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Isolation, characterization and *in-silico* molecular docking simulation of the bioactive compound that relates to the anti-diabetic activity of *Lepidium Sativum* L. (Brassicaceae)

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Abstract
Diabetes mellitus is a deteriorating chronic endocrine disorder that is characterized by elevated blood glucose levels that impact fats, protein and carbohydrate metabolism. This study was to determine the bioactive compound that is related to the anti-diabetic activity of Lepidium sativum and propose a mechanism of action for the activity. Using bioassay-guided fractionation, column chromatography, NMR, IR and *In silico* molecular docking, the bioactive principle was isolated, its chemical structure determined and its mechanism of action proposed. Results obtained from NMR and IR supported the proposed structure 2-methoxy-4-(2-propenyl) phenol (eugenol) to be the bioactive compound. Administration of 200 mg/kg eugenol from *L. sativum* to streptozotocin-induced diabetic rats showed a significant reduction in blood glucose levels. The modeling results showed that eugenol acted by binding to glucokinase and Protein Tyrosine Phosphatase 1B enzymes due to their high binding affinities.

Keywords: Diabetes mellitus, *Lepidium sativum*, bioactive principle, blood glucose and eugenol

Introduction

Diabetes mellitus (DM) is a notable deteriorating disease in our society afflicting many lives both in developing and developed countries [1]. It is an endocrine disorder that is characterized by elevated blood glucose levels that affect fats, protein, and carbohydrate metabolism [2]. Nature has been a major source of medicinal plants and a remarkable number of novel drugs have been isolated from it. The use of plants in traditional medicine has been the foundation of many isolations and a significant world population rely on traditional medicine as a source of medical care [3]. In the management of diabetes mellitus, many treatments that involve the use of medicinal plants are advocated [4]. *Lepidium sativum* is a medicinal plant that is used by the natives in Jos Plateau state Nigeria as medication for diabetes. *Lepidium sativum* commonly referred to as garden cress belongs to the family Brassicaceae (Cruciferae). In Northern Nigeria, it is called làbsúr or làfusûr by the Berom and Hausa communities. Garden cress has a typical pungent odor due to the volatile oils it contains and has been employed to treat various conditions such as respiratory disorders, bone fractures, inflammation, and muscle pain. The plant is reported to have antihypertensive, hepatoprotective, diuretic, antimicrobial, antioxidant and laxative properties. *L. sativum* can grow in various climates and soil types, it is tolerant to low acidic conditions, and requires limited irrigation and farming equipment [5]. The use of the aqueous extract of *L. sativum* as an antidiabetic agent has been scientifically determined [6]. The aim of this study was to isolate, characterize and propose the mechanism of action of the active principle in *L. sativum* through in-silico molecular docking, responsible for lowering blood glucose levels in streptozotocin-induced diabetic rats.

Materials and Methods

Plant Extraction

Dried whole plant of *L. sativum* was powdered (200 g) was macerated with 70% methanol at room temperature for 72 hours. A dried crude extract was obtained by evaporating to dryness with a rotary evaporator and the yield was determined. Subsequently, successive sequential extractions were carried out to yield n-hexane, chloroform and methanol fraction extracts. The extracts collected were allowed to dry at room temperature for 3-4 days and their yields were determined. The dried extracts were refrigerated until required.
The methanolic fraction emerged with the most potent hypoglycemic activity following the administration of the various solvent extracts to diabetic rats.

Animals
The adult male albino Wistar rats with body masses between 180-220 g used for this study were purchased from the animal house, Department of Pharmacology, University of Jos, Jos Nigeria. The rats were allowed free access to standard pellet feed and water *ad-libitum* and kept in standard cages and laboratory conditions. The animal experiments with reference approval F17-00379 were in compliance with the condition of the institutional animal care and use (IACU) in collaboration with the office of Laboratory Animal Welfare (OLAW).

Experimental animals
Two hundred and fifty male rats were obtained for the biological assay guide in the fractionation process. The animals were allowed to acclimatize for three weeks. Diabetes was induced after fasting by administering streptozotocin (55 mg/kg). The animals were left for 72 h after which their blood glucose levels were determined using the On Call plus Glucometer. The rats whose blood glucose levels were ≥ 200 mg/dl were considered to be diabetic [6].
Following the induction of diabetes, the animals with diabetes were randomly distributed into four groups of five animals and administered their diets, methanol fractions of *L. sativum*, or standard drug for 28 days as follows:
Group A: Normal Control rats: Rats that were non-diabetic and non-treated.
Group B: Diabetic Control Rats: Rats with diabetes and non-treated.
Group C: Diabetic treated rats: Rats with diabetes and treated with *L. sativum* fractions and sub-fractions (100 mg/kg).
Group D: Diabetic treated rats: Rats with diabetes treated with *L. sativum* fractions and sub-fractions (200 mg/kg).
Group E: Diabetic treated rats: Rats with diabetes treated with glibenclamide (2 mg/kg).

Chemicals and reagents
The solvents used for the study were sourced from Sigma-Aldrich (St. Louis, MO, USA).

Bioassay guided fractionation
The wet method was used to set up the stationary phase with Silica gel (l00 g, 40-63μ µm) in a glass tube making an allowance of 60 cm at the top of the column. The methanol fraction extract of *L. sativum* (3 g) pre-adsorbed on silica was loaded and steadied for 3 hours before elution began. Vials of 40 ml fractions were collected; 1-25 vials were collected using hexane-methanol (9:1), 26-60 were collected using hexane-methanol (8:2) and 61- 65 vials were collected using 100% methanol.
Fractions were dried under pressure, monitored on TLC, visualized with the aid of 254nm and 365 nm UV light and *p*-Anisaldehyde was applied. Fractions with similar R_f-values were pulled together as follows: Fraction 1 (1-3 vials), Fraction 2 (4-8 vials), Fraction 3 (9-13 vials), Fraction 4 (14-18 vials), Fraction 5 (19-23 vials), Fraction 6 (24-28 vials), Fraction 7 (29-33 vials), Fraction 8 (34-38 vials), Fraction 9 (39-43 vials), Fraction 10 (44-48 vials), Fraction 11 (49-53 vials) and Fraction 12 (54-58 vials).
The fractions (1-12) were administered to streptozotocin-induced diabetic rats and hypoglycemic effects were observed from fractions 6 and 7. Fractions 6 (24-28 vials) and 7 (29-33 vials) were returned to the column for further separation. Seven sub-fractions: 13 (A, B, C, D, E, F, G) were collected and again monitored on TLC plates. The sub-fractions were administered to streptozotocin-induced diabetic rats and sub-fraction 13E showed a hypoglycemic effect.

Nuclear Magnetic Resonance (NMR)
1D and 2D NMR spectroscopy were conducted. With the aid of ARX-400 NMR spectrometers, NMR spectra were captured at 300 °K.
The attenuated total reflectance (*ATR*) spectrophotometer was employed to conduct Infrared Spectrometry (IR).

Molecular Docking
The molecular docking simulations were performed at Jaris Computational Biology Center, Jos, Nigeria. The enzymes alpha-amylase (2QV4), alpha-glucosidase (7K9N), glucokinase (1V4S), protein tyrosine phosphatase 1B (PTP1B) (1T4J) and hexokinase (HK-II) (5HEX) were modeled and the simulations were run with eugenol [7].

Results

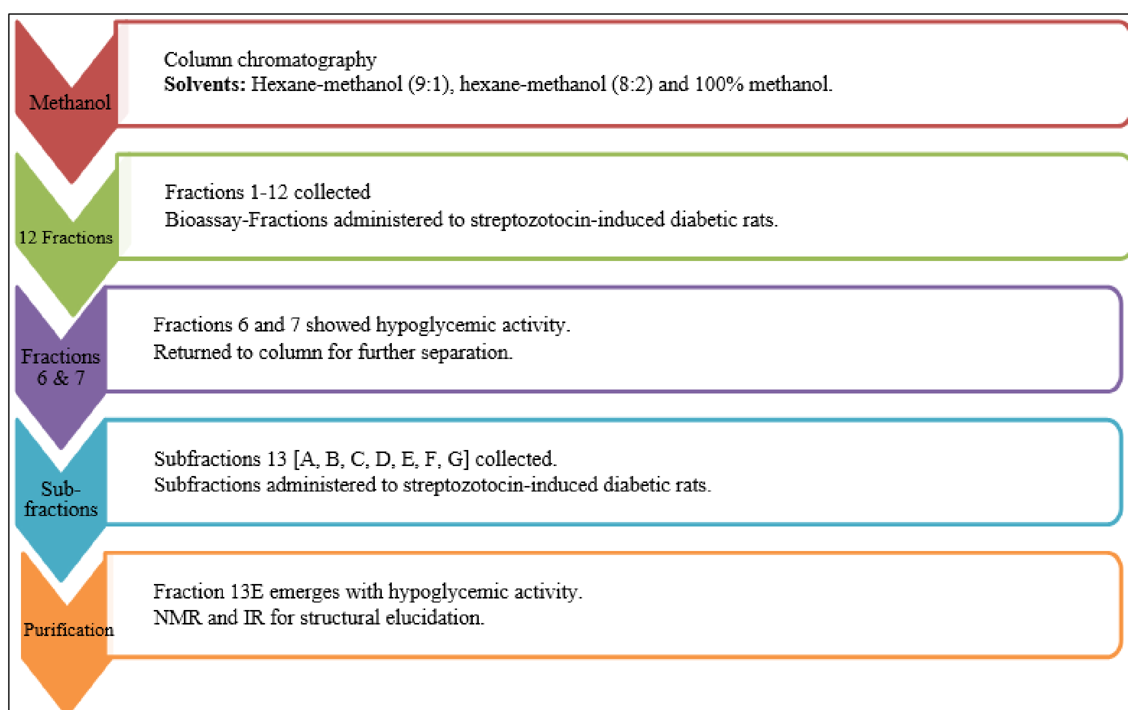

Fig 1: Bioassay guided fractionation and structural elucidation

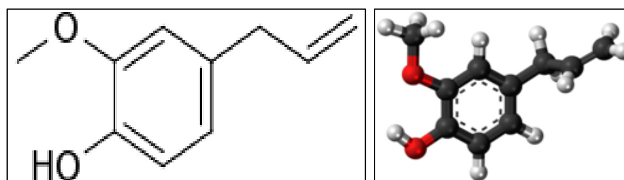

Fig 2: Structure of the Active Principle-2-methoxy-4-(2-propenyl) phenol (Eugenol)

Table 1: The effect of Eugenol from *L. sativum* on Blood Glucose

Treatment Groups	Glucose (mmol/L)
Normal Control (A)	**5.07 ±0.08**
Diabetic Control (B)	19.77 ±0.62[a]
Diabetic Treated with 100 mg/kg Eugenol (C)	9.09±0.62[ab]
Diabetic Treated with 200 mg/kg Eugenol (D)	7.45±0.10 [b]
Glibenclamide Treated (E)	4.22±0.56 [b]

Values are mean± S.D for five determinations.
a = Significant difference at $p<0.05$ when compared to normal control.
b = Significant difference at $p<0.05$ when compared to diabetic control.

Table 2: Binding Affinities of Eugenol against Selected Glucose Metabolism Enzymes

Title	Docking score (kcal/mol)
1T4J-Protein Tyrosine Phosphatase 1B	-6.0
1V4S-Glucokinase	-6.0
5HEX-Hexokinase 2	-5.5
2QV4-Alpha-Amylase	-5.5
7K9N-Alpha-Glucosidase	-5.9

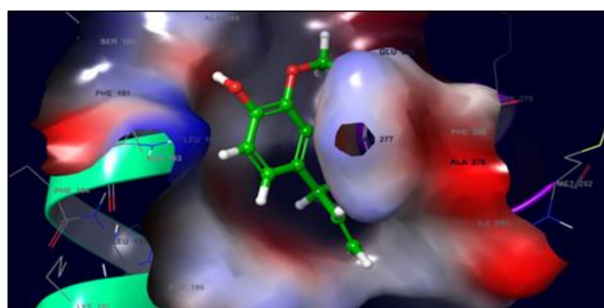

Fig 3: The 3D Interaction of Eugenol and Protein Tyrosine Phosphatase 1B (PTP1B)

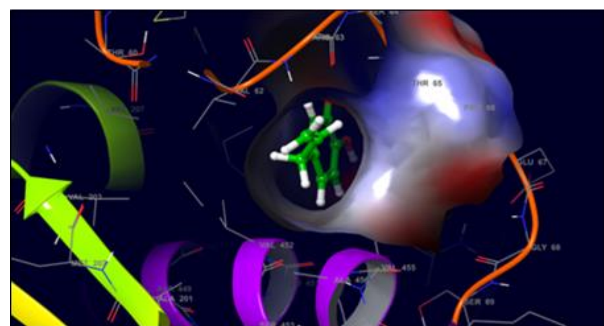

Fig 4: The 3D Interaction of Eugenol and Glucokinase

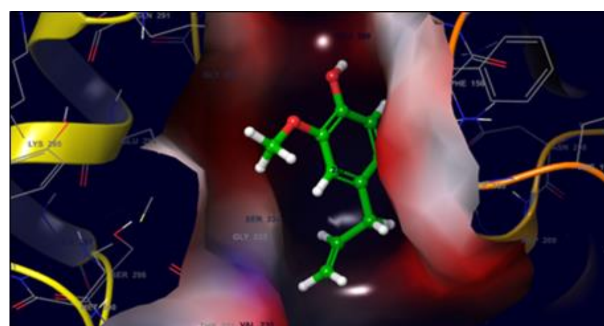

Fig 5: The 3D Interaction of Eugenol and Hexokinase II

Fig 6: The 3D Interaction of Eugenol and Alpha-Amylase

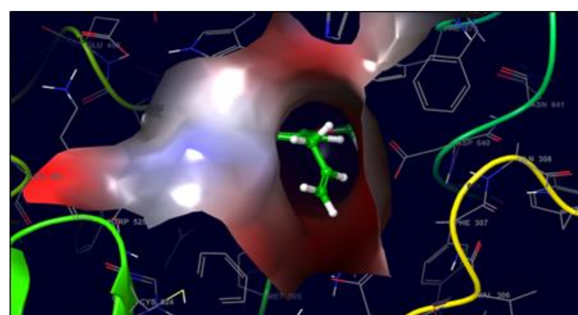

Fig 7: The 3D Interaction of Eugenol and Alpha-Glucosidase

Discussion

The isolated compound was ivory-colored and gel-like substance with a distinctive smell. 12 protons were visible from the [1] H NMR spectrum; three protons signaled downfield at 6.66, 6.67 and 6.84 ppm (1H each) with an aromatic ring at position 3 and the singlets at 3.31ppm proposing a methoxy group on the aromatic ring. There was a $-CH_2$ attached to an aromatic ring at the doublet at 3.29 ppm (2 protons). The downfield doublets at 5.07 ppm and 5.08 ppm (1 H each) proposed an exocyclic (outside the ring) double bond which was reinforced by one proton multiplet at 5.94 ppm for a methyl proton. These results aligned with the literature reported [8, 9].

There were 10 carbon atoms from the [13] C NMR spectrum of the isolated compound; three quaternary, four tertiary, two secondary and one methyl carbon from the DEPT 90 and DEPT 135. From the [1] H NMR results, for the three signals linked to the aromatic protons; one was singlet while two were doublets with proposed switched positions at 1, 2 and 4 on the aromatic ring. Position 1 was replaced with the hydroxyl group, 2 with a methoxy group while position 4 had the allyl group [9].

The NMR and the IR data collected supported the proposed structure of 2-methoxy-4-(2-propenyl) phenol also known as eugenol to be the bioactive compound.

Eugenol significantly reduced the glucose level of diabetic rats when administered at 200 mg/kg when compared to the glucose level of the diabetic control group of rats and therefore could be responsible for the antidiabetic property of the plant. The reduction was close to the estimated normal range of fasting blood glucose (FBG) and postprandial blood glucose (PBG) levels in rats. The work of [10] revealed (3.95

+/- 1.31) mmol/L as the average FBG and (5.65 +/- 1.63) mmol/L as the average PBG of normal Wistar rats. A maximum limit of 6.2 mmol/L for FBG and 7.9 mmol/L for PBG were also availed.

Glucokinase showed the highest binding affinity followed closely by PTP1B. Their interaction with eugenol implied that the binding interaction could occur spontaneously. Alpha-Glucosidase, alpha-amylase, and alpha-glucosidase enzymes showed limited potential interactions with eugenol.

Glucokinase catalyzes the conversion of glucose to glucose-6-phosphate an important step in glycolysis. It acts as a glucose sensor and a rate-limiting step for glycogen synthesis and glucose catabolism. The glucokinase active sites had about 15 amino acid residues. The hydroxyl group of Eugenol formed a hydrogen bond with the amino acid TRY 215 and bound at the allosteric site of the enzyme as an agonist. The binding of eugenol to the allosteric site is posited to activate the enzyme and allow the active site for glucose metabolism. This is supported by the research work of [7], where the binding simulation with essential oil constituents; beta-caryophyllene and eugenol showed high affinities for the glucokinase enzyme.

The PTP1B enzyme had the residues ALA 189, SER 190, LEU 192, ASN 193, PHE 196, ILE 281, PHE 280, GLY 277, GLU 276 and LEU 232. There was a hydrogen bond formation between the hydroxyl group of Eugenol and the residue ALA 189 and a Pi-Pi stacking between eugenol and the residue PHE 280. The binding of eugenol to the active site of PTP1B served an antagonistic effect and inhibited the binding of the phosphate group on insulin receptors to the enzyme. This allowed the insulin receptor to stay phosphorylated, GLUT 4 transporters get translocation to the cell surface, increased glucose uptake by GLUT4 transporters, and lowered blood glucose levels [7].

The mechanism of action of eugenol to decrease elevated blood glucose levels was proposed to be by the binding of eugenol to the allosteric site of glucokinase and the active site of PTP1B in the glucose metabolic pathway to allow for glucose metabolism and enable glucose uptake by GLUT 4.

Conclusion

This study was undertaken to isolate the active principle, elucidate the structure and propose a mechanism for the bioactive principle. Standard methods for diabetes induction, spectroscopic analysis (Nuclear Magnetic Resonance and Infra-Red spectroscopy), and in silico molecular docking were employed. The isolated and characterized compound was determined to be eugenol. The mechanism of the antidiabetic effect of eugenol from *L. sativum* was proposed to be due to the high-affinity binding of eugenol with glucokinase and PTP1B to increase glucose uptake by GLUT 4 and allow glucose metabolism. This to the best of my knowledge is the first study to isolate the bioactive principle from *L. sativum* plant responsible for its antidiabetic effect and has opened up the chemistry of the plant.

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