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Evaluation of the effects of *Citrus sinensis* seed oil on blood glucose, lipid profile and liver enzymes in rats injected with alloxan monohydrate

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ABSTRACT

Objective: To evaluate the effects of *Citrus sinensis* seed oil on blood glucose, lipid profile and some liver enzymes activities in alloxan induced diabetic rats.
Methods: About 120 mg/kg body weight alloxan monohydrate was injected intraperitoneally into 18 adult male albino rats weighing 180-200 g, which has been acclimatized in our laboratory for two weeks. Approximately 72 h after the alloxan injection, the rat became hyperglycaemic with blood glucose above 200 mg/dL. The diabetic rats were randomly assigned into three diabetic and one control groups of six rats each: normal control, diabetic treated with 1000 mg/kg body weight of emulsified seed oil; diabetic control, diabetic treated with 150 mg/kg body weight of metformin hydrochloride. Both controls received weight-checked solution of 4.8% v/v Tween-80 in distilled water. All injections in all groups were done intraperitoneally once daily for 28 d. The blood glucose estimation was done every week, with one touch glucometer as well as the weight checked with animal weighing balance. Lipid profiles and some liver enzymes activities (AST, ALT and ALP) were analysed using test kits and spectrophotometer. Data obtained were analyzed using One way ANOVA and *post hoc* test done using graph pad prism-version 6.
Results: The results of this study indicated that *Citrus sinensis* seed oil was able to reduce blood glucose significantly ($P<0.001$) in the early weeks of the study when compared with both the diabetic control group and the metformin-treated group. The seed oil significantly lowered serum triglyceride, the serum LDL-cholesterol, total cholesterol and VLDL-cholesterol; the activities of all the liver enzymes assayed ($P<0.05$) but significantly increased the HDL-cholesterol in the diabetic oil-treated rats as compared to diabetic control ($P<0.05$).
Conclusions: However, further studies need to be carried out to show its mechanism of action and to isolate the active ingredient in the *Citrus sinensis* seed oil that is responsible for these actions.

1. Introduction

Diabetes mellitus (DM) is a metabolic disorder of the endocrine system causing hyperglycemia with disturbance of carbohydrate, fat and protein metabolism[1]. Chemical agents such as alloxan induce diabetes specifically by damaging β-cells or causing temporary inhibition of insulin production and or diminishing the metabolic efficacy/efficiency of insulin in target tissues[2,3]. Currently available agents use to lower blood glucose such as sulfonylurea, biguanide and alpha-glycosidase inhibitors *etc*, besides being expensive, produce serious side effects thus limiting their use during pregnancy. Plants and plants products present some hope to scientist serving as an alternative avenue of discovery from the main stream approach of finding solutions to diseases that have proven very resistant to conventional drugs[4,5]. These products range from aqueous extracts, ethanol extracts *etc* to oil extracts from plants parts like flowers, fruits and seeds. For example, administration of aqueous seed oil extracts of *Khaya*

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senegalensis produce a significantly lowered blood glucose[5]. Finding has also shown that oral administration of *Barbati* seed oil extract significantly lowered the blood glucose level to near normal in an alloxan induced model[6]. The oil from seed of *Citrus sinensis* has been extracted and found to have antifungal effects on leather products[7]. Also recently, the physiochemical characteristics and composition of the oil from the seed of *Citrus sinensis* have been estimated[8-10]. These studies have reputed *Citrus sinensis* seed oil to be a good source of essential fatty acids with linoleic, oleic, and linolenic acids the most predominant unsaturated fatty acids present[11], and also of good industrial utility[8]. African sweet orange seed oil contains about 56% of oleic acid[8]; a monounsaturated fatty acid associated with improved peripheral insulin sensitivity and improved glycaemic control[1,12]. Another study at Saudi King University estimated 25% of oleic acid in *Citrus sinensis*[9]. Tocopherols (an antioxidant) are also present in citrus seed oil with α-tocopherol predominating[12]. However, no such scientific data is available regarding the effect of the oil as a blood glucose lowering agent and a possible antihyperlipidemic agent. In this present study therefore, we sort to evaluate the effect of *Citrus sinensis* seed oil effects on lipid profiles, liver enzyme activities and blood glucose of the test animals.

2. Materials and methods

2.1. Collection and identification of plant

Seed of *Citrus sinensis* (riped) were collected from fruits of a *Citrus* tree from Otolo town in Nnewi North Local Government Area of Anambra State in January, 2012, a dry season. The identification was done by Mr. Ugwuzor P. O. (with herbarium number NAUH 99) of the Department of Botany, Nnamdi Azikiwe University Awka, Anambra State, Nigeria.

2.2. Extraction of the seed oil

The oil from the seeds of *Citrus sinensis* was extracted using the batch solvent extraction method. The seeds were washed, air-dried and crushed. A total of 2.5 kg of coarsely crushed seeds were immersed in 4 liters of *n*-hexane for 12 h with intermittent vigorous shaking. At the end of the 12 h, the organic solvent and the aqueous phase were allowed to separate by gravity. The organic solvent was then decanted and the concentration of lipids in the solvent was determined. This was done by evaporating the solvent using simple vacuum distillations, to exclude any contamination from the solvent of extraction. Extracted seed oil was then stored in the refrigerator (4 °C) for physiochemical analysis.

2.3. Physiochemical properties of oil

Percentage oil yield was calculated as follows:

$$\text{Oil content (\%)} = \frac{\text{Oil weight}}{\text{Sample weight}} \times \frac{100}{1}$$

pH determination was done using a pocket-sized (pH) meter (Hana instrument). Determination of Specific Gravity (SG) was calculated as follows:

$$\text{SG} = \frac{\text{Mass of oil}}{\text{Mass of equal vol. of water}}$$

Solvent miscibility was done by physical observation of the uniform blending of oil sample in acid (HCL), alkali (NaOH), aqueous ammonia (NH_3), ether and water at room temperature, 40 ℃, 50 ℃ and 80 ℃ respectively[8].

Acid value (AV) was determined by weighing and transferring 10 g of pure oil into 250 mL conical flask; 100 mL of ethanol was added to the oil by means of pipette, and then heated on a steam bath for 3 min. The flask was cooled and the content titrated with 0.1 mol/L alcoholic potassium hydroxide (KOH) solution using phenolphthalein as an indicator[13,14]. The acid value (AV) was calculated using the expression AV =

$$\frac{\text{Molecular wt.KOH} \times \text{Vol.KOH}}{\text{10 g of oil}} = \frac{56.1 \times 0.1 \times \text{Vol. KOH used}}{\text{10 g of oil}}$$

Where, 56.1=Molecular weight of KOH, 0.1=Normality of KOH.

Percentage of free fatty acid (%FFA) (as oleic acid) was determine by multiplying the acid value (AV) by a factor 0.503[15].

Thus % FFA=0.503 × Acid value

2.4. Preparation of oil – water emulsion

A standard volume ratio of 4:2:1 (oil: emulsify agent: water respectively) was done accordingly[16]. A volume of 1 mL of the milky emulsion obtained was diluted with 5 mL of distilled water so that the concentration of seed oil obtained was 0.1 g/mL of emulsion (specific gravity of oil equals 0.93). This emulsion concentration was used for the study and it was always prepared fresh and given intra-peritoneally (IP).

2.5. Animal experiment

A total of 42 albino wistar rats of both sexes were used

for this study. They were reared in animal house of the Department of Pharmacology NAU. Rats weighed between 180-200 g were used for the experiment. The rats were fed with feed freshly prepared in the University and had access to drinking water *ad libitum*. They were grouped with distinct identity and acclimatized for two weeks at a room temperature of 25-28 ℃ and a relative humidity of 70%-75%. About 18 animals out of the 42 animals were used for acute toxicity test study of the seed oil extract.

2.6. Acute toxicity study

Acute toxicity of *Citrus sinensis* seed oil was carried out using modified Lorke[17]. The study was carried out in two phases. In phase one of the study, 9 albino wistar rats of both sex randomized into 3 groups of 3 rats each and were injected 600, 800 and 1 000 mg/kg body weight of the oil/water emulsion intraperitoneally (IP). The rats were observed for signs of toxicity which including paw-licking, salivation, stretching of the entire body, weakness, sleep, respiratory distress, coma and death in the first 4 hours and subsequently daily for 7 d. In the second phase of the study, another fresh set of 9 rats of both sex were given 1 500, 2 000 and 2 500 mg/kg body weight of the emulsion intraperitoneally (IP) based on the result of the first phase. The IP median Lethal dose was calculated using the formula LD_{50} = √Minimum toxic dose × maximum tolerated dose.

2.7. Induction of hyperglycaemia (diabetes)

Hyperglycaemia was induced by intraperitoneal (IP) injection (single dose) of a freshly prepared aqueous solution of alloxan monohydrate (120 mg/kg body weight in 0.9% w/v normal saline) to overnight (12 h) fasted normal rats[18]. Alloxan monohydrate is one of the chemicals used to induce hyperglycaemia (diabetes) by partial destruction of the β-cells of langerhan, mediated by generation of reactive oxygen species[19-21]. The destruction of the pancreatic β-cells of langerhan leads to decrease in insulin production and release and a consequent hyperglycaemia. Blood was collected from the tail vein for glucose estimation. Blood glucose level was checked by using one-touch glucometer (ACCU-CHEK, Germany) and confirmed after 72 h of alloxan injection. Rats that showed fasting blood glucose (FBG) ⩾ 200 mg/dL in addition to polydypsia and polyuria, were considered to be hyperglycaemic after 72 h and were selected for the studies[22].

2.8. Hyperglycaemic, hyperlipidaemic and enzyme studies (anti-diabetic)

Total of 24 rats (18 hyperglycaemic, 6 normal, all males) were fasted overnight and randomly assigned into 3 groups of hyperglycaemics, and one control group (n=6) as follows. Normal control rats -(NRcon) without alloxan injection but administered with vehicle/weight checked volume of 4.8% v/v Tween-80 in distilled water ip only. DRmet group alloxan injected rats, but treated with metformin administered ip at a dose of 150 mg/kg body weight[23]. Metformin used as a standard drug is an insulin-sensitizing agent with potent anti-hyperglycaemic properties. The anti-hyperglycaemia properties of metformin is mainly attributed to suppressed hepatic glucose production especially gluconeogenesis and increased peripheral tissue insulin sensitivity[24]. DRcon group-alloxan injected control rats administered with vehicle ip to equalize stress. DRoil group alloxan injected rats but treated with 1 000 mg/kg body weight of oil in the emulsion ip. All doses were started 72 h, after alloxan injection and the above treatment were given once daily for 28 d. Blood glucose levels were estimated every 7 days.

2.9. Body weight, blood glucose and serum lipid profile estimations

The body weight of each rat in all the groups were estimated using Camry animal weighing balance (China). Animals were weighed on Days 0,7,14, 21 and 28 after injection of alloxan to detect changes in body weight[25].

Blood samples were obtained by tail vein puncture of both normal and alloxan injected rats and measured with single touch glucometer. These glucose determinations were done on Days 0, 7, 14, 21 and 28 by glucose oxidase method of single touch glucometer (ACCU - CHEK, Germany) and expressed in milligrams per deciliter (mg/dL).

The serum total cholesterol (TC), triglycerides (TG) and high density lipoprotein (HDL) were determined using spectro-photometer at a wavelength of 500 nm and enzymatic kits (Bio-system, Barcelona Spain). Low density lipoprotein (LDL) and very low density lipoprotein (VLDL) values were calculated by Friedwalds formula[26].

After 28 days of treatment, the overnight fasted animals were sacrificed under ether anesthesia and blood collected by cardiac puncture. Serum was obtained immediately by centrifugation (15 min at 400 r/min) of the blood samples, which was used for the assay of serum lipid profile and some serum liver enzymes.

2.10. Estimation of some liver enzyme activities

Alanine (ALT) and aspartate (ASP) transaminases activities in serum were determined according to the method of Reitman's and Frankel[27], whereas alkaline phosphatase activity in serum was assayed using reagent kit (Randox Company, United Kingdom). The activities of the enzymes were estimated using spectrophotometer at a wave length of 545 nm after incubation at 37 ℃.

2.11. Statistical analysis

Data was expressed as mean±standard deviation. Statistical comparison were performed by One way analysis of variance (ANOVA), followed by *post-hoc* test. Results were considered to be significant when *P*-values were less than 0.05 ($P<0.05$).

3. Results

The oil is golden orange in colour and in liquid state at room temperature. The content of the crude oil in the seeds is 33 V/100 g of dry weight. It has a specific gravity of 0.93, less dense than water, with a refractive index of 1.47 and pH is 6.9.

Table 1
The results for the physicochemical analysis of the *Citrus sinensis* oil.

Physicochemical parameters	Values for oil
Colour	Golden-orange
Oil content (% v/w)	33
Specific gravity	0.93
Refractive index	1.47
pH	6.9
Acid value (mg KOH/g)	14.56
Saponification value (mg KOH/g)	196.35
Free fatty acid (%)	7.32
Solubility in solvent	Soluble in water

Table 2
The result of the fasting blood glucose of the groups per week (mg/dL).

Groups	Day 0	Day 7	Day 14	Day 21	Day 28
NRcon.	84.83±18.3^a	89.33±4.3^a	86.17±18.04^a	92.67±13.8^a	84.00±8.3^a
DRmet.	297.8±61.8^b	275.5±57.8^b	221.2±28.1^b	164.5±79.9^b	121.8±18.7^b
DRcon	365.7±12.6^b	398.7±11.6^c	492.7±59.9^c	517.5±45.8^c	427.0±59.6^c
DRcoil	416.8±11.1^b	87.17±1.5^a	109.8±29.6^d	134.2±34.1^d	138.8±18.7^d

Values are given as mean±standard deviation (SD). Values in the same column not sharing a common superscript letter differ significantly at $P<0.05$.

Table 3
The lipid profile for each treatment group at the end of the treatment (mg/dL).

Groups	TC	TG	HDL-C	LDL-C	VLDL-C
NRcon.	182.5±1.9^a	65.3±7.3^a	38.8±1.2^a	130.0±2.4^a	13.00±1.5^a
DRmet.	205.8±11.6^b	174.8±30.7^b	38.2±2.2^a	132.7±14.3^a	34.9±6.1^b
DRcon	470.2±18.4^c	238.9±22.6^c	12.8±2.9^c	409.6±14.1^c	47.8±4.5^c
DRcoil	292.0±11.2^d	185.2±4.7^b	37.0±0.5^a	140.8±10.8^a	14.1±1.0^a

Values are given as mean±standard deviation (SD). Values in the same column not sharing a common superscript letter differ significantly at $P<0.05$.

Table 4
The liver enzyme activities at the end of the treatment (U/l).

Groups	ALP	AST	ALT
NRcon.	325.7±9.7^a	36.5±0.6^a	43.2±1.9^a
DRmet.	401.8±86.7^b	43.0±3.1^b	65.8±2.1^b
DRcon	1499.0±9.13^c	84.5±6.6^c	93.3±2.6^c
DRcoil	345.0±11.9^d	52.0±0.6^d	44.7±4.4^d

Values are given as mean±SD. Values in the same column not sharing a common superscript letter differ significantly at $P<0.05$.

Table 5
The mean body weight (g) of each group per week.

Groups	Day 0	Day 7	Day 14	Day 21	Day 28
NRcon.	155.0±13.8^a	161.7±13.0^a	166.7±12.2^a	169.2±12.0^a	173.3±12.2^a
DRmet.	184.2±7.8^b	187.5±7.8^b	190.8±7.2^b	195.0±6.7^b	199.2±6.3^b
DRcon	190.8±8.9^c	190.0±10.4^c	180.0±12.3^c	174.0±15.4^c	170.0±12.3^a
DRoil	180.8±12.6^d	185.8±16.8^b	190.2±15.8^b	190.0±15.5^b	192.8±13.7^d

Values are given as mean±SD. Values in the same column not sharing a common superscript letter differ significantly at $P<0.05$.

3.1. Acute toxicity test

Signs of toxicity were first noticed after 3-5 h of oil emulsion administration within this period. There were decreased loco-motor activity and sensitivity to touch and pain, also was decreased feed and fluid intake and prostration was noted after 4 h of oil emulsion administration. In the final phase, the mortality rates for 1 500, 2 000 and 2 500 mg/kg body weight of extract were 0%, 67% and 100% respectively. The LD_{50} was calculated as the square root of the products of minimum toxic dose and the maximum tolerated dose giving a value of 1 732 mg/kg[17].

There was no significant difference in fasting blood glucose (FBG) among the alloxan injected groups before commencement of the study.

3.2. Enzyme activities

The serum enzymes (AST, ALT and ALP) levels of diabetic rats increased significantly as compared to non diabetic control rats (Table 4); After 28 days of *Citrus sinensis* seed oil and metformin administrations, the serum enzymes (AST, ALT and ALP) levels of treated diabetic rats significantly decreased/reduced ($P<0.05$) as compared to diabetic control rats.

4. Discussion

In this study the quality of the oil extract from the seed of local sweet orange using *n*-hexane in a batch method, was assessed using parameters such as pH values, specified gravity, solubility in various solvent, percentage oil content and calculated percentage free fatty acid. N-hexane was used for the extraction because most studies favored the

use of non-polar solvent as an extraction solvent[10,28]. In the present study, specific gravity of 0.93 showed that the oil extract of *Citrus sinensis* is less dense that water and pH value of 6.9 suggest that it is slightly acidic. The color of the oil is golden yellow which is consistent with the works earlier done[8,10]. Therefore, it may be an indication of the presence of carotene which contains vitamins A and E; giving the oil an important medicinal value. The percentage yield of the oil from local sweet orange seed is 33% which is consistent with earlier works[10,29].

Intra-peritoneal administration of *Citrus sinensis* seed oil significantly decreased the fasting blood glucose in the oil-treated diabetic rats compared to the other groups at Day-7 fasting blood glucose estimation. After Day 7, there was a gradual increase in the fasting blood glucose as seen in estimations at Days 14, 21 and 28. However, this gradual increase in fasting blood glucose of oil-treated diabetic group showed a better glucose control devoid of much swing when compared with the group receiving metformin. This suggests a better blood glucose control by the seed oil extract than metformin. The reduction in blood glucose could be due to the presence of monounsaturated fatty acids (MUFAs) as reported in an earlier work[30]. MUFA rich diets are capable of lowering blood glucose[1]. African *Citrus sinensis* seed oil has been shown to contain about 56% of a MUFA called oleic acid[8].

Under normal circumstances, insulin activates uptake of fatty acids into adipose tissues, increases triglycerides synthesis and inhibit lipolysis. In insulin-deficiency (alloxan injection), the concentration of serum free fatty acids is elevated as a result of free acid esterification-triglyceride lipolysis cycle is shifted in favor of lipolysis[31]. This condition leads to dyslipidemia characterized by hypertriglyceridemia, decreased serum levels of HDL - cholesterol, elevated serum of LDL-cholesterol and VLDL choresterol[24,25]. In this study, lipid profile values of oil-treated group were not significantly different when compared to that of the group that received the standard drug. This suggests that the oil extract could have a similar effect to metformin in its mechanism of action as an anti-hyperglycaemia agent with good lipid profile control (anti-hyperlipidimia). The effect of *Citrus sinensis* seed oil on the serum lipid profile might also be attributed to the possibility of the oil possessing peroxisome proliferator-activated receptors (PPARs) activating ability. PPARs are ligand-activated nuclear receptors that respond to several exogenous and endogenous ligands by modulating genes related to lipid, glucose, and insulin homeostasis. PPARγ (an isoform), expressed in adipose tissue and liver, regulates lipid storage and glucose metabolism and is the target of type 2 diabetes drugs, thiazolidinediones[32].

The liver is an important insulin-dependent tissue which plays a pivotal role in glucose and lipid homeostasis and is severely affected during dysregulation[30]. Therefore, increase in the activities of transaminases (ALT) (AST) and alkaline phosphates (ALP) in dysregulation may be due to leakage of these enzymes from the hepatic cells into the blood stream[33]. In this study, the groups treated with *Citrus sinensis* seed oil showed a reduction in the activity of these serum enzymes compared to metformin treated group. This *Citrus sinensis* seed oil consequently, may alleviate or completely protect the liver from damage caused by insulin dysfunction (hyperglycaemia, dyslipidemia). The golden yellow color of the seed oil extract may contain carotene, vitamins A and E; this nutracenticals have antioxidant properties and therefore may improve glyceamic control in the oil treated rats.

Also worthy of note is the weekly average weight of the animals within the period of treatment. There was a steady increase in weight in all the groups with the exception of the diabetic control suggesting a good control of hyperglycemia; dyslipidimia and addition of adipose tissue for the groups treated with metformin and seed oil extract. This could further strengthen the argument for the use of seed oil extract as an anti-diabetic agent as done in folk medicine.

In conclusion therefore, the study calls attention to the therapeutic benefits in the use of *Citrus sinensis* seed oil as a good neutraceutical for control of hyperglycaemia, hyperlipidemia and possible hepato protective benefits as demonstrated in this work. However, further studies also need to be carried out to elucidate the mechanism of action and to isolate the active ingredients.

Conflict of interest statement

The authors report no conflict of interest.

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