this extract could be attributed. Cardiac protection is designated usually to inhibition of hydroxymethylglutaryl Coenzyme A (HMG CoA) reductase which is considered the key regulatory enzyme in cholesterol biosynthesis (Kothare, et al., 2007). This enzyme plays an important role in reducing the absorption of cholesterol by the intestinal wall and/or acting by an induction of LDL-receptors within the peripheral tissue (Danesh, et al., 2004). After 7 days treatment with *O. anatolica* extract, a significant reduction in the serum level of CK was observed. This reduction suggested that this plant may likely cardio-protective compounds hence **CK low levels are a strong indictor of cardiac protection, prevention damage to cardiac muscle and it is therefore indicative in determination of myocardial injury.** The concomitant decrease in the serum CK together with cholesterol lowering effect of this extract, further confirms the cardio-protective effects of the plant. Several studies had been emploid and reported such effect of other compounds on these enzymes (Mahanta, et al. 2001). treatment with *O. anatolica* extract reduces the blood serum titer of TnI also indicating cardio protective mechanisem of this extract. These results are consistent with those reported by Jacquet et al. **(1998)** who noted that TnI assessment especial when elevated is reliable for the identification of excessive myocardial necrosis.

CONCLUSION

The data suggest an important role of *O. anatolica* in the stimulation of insulin secretion the normalization of glucose elevation in rats where the levels of free radicals were decreased in plasma. This indicates that this plant could have some biological structure that may act to increase secretion of glucagon-like peptide-1 or acting on the pancreatic β-cells to stimulate insulin secretion. *O. anatolica* extract reduce serum blood glucose, cholesterol levels without altering the normal values of other cardiac or liver enzymes and with no evident of organ toxicity. This could be an indicator of the important role as cardio and hepato protective role played concomitant with an important hypoglycemic effect.

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