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Effect of Solvents Fractions of *Cucurbita maxima* Cuticular Lipids on Metabolic Biomarkers of Cardiovascular Disease

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

This study was aimed at studying the effect of solvents (n-hexane, chloroform and methanol) fractions of *Cucurbita maxima* (pumpkin) cuticular lipids on metabolic biomarkers of cardiovascular disease using L-NAME induced hypertensive rats. A total of thirty-six (120-150 g) albino rats were randomly selected and placed into twelve groups of three rats each were used for this study. Each rat was weighed and tagged and thereafter weighed weekly for five weeks of the experiment. Rats were induced with hypertension using 40mg/Kg body weight/24hours. Other rats were placed on normal feeds and water while biomarkers were assayed and recorded on weekly basis. Group I served as normal control, Group II were hypertensive control, Group III were induced with hypertension and administered with standard drug while Groups IV-XII were induced with hypertension and administered with varying doses of n-hexane, chloroform and methanol fractions. A slight alteration on metabolic biomarkers between the normal control group and hypertensive control group was recorded, which was reversed by the administration of methanol fraction. Thus, cuticular lipids from *Cucurbita maxima* might have some anti-hypertensive potentials.

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1. INTRODUCTION

Cucurbita maxima is an annual climbing plant that requires a rich, well-drained moisture retentive soil and a very warm, sunny and sheltered position [1]. It prefers a pH of 5.5 to 5.9, but tolerates up to 6.8, dry periods with a relatively low humidity favour the best growth. A frost-tender annual plant, it is widely cultivated in tropical and temperate zones for its edible fruit, there are very many named varieties differing considerably in their fruits. It can be cultivated the whole year round and many forms require a temperature range of 20 - 27°C during the growing season, but there are some forms that tolerate cooler conditions and these succeed outdoors most years. Most cultivars are relatively insensitive to day-length. Cucurbita species can be differentiated from each other by their fruit stalk, it is angular and polygonal in some cultivators but thick, soft and round in others. Most species mature between 90 and 125 days [2].

Traditional medicinal plants are a therapeutic resource used by population of all continents of the world specifically for healthcare, which may also serve as a starting material for drugs [3]. Popularity of pumpkin in various systems of traditional medicine for several ailments (antidiabetic, antihypertensive, antitumor, immunomodulation, antibacterial, anti-hypercholesterolemia, intestinal anti-parasitia, anti-inflammation and antalgic) was focused the investigators' attention on this plant [4]. The fruit pulp of pumpkin is used as a sedative, emollient and refrigerant, poultice, applied to burns, inflammations, boils, and burns, and also used as diuretic, anthelmintic (for tapeworm). Because of their zinc content and anti-mitotic effect, seeds are used to arrest enlargement of prostate gland. Also used in cystitis and minor kidney dysfunction [5,6,7]. However, most of this traditional claims are yet to be proven scientifically.

Cardiovascular diseases (CVD) are an unavoidable topic when discussing health related issues, particularly in developed societies. CVD is the leading cause of mortality in these countries [8], assuming a progressively more important role in developing countries and even in less developed countries. In epidemiological terms, coronary heart disease and hypertension represent the most significant expressions of cardiovascular disease and were the main causes of mortality and morbidity worldwide, accounting for one third of total mortality in the year 2015 (AHA, 2015).

Hypertension is one of the major risk factors for cardiovascular diseases. It has become a major public health issue in most developed and developing countries [9]. According to the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure, high blood pressure (BP) is defined as systolic blood pressure (SBP) greater than 140mmHg and/or diastolic blood pressure (DBP) greater than 90 mmHg [10]. Patients with SBP ranging between 120 mmHg and 139 mmHg, or DBP of 80mmHg to 89 mmHg, are categorized as prehypertensive. They have a higher risk of developing hypertension and therefore require medical intervention [10].

Most cardiovascular diseases can be prevented by addressing behavioral risk factors such as tobacco use, unhealthy diet and obesity, physical inactivity and harmful use of alcohol using population-wide strategies. People with cardiovascular disease or who are at high cardiovascular risk (due to the presence of one or more risk factors such as hypertension, diabetes, hyperlipidemia or already established disease) need early detection and management using counselling and medicines, as appropriate [11].

Several plants' cuticular lipids studied e.g. apple, mangoes, sugarcane have been shown to contain triterpenoids the have beneficial effects in preventing or mitigating the severity of non-communicable diseases including cardiovascular diseases [12]. Among the studied plants are domesticated edible and non-edible plants in other part of the world but none of domesticated plants in northern Nigeria have been studied. This work was done to assess the effect of cuticular lipids from *Cucurbita maxima* fruit (Common name; Pumpkin, Kabewa in Hausa Language) on L-NAME induced CVD.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Plant material

Cucurbita maxima was cultivated through a surrogate for this study in order to harvest

samples. This provided an opportunity for adequate collection of good fruits samples under specified climatic and weather conditions. A total of 30 fresh fruits without scratch marks were subjected to extraction.

2.1.2 Experimental animals

Male and female albino rats weighing between 100 – 150 g were purchased from animal house of Biological Sciences Department, Bayero University, Kano. The animals were housed in well-ventilated cages in the animal house of the Biological Sciences Department, Bayero University, Kano. The rats were allowed to acclimatize for one week prior to the experiment and had access to food and clean water *ad libitum*. Principles of laboratory animal care (NIH, 1996) and ethical guidelines for investigation of experimental pain in conscious animals (Zimmermann, 1983), were observed during the experimentation.

2.2 METHODS

2.2.1 Extraction of plant cuticular waxes

Plant cuticular waxes were extracted by dipping the fruit in two portions of chloroform for 60 secs and 30 secs respectively at room temperature [13,14,15]. The two-step extraction with the use of two portions of solvent improved recovery. The extract was filtered using cellulose acetate filter with pore size 0.45µm under vacuum. Extracts were evaporated at 50^{o}C to dryness weighed and kept at -20^{o}C, until required for further work.

2.2.2 Fractionation of cuticular waxes

Crude fruit extract from was subjected to fractionation using the methods described by Kolattukudy [16] with some modifications. The crude extract (3.0 g) was re-dissolved in 50ml of chloroform and added to 3 g of Celite, it was then thoroughly mixed and dried. A dry column (3 x 4 cm) was made using silica Gel H (TLC grade 40-60 mesh). The celite/lipid extract was loaded on top of the column. 30 ml each of n-hexane, chloroform and methanol were then passed through the column [17].

Elution was done under negative pressure at -100 to -150mmHg using manual vacuum pump (Model HT05040) with maximum flow of 1ml per minute. All eluents were evaporated under vacuum and the analytes were named *n*-hexane, chloroform and methanol fractions respectively.

2.2.3 Study design and treatment of experimental rats

A total of thirty-six (120-150 g) albino rats were randomly selected and placed into twelve groups of three rats each were used for this study. Each rat was weighed and tagged and thereafter weighed weekly for five weeks of the experiment. Rats induced with hypertension were administered with 40mg/Kg body weight/24hours of L-NAME in distilled water with some adjustment to accommodate variations in weight for the duration of the experiment. Other rats were placed on normal feeds and water. Biomarkers were assayed and recorded on weekly basis. Administration of extract according to the experimental design was commenced on third after the biomarkers were found to have exceeded normal values.

Various doses of extract and L-NAME were co-administered to induced group except those on standard treatment, normal rats and the control groups as per the experimental design.

Group I: Normal Rats
Group II: Induced no Treatment
Group III: Induced; administered with Lisinopril at 0.07 mg/Kg body weight
Group IV: Induced; administered with 250 mg/Kg n-Hexane fraction
Group V: Induced; administered with 250 mg/Kg Chloroform fraction
Group VI: Induced; administered with 250 mg/Kg Methanol fraction
Group VII: Induced; administered with 500 mg/Kg n-Hexane fraction
Group VIII: Induced; administered with 500 mg/Kg Chloroform fraction
Group IX: Induced; administered with 500 mg/Kg Methanol fraction
Group X: Induced; administered with 750 mg/Kg n-Hexane fraction
Group XI: Induced; administered with 750 mg/Kg Chloroform fraction
Group XII: Induced; administered with 750 mg/Kg Methanol fraction

The biomarkers assayed during the five weeks of the experiments are metabolic biomarkers, i.e: Glycated Haemoglobin (HbAIc), Total Cholesterol (TC), High Density Lipoprotein Cholesterol (HDL-C), Low Lipoprotein Cholesterol (LDL-C) and Triacylglycerides (TAG).

2.2.4 Biomarker assays

Biomarker analysis were carried out using colloidal gold immune-chromatographic assay

using "RL-A2000 quantitative Point of Care Testing (POCT) device (by Hangzhou Realy Tech. Co.), for HbA1c analysis. Dry Chemistry Analyzer "CardioCheck© P.A model 3231825" device was used for lipid profile analysis.

2.4.5 Statistical analysis

Results were expressed as mean ± standard deviation and analyzed using ANOVA, with p value <0.05 considered significant, a component of GraphPad Instat3 Software version 3.05 by GraphPadInc was used.

3. RESULTS

The Effect of L-NAME administration on HbA1c percentage is shown in Table 1. Although there is variation in the level of HbA1c between groups, no significant difference (p>0.05) was observed between the normal control group, test control and *C. maxima* fraction administered groups.

Triglyceride levels of L-NAME and fractions administered groups was presented in Table 2. Although there was variation in its level within the period of the experiment, no significant difference (p>0.05) was recorded in between the groups.

Total cholesterol levels of hypertensive induced rats administered with fractions of *Cucurbita maxima* fruit were presented in Table 3. Although there is variation in its level within the period of the experiment, no significant difference (p>0.05) was recorded in between the weeks across all groups.

Effect of L-NAME and fraction administration on HDL-C was presented in Table 4. No significant difference (p>0.05) was observed in between the weeks across all the groups.

Table 5 presents the effect of administration of fractions from *Cucurbita maxima* fruit on LDL-cholesterol. No significant difference (p>0.05) was seen in between the weeks across all groups.

4. DISCUSSION

L-NAME-induced hypertension is a well-established experimental model for induction of hypertension in laboratory animals. Successful induction of hypertension was recorded after three weeks administration of 40 mg/kg body weight of L-NAME. This could be due to decrease in NO and progressive increase in BP as a result of L-NAME administration [18].

L-NAME, a structural analog of L-arginine is metabolized by non-enzymatic hydrolysis into the active form, N omega-nitro-L-arginine (L-NOARG), which competitively binds to endothelial NOS [19]. NOS inhibition attenuates both the synthesis and metabolism of NO, the smallest gaseous intercellular signaling molecule mediating the vascular relaxation. Subsequently, NO deficiency leads to systemic vasoconstriction and hypertension [20]. As L-NAME model mimics hypertension in human, it is very suitable to study the cardiovascular effects of new agents using L-NAME [18].

Table 1. Blood level of HbA1c in rats administered with L-NAME and solvent fraction of *C. maxima*

HbA1c (%)	1st week	2nd week	3rd week	4th week	5th week
Normal Rats	4.26± 0.63	4.16±0.28	4.20±0.51	4.20±0.34	4.16 ± 0.55
Induced no Treatment	3.96±0.11	4.00±0.00	3.96±0.05	4.00±0.00	3.83±0.05
Induced on Standard Treatment	4.00 ± 0.10	4.00 ±0.00	3.96 ± 0.20	3.93 ± 0.25	4.16 ± 0.55
Induced on 250 mg/Kg (n-Hexane)	4.32±0.51	4.13±0.30	4.33±0.63	4.120±0.16	4.06±0.29
Induced on 250 mg/Kg (Chloroform)	4.10±0.30	3.98±0.41	4.45±0.31	4.33±0.90	4.63±0.64
Induced on 250 mg/Kg (Methanol)	4.42±0.11	4.20±0.30	4.16±0.16	5.10±0.15	4.04±0.19
Induced on 500 mg/Kg (n-Hexane)	4.50±0.34	4.20±0.54	3.90±0.29	4.10±0.53	4.62±0.77
Induced on 500 mg/Kg (Chloroform)	4.21±0.70	4.30±0.61	4.13±0.65	4.13±1.00	4.20±0.76
Induced on 500 mg/Kg (Methanol)	4.10±0.30	4.08±0.28	4.33±0.13	4.66±0.42	4.53±0.44
Induced on 750 mg/Kg (n-Hexane)	4.23±0.42	4.20±0.30	4.61±0.42	4.06±0.52	4.23±0.77
Induced on 750 mg/Kg (Chloroform)	4.32±0.30	4.16±0.72	4.66±0.80	4.43±0.40	4.10±0.76
Induced on 750 mg/Kg (Methanol)	4.35±0.20	4.13±0.33	3.80±0.58	4.66±0.21	4.43±0.82

Results are expressed as mean ± SD (n=3)

Table 2. Triglyceride levels of L-NAME induced hypertensive rats administered with solvents fractions of *C. maxima* Fruit

TAG (µmol/L)	1st week	2nd week	3rd week	4th week	5th week
Normal Rats	114.33±1.15	114.26±2.51	115.00±1.00	114.66±2.51	115.00±1.00
Induced no Treatment	110.00±5.00	116.67±7.64	110.00±5.00	116.67±7.64	110.00±5.00
Induced on Standard Treatment	114.60±0.57	116.66±1.52	115.23±1.15	116.66±5.68	111.66±2.08
Induced on 250 mg/Kg (n-Hexane)	105.66±6.03	110.00±5.00	117.00±7.20	110.00±5.00	105.00±5.00
Induced on 250 mg/Kg (Chloroform)	116.33±4.50	105.00±5.00	118.00±2.60	106.33±2.33	105.00±3.60
Induced on 250 mg/Kg (Methanol)	112.67±6.61	110.00±5.00	111.33±7.09	107.00±2.00	106.00±1.72
Induced on 500 mg/Kg (n-Hexane)	117.66±5.04	109.66±0.57	116.66±2.06	106.33±2.30	111.66±3.51
Induced on 500 mg/Kg (Chloroform)	125.65±5.29	112.33±10.69	117.33±2.51	108.66±7.09	105.33±8.38
Induced on 500 mg/Kg (Methanol)	114.33±8.83	112.00±6.23	116.66±3.52	107.33±5.50	112.66±4.73
Induced on 750 mg/Kg (n-Hexane)	106.66±10.69	114.00±5.29	107.33±4.62	109.66±10.20	116.33±3.71
Induced on 750 mg/Kg (Chloroform)	115.00±0.00	115.00±0.00	118.66±2.08	108.66±14.15	108.33±9.45
Induced on 750 mg/Kg (Methanol)	115.00±0.00	115.00±0.00	107.66±6.80	111.00±1.00	108.33±7.62

Results are expressed as mean ± SD (n=3)

Table 3. Total cholesterol levels of L-NAME induced hypertensive rats administered with solvents fractions of *C. maxima* fruit

TC	1st week	2nd week	3rd week	4th week	5th week
Normal Rats	125.66±5.132	132.00±9.16	119.33±17.17	132.00±9.16	119.33±17.17
Induced no Treatment	125.66±5.132	118.66±7.56	117.56±11.05	112.00±9.16	109.33±9.26
Induced on Standard Treatment	123.66±2.51	155.66±10.16	112.67±11.67	115.00±9.01	113.33±10.17
Induced on 250 mg/Kg (n-Hexane)	106.66±9.07	116.33±4.93	118.00±10.40	111.67±7.63	106.00±4.35
Induced on 250 mg/Kg (Chloroform)	122.45±9.07	114.67±5.03	119.67±3.51	108.33±2.89	103.33±5.77
Induced on 250 mg/Kg (Methanol)	106.00±7.00	115.66±5.13	113.33±8.93	108.00±2.88	108.00±2.88
Induced on 500 mg/Kg (n-Hexane)	114.67±6.03	110.33±3.51	120.33±2.51	108.33±2.88	106.33±5.50
Induced on 500 mg/Kg (Chloroform)	115.66±6.11	105.66±5.13	107.66±4.04	108.33±2.88	103.00±5.77
Induced on 500 mg/Kg (Methanol)	115.33±6.66	105.66±5.13	107.66±4.04	133.67±31.94	105.33±5.13
Induced on 750 mg/Kg (n-Hexane)	110.33±4.51	112.00±5.10	111.67±6.67	107.33±3.67	109.54±5.10
Induced on 750 mg/Kg (Chloroform)	117.33±5.23	108.66±2.23	107.10±4.13	109.54±8.52	105.66±7.45
Induced on 750 mg/Kg (Methanol)	120.33±7.74	113.33±6.67	110.00±5.00	117.66±8.45	109.55±6.10

Results are expressed as mean ± SD (n=3)

Table 4. HDL-C levels of L-NAME induced hypertensive rats administered with solvents fractions of *C. maxima* fruit

HDL-C (mg/dl)	1st week	2nd week	3rd week	4th week	5th week
Normal Rats	59.33±2.08	55.33±1.52	56.66±2.08	55.33±1.52	56.66±2.08
Induced no Treatment	61.67±2.98	62.00±4.35	55.33±1.52	56.67±2.08	52.00±2.00
Induced on Standard Treatment	57.33±2..52	56.66±1.98	57.33±2.03	50.33±1.87	51.34±2.47
Induced on 250 mg/Kg (n-Hexane)	59.33±2.08	55.33±1.52	56.67±2.08	55.00±1.00	52.00±2.00
Induced on 250 mg/Kg (Chloroform)	60.66±0.57	58.66±1.53	54.00±3.60	55.00±1.00	54.00±2.00
Induced on 250 mg/Kg (Methanol)	60.00±1.00	59.00±1.70	54.00±1.00	54.33±2.08	53.66±1.52
Induced on 500 mg/Kg (n-Hexane)	60.66±1.53	59.00±2.70	52.33±3.21	54.33±4.50	51.00±1.00
Induced on 500 mg/Kg (Chloroform)	61.00±1.73	58.33±4.62	52.00±1.73	56.33±0.57	53.67±3.25
Induced on 500 mg/Kg (Methanol)	60.66±2.08	58.33±5.50	54.33±1.15	55.66±2.52	54.00±3.60
Induced on 750 mg/Kg (n-Hexane)	59.33±2.08	59.33±2.08	55.00±1.00	54.33±4.50	52.66±2.30
Induced on 750 mg/Kg (Chloroform)	59.33±2.08	55.33±1.53	54.00±1.73	56.66±0.57	54.66±1.52
Induced on 750 mg/Kg (Methanol)	59.33±2.08	55.33±1.53	53.00±2.00	54.67±2.88	55.66±2.08

Results are expressed as mean ± SD (n=3)

Table 5. LDL-C levels of L-NAME induced hypertensive rats administered with solvents fractions of *C. maxima* fruit

LDL-C (mg/dl)	1st week	2nd week	3rd week	4th week	5th week
Normal Rats	42.80±1.00	53.80±1.55	39.70±1.52	53.70±2.00	39.70±1.15
Induced no Treatment	42.00±3.60	33.30±2.51	40.20±2.13	32.00±2.88	35.30±1.72
Induced on Standard Treatment	43.35±2.08	75.70±1.00	32.30±6.49	41.30±2.08	39.65±1.73
Induced on 250 mg/Kg (n-Hexane)	26.20±1.85	39.00±2.08	39.97±2.04	34.70±2.61	33.00±1.15
Induced on 250 mg/Kg (Chloroform)	38.50±1.00	35.00±1.00	42.10±2.08	32.06±1.35	28.33±2.53
Induced on 250 mg/Kg (Methanol)	32.47±1.00	36.66±1.00	37.00±0.57	32.32±4.50	33.14±0.57
Induced on 500 mg/Kg (n-Hexane)	30.50±3.00	29.4±2.08	44.70±2.08	32.73±4.13	33.00±1.52
Induced on 500 mg/Kg (Chloroform)	29.53±2.08	24.86±3.60	33.20±2.30	30.27±0.58	28.26±1.52
Induced on 500 mg/Kg (Methanol)	31.8±1.15	23.93±3.51	30.00±4.05	56.50±5.52	28.88±1.73
Induced on 750 mg/Kg (n-Hexane)	29.67±1.15	29.87±3.50	35.00±3.22	31.07±4.16	33.6±0.57
Induced on 750 mg/Kg (Chloroform)	35.00±3.45	33.30±2.52	29.37±1.15	31.15±1.00	29.33±0.58
Induced on 750 mg/Kg (Methanol)	38.00±5.20	35.00±2.52	35.50±3.13	40.80±3.21	32.20±2.28

Results are expressed as mean ± SD (n=3)

HbA1c level is an independent risk factor for cardiovascular events [21]. There is also evidence that the association of HbA1c level with mortality from all causes and CVD can be found at lower levels than the diabetic threshold [22]. A metaanalysis showed that HbA1c level is an independent predictor of mortality in patients with coronary artery disease without established diabetes but not in those with established diabetes [23]. Currently, the association between chronic hyperglycemia and cardiovascular complications is not well defined. Several observational studies have demonstrated that a higher HbA1c level is associated with increased risk of CVD in diabetes [24]. Thus, an elevated HbA1c level might contribute to the development of CVD.

Although a positive correlation between level and the progression of CVD was reported, no significant change was recorded in the level of HbA1c throughout the period of the study. This is contrary to most reports that HbA1c level increases in CVD [25]. The observed HbA1c result may be due to different pathophysiology in the induction of CVD as most reported high HbA1c level were in patients with underlying condition of diabetes mellitus [24]. In his conclusion, Lyons and Basu [26] report that there have already been numerous studies on the use of HbA1c level as a prognostic marker for CVD outcome and mortality, but the individual studies have been controversial, so there is uncertainty regarding its use.

Hypertension induction was accompanied by slight increase in serum total cholesterol, triglyceride and LDL-cholesterol levels in L-NAME administered groups. Increase in these parameters have been positively related to hyperlipidemia and the risk of cardiovascular diseases (Ma et al., 1999). Austin (2007) and Alhassan et al. (2016) also reported the positive correlation between CVDs and elevated level of total cholesterol, LDL-cholesterol, VLDL-cholesterol and reduced HDL-cholesterol and/or elevated triglycerides.

The effect of oral administration of varying doses of *C. maxima* fractions on L-NAME hypertensive rats shows a slight non-dose dependent hypolipidemic activity in methanol fraction. The results for the effects on total cholesterol and triglycerides no significant changes in their concentrations in all tests groups administered with the fractions compared to positive control. Thus it may be suggested that the plant may have affected cholesterol biosynthesis which resulted in the reduction in the level of cholesterol in the blood.

HDL-cholesterol levels are reported to correlate inversely with the risk of coronary heart disease (Stensvold et al., 1992). In the current study, a non-significant increase in level of HDL-cholesterol compared to hypertensive control was observed in methanol fraction administered group. The association between a low level of HDL-cholesterol and an increased risk of cardio vascular diseases has been well established through epidemiology and clinical studies (Assmann and Gotto, 2014). A study in humans provides support for the proposition that raising the level of HDL-cholesterol is of substantial therapeutic advantage (Nissen et al., 2013). This was a small study in which a preparation of reconstituted HDL was infused into human subjects. The result was consistent with a profound protective action of HDL-cholesterol on the basis of the epidemiologic data. In this study, methanol fraction led to a slight elevation of serum HDL-cholesterol, indicating its promising role against CVD.

The exact mechanism of action of the active principle in this fraction on lipid metabolism may be by lowering plasma and hepatic cholesterol concentrations by suppressing/inhibiting HMG-CoA reductase an enzyme that catalyzes the committed step in cholesterol synthesis (Seenivasan et al., 2011). Another possible mechanism may be through antioxidant properties of this fraction, thus preventing the oxidation of LDL-cholesterol and the expression of cellular adhesion molecules and monocyte recruitment [5].

5. CONCLUSION

The research concludes that pumpkin cuticular waxes might possess anti-hypertensive activity as evident from the reduction of serum cholesterol and triglycerides. However further ascertain this claim, further study on its effect on diagnostic biomarkers of CVDs should be conducted.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Animal Ethic committee approval has been taken to carry out this study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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