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Autranella congolensis Extract Prevents Atherogenic Dyslipidemia in Diabetic Rats via Modulation of Global Hepatic DNA Methylation

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Abstract: DNA methylation is an epigenetic mechanism involved in the regulation of blood lipid levels which contribute to the cardiovascular risk in diabetic patients. Active agents that target atherogenic dyslipidemias epigenetically are therefore of paramount interest for prevention of cardiovascular complications in these patients. This study aims at evaluating the antiatherogenic effect of hydroethanolic extract of *A. congolensis* (HEEAC) on diabetic rats. Diabetes was induced by a single dose of 50 mg/kg b.w streptozotocin. Diabetic rats were then treated with 150 mg/kg b.w of HEEAC or with atorvastatin 10 mg/kg b.w (reference drug) for 28 days. At the end of the experimental period, rats were sacrificed, blood samples and liver tissues were collected and the plasma concentrations of triglycerides (TG), total cholesterol (TC), HDL-cholesterol and LDL-cholesterol were assessed. The atherogenic indices were also calculated and the liver and blood DNA extracted to determine DNA methylation. Comparing to untreated diabetic rats, the HEEAC treated group showed significant lower values of TG 245.98 ± 41.39 vs 57.88 ± 10.64 mg/dL ($p < 0.05$), TC 159.88 ± 17.56 vs. 87.77 ± 9.51 mg/dL ($p < 0.05$), LDL-C 94.51 ± 0.66 vs 48.71 ± 1.45 mg/dL ($p < 0.05$) and higher values of HDL-C 19.55 ± 1.6 vs 27.49 ± 1.45 ($p < 0.05$). Atherogenic indices were significantly lowered in HEEAC-group. The effects of HEEAC on lipid profile and atherogenic indices were significantly higher than atorvastatin. Also, the percentage of global hepatic DNA methylation (0.34±0.033 vs 0.63±0.023 %) was significantly increased in HEEAC-group compared to untreated diabetic group. DNA methylation profile in HEEAC-group correlated negatively and significantly ($P < 0.01$) with LDL-C and TC levels. HEEAC prevents atherogenic dyslipidemia in diabetic rats by targeting global DNA-methylation status in diabetic rats.

Keywords: Diabetes, DNA Methylation, Liver, Lipid Profile, Atherogenic Risk, *Autranella congolensis*

1. Introduction

Diabetes is a metabolic disease characterized by persistent hyperglycemia resulting from a defect in the action and/or secretion of insulin [1]. The incidence of this chronic metabolic disease has exponentially increased over the last 30 years and affects now more than 425 million people worldwide [1]. Diabetes patients are most commonly affected by cardiovascular diseases in terms of mortality and morbidity [2]. Particularly in diabetic patients, blood lipid abnormalities including low levels of HDL-cholesterol, high levels of LDL-cholesterol, triglycerides (TG), and total cholesterol (TC) are considered as heritable and modifiable factors leading to a higher risk of cardiovascular diseases [3]. Proper control of these risk factors is consequently essential as the disease's prevalence rises. In addition, it was shown that genes involved in the regulation of cholesterol and fatty acid metabolism had a relationship with DNA methylation and lipid levels [4].

DNA methylation is an epigenetic mechanism which modulates transcriptional regulation without changing the underlying DNA sequence [5]. It consists in the addition of a methyl group from the principal methyl donor, S-Adenosyl-methionine (SAM), to the cytosine at the CpG dinucleotide residues (to form 5-methylcytosine). The addition of methyl groups to the cytosine is directly catalyzed by DNA methyltransferase families (DNMT) which are the major players as epigenetic modifiers [5-7]. Several genes were previously reported with altered methylation profiles in diabetes. Some of these genes such as *ABCG1 (*ATP-binding cassette sub-family G member 1) and *SREBF* (sterol regulatory element binding transcription factor 1; regulator of hepatic lipogenesis) are implicated in lipid metabolism pathways [4]. In addition, several studies have shown that, DNA methylations of these genes are associated with plasma lipids concentration in diabetes [8]. Herein, light is shed on active agents that target cardiovascular risk factors in diabetic patients via epigenetic mechanisms. Recently, the potential of dietary polyphenols to modulate epigenetic events in human health has become evident [9].

Polyphenols are the largest class of plant secondary metabolites which are mainly found in fruits and vegetables. In addition to their antioxidant or anti-inflammatory activities, polyphenols such as flavonoids might modulate epigenetic alterations including modulation of DNA methylation status [10, 11]. Sapotaceae like *Baillonella toxisperma* or *Autranella congolensis* are dietary flavonoid-rich plants with several biological activities [12, 13]. Indeed, it has been shown that hydroethanolic extract of *Autranella congolensis* reduces significantly plasma TC and TG and maintain brain lipid membrane composition and fluidity at a healthy state in Alzheimer's rats [13]. Therefore, the present study aimed to evaluate the preventive effect of hydroethanolic extract of *A. congolensis* (HEEAC) on atherogenic dyslipidemia via its impact on the methylome in diabetic rats.

2. Materials and Methods

2.1. Plant Material and Hydroethanolic Extract Preparation

The plant material was harvested in forests located in the East and Center region of Cameroon. The hydroethanolic extract of *A. congolensis* (HEEAC) was prepared like previously described by Ngoumen et *al*. [13]. Briefly, 200 mg of fine powder of stem barks were dissolved in 1000 mL of ethanol/water (50:50 v/v) extractant for 48 h at room temperature, and then filtered. The obtained filtrate was evaporated, and the crude extract was stored until used.

2.2. Animals and Experimental Design

Twenty (20) rats of Wistar strain weighing between 250 and 300 g were provided by the Laboratory of Nutrition and Nutritional Biochemistry (LNNB) (Department of Biochemistry, University of Yaoundé I). Animals were kept in cages in the animal house of the laboratory at a constant room temperature (22 ± 1°C) under a 12 h Light/12h Dark cycle. Food and water were provided *ad libitum*.

Rats were firstly divided into two groups

a) Group 1 or control group (5 rats): fed with normal diet

b) Group 2 (15 rats): fed with normal diet, after 2 weeks, the animals (fasted for 12 h) except those of control group received a single intraperitoneal (*i.p*.) dose of Streptozotocin (STZ) (50 mg/kg) freshly dissolved in citrate buffer (100 mM; pH 4.5; 150 mM NaCl). One hour later, rats received a glucose solution (20% w/v) to avoid hypoglycemic shock. After 48 hours, glucose level was measured using a glucometer (Gluco-Plus brand TM) by the sampling of blood from the tail vein of the animals. Animals with blood sugar levels greater than or equal to 200 mg/dL were considered as diabetic [15]. Subsequently, diabetic rats were further divided into three groups of five rats each:

Group 1: diabetic rats (untreated diabetic group)

Group 2: diabetic rats + oral administration of 150 mg/kg body weight of hydroethanolic extract of *A. congolensis* for 28 days.

Group 3: diabetic rats + oral administration of 10 mg/kg body weight of atorvastatin (reference drug) for 28 days.

Control group and diabetic untreated group were orally administered distilled water for 28 days.

At the end of experimental period, animals were euthanized by decapitation after 12 hours of fasting. Blood was collected in Ethylene Diamine Tetra Acetate tubes and centrifuged at 3000 rpm for 10 min to obtain the plasma [14]. The liver was removed and taken into a previously prepared dimethylsulfoxide (DMSO) saline solution for further DNA extraction.

2.3. DNA Extraction and Global DNA Methylation Analysis

DNA extraction

DNA was extracted according to the protocol described by Coombs et *al*. [15]. By using spectrophotometry on a NanoDrop spectrophotometer (Thermo Scientific), the quality and integrity of DNA were evaluated. Only

samples having OD 260/280 ratios between 1.8 and 2.1 were used.

Global DNA methylation analysis

The hepatic methylome was analyzed using the methylated DNA immunoprecipitation method from the MethylFlash™ Global DNA Methylation (5-mC) ELISA Easy Kit (catalog: P-1030) according to the supplier's protocol. The percentage of 5-methylcytosine was calculated using the following formula:

$$\%5mC=\left(\frac{(\text{OD sample}-\text{OD negative control})}{\text{slope x S}}\right)\times 100$$

S: amount of DNA in the sample (ng)

2.4. Plasma Lipid Profile

Triglycerides (TG), total cholesterol (TC) and high-density lipoprotein cholesterol (HDL-C) levels were quantified using CHRONOLAB kit according to the supplier's protocol. Low density lipoprotein cholesterol (LDL-C) were determined using the formula described by Friedewald et *al*. [16]:

$$\text{LDL-C (mg/dL)} = \text{TC} - (\text{HDL-C} + \text{TG}/5)$$

2.5. Atherogenic Risk Indices

Atherogenic risk indices were calculated from the following formulae:

$$\text{CRR (Cardiac Risk Ratio)} = \text{TG/HDL}-\text{C [17]}$$

$$\text{AC (Atherogenic Coefficient)} = \text{TG-HDL}-\text{C/HDL}-\text{C [18]}$$

$$\text{sd-LDL predictor (Small, dense low-density lipoprotein)} = \text{TG/LDL-C [19]}$$

2.6. Plasma Aspartate Amino Transferase (ASAT) and Alanine Amino Transferase (ALAT) Activities

Both enzymes were assessed according to the method described by Reitman and Frankel [20].

Oxidative stress markers assessment

MDA levels were evaluated according to the method described by Yagi [21], the activity of catalase was evaluated according to the method described by Sinha [22] and superoxide dismutase (SOD) activity measured according to the method described by Misra and Fridovich [23].

2.7. Statistical Analysis

Statistical Package for Social Science (SPSS) version 20.0 for Windows was used for statistical analysis of the results. The one-factor Analysis Of Variance (ANOVA) test followed by a post hoc test (LSD) was performed to compare the means of the different groups. All results with $p< 0.05$ were considered significant. Pearson correlation was used to assess the relationship between the different parameters. Results were expressed as mean ± standard deviation.

3. Results

3.1. Effect of HEEAC on the Levels of Body Weight Variation

The effect HEEAC on the entire body weight was measured in rats (Table 1). The average body mass of the diabetic group was significantly ($p < 0.05$) decreased (-14.27%) compared to control group. Decreased body weight in HEEAC group (150 mg/kg) (-1.08 %) was significantly lower than the diabetic group and the reference drug group (-14.05%).

Table 1. *Effect of HEEAC on level of body weight variation in diabetic rats.*

Groups	Initial body weight (g)	Final body weight (g)	% of body weight variation
Control	294.6 ± 17.30	297.6 ± 20,52	1.01[a]
Diabetic Rats + H_2O 5 mL mg/kg b.w	284.6 ± 17.47	244 ± 20.22	-14.27[b]
Diabetic Rats +150 mg/kg b.w. of HEEAC	284.75 ± 17.34	281.67 ± 7.76	-1.08[a]
Diabetic Rats +10 mg/kg b.w. of atorvastatin	298.6 ± 14.6	256.4 ± 11.04	-14.05[b]

Values are expressed as mean ± standard deviation HEEAC: hydroethanolic extract of *Autranella congolensis*; Numerical values in the same column assigned by different letters (a, b) are significantly different ($p<0.05$).

3.2. Effect of HEEAC on Global DNA Methylation Profile

The effect of HEEAC on global DNA methylation was assessed (table 2). The level of methylated DNA in liver was significantly ($p< 0.05$) decreased to nearly 2-fold in diabetic group compared to control group. Meanwhile, administration of HEEAC led to a 2-fold increase in the level of DNA methylation relative to diabetic group. In the blood, no significant difference was found in the level of global DNA methylation.

Table 2. *Effect of HEEAC on global methylated DNA (5-mC) percentage in the blood and liver of diabetic rats.*

Groups	Global DNA methylation (%) in blood	Global DNA methylation (%) in liver
Control	0.48±0.071[a]	0.59±0.068[b]
Diabetic Rats + H_2O 5 mL mg/kg b.w	0.43±0.033[a]	0.34±0.033[a]
Diabetic Rats +150 mg/kg b.w. of HEEAC	0.44±0.061[a]	0.63±0.023[b]
Diabetic Rats +10 mg/kg b.w. of atorvastatin	0.51±0.003[b]	0.44±0.062[a]

Values are expressed as mean ± standard deviation. Numerical values in the same column assigned by different letters (a, b) are significantly different ($p<0.05$).

3.3. Effect of HEEAC on Blood Lipid Profile

The effects of HEEAC on blood lipids profile were assessed in rats. The results (Table 3) show that total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and triglycerides (TG) were significantly ($p<0.05$) increased whereas high-density lipoprotein cholesterol (HDL-C) decreased in diabetic group relative to control group. The levels of TC, LDL-C and TG were significantly diminished whereas HDL-C value was significantly increased after HEEAC treatment compared to untreated diabetic group. Furthermore, significant differences were found between HEEAC-group and control for all dyslipidemia parameters. Cardiac risk ratio and atherogenic coefficient were significantly ($p<0.05$) higher in diabetic rats compared to control group. On the other hand, when HEEAC was administered to diabetic rats, the results showed that cardiac risk ratio was significantly lowered 7-fold; atherogenic coefficient was lowered 12-fold and predictors of increase sd-LDL were significantly diminished 3-fold compared to untreated diabetic rats. HEEAC showed more effect than the reference drug. Furthermore, atherogenic risk indices were significantly lower in HEEAC-group than control group.

Table 3. *Effect of HEEAC on lipid profile makers in diabetic rats.*

Groups	TC (mg/dL)	HDL-c (mg/dL)	LDL-c (mg/dL)	TG (mg/dL)	Cardiac Risk ratio	Artherogenic coefficient	Predictors of increased sd-LDL
Control	126.33 ± 1.18^{a}	19.55 ± 1.6^{a}	87.4 ± 1.6^{a}	96.89 ± 4.95^{a}	5.0 ± 0.12^{a}	3.6 ± 0.47^{a}	1.01 ± 0.028^{a}
Diabetic Rats + H_2O 5 mL mg/kg b.w	159.88 ± 3.5^{a}	16.17 ± 0.66^{a}	94.1 ± 0.66^{b}	245.98 ± 8.27^{b}	15.0 ± 2.4^{b}	13.4 ± 2.3^{b}	3.4 ± 0.14^{b}
Diabetic Rats +150 mg/kg b.w. of HEEAC	87.77 ± 1.92^{b}	27.49 ± 1.45^{b}	48.71 ± 1.45^{c}	57.88 ± 10.64^{c}	1.9 ± 0.03^{c}	1.1 ± 0.062^{c}	0.97 ± 0.036^{a}
Diabetic Rats +10 mg/kg b.w. of atorvastatin	116.42 ± 4.062^{a}	34.56 ± 2.12^{b}	63.66 ± 4.78^{b}	90.96 ± 7.17^{a}	0.78 ± 0.26^{d}	2.37 ± 0.98^{d}	1.43 ± 0.04^{b}

Values are expressed as mean ± standard deviation TG, triglycerides; TC, total cholesterol; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol. Numerical values in the same column assigned by different letters (a, b) are significantly different ($p<0.05$).

3.4. Correlation Between Global DNA Methylation and Lipid Profile Markers

The correlations between global DNA methylation and lipid profile markers in HEEAC-group were assessed (table 4). The results showed significant ($p<0.01$) negative correlation ($r=-0.961$) between the global hepatic DNA methylation and total cholesterol levels. Significant ($p<0.01$) correlation ($r=-0.978$) was also found between the global hepatic DNA methylation and LDL cholesterol level.

Table 4. *Correlation between global DNA methylation and lipid profile markers in HEEAC-treated diabetic rats.*

Groups	r (TC)	r (TG)	r (HDL)	r (LDL)
Control	0.3	-0.72	-0.12	0.3
Diabetic Rats + H_2O 5mL /mg/kg b.w	0.27	0.28	0.37	0.17
Diabetic Rats +150 mg/kg b.w. of HEEAC	-0.961**	-0.24	-0.11	-0.978**

TG, triglicerides; TC, total cholesterol; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol; sd-LDL: Small dense low-density lipoprotein; r: Correlation coefficient * *$p<0.05$; **: $p<0.01$*

3.5. Effect of HEEAC on Glucose Levels, Plasma Transaminases and Oxidative Stress Markers in Liver in Diabetic Rats

Table 5. *Effect of HEEAC on oxidative stress makers in liver, plasma glucose level and transaminases activities.*

Groups	Liver MDA (µM)	Liver Catalase (mmol/min/g of proteins	Liver SOD (mmol/min/g of proteins)	Blood glucose (mg/dL)	Plasma ALAT (UI/ml)	Plasma ASAT (UI/ml)
Control	1.99 ± 0.12^{a}	0.06 ± 0.016^{a}	$0.9 \pm 0{,}009^{a}$	$73.2 \pm 5{,}18^{a}$	69.26 ± 6.85^{a}	72.98 ± 7.76^{a}
Diabetic Rats + H_2O 5 mL mg/kg b.w	3.8 ± 0.37^{b}	0.086 ± 0.03^{b}	0.72 ± 0.02^{b}	427.4 ± 45.65^{b}	123.82 ± 10.17^{b}	82.74 ± 9.8^{a}
Diabetic Rats +150 mg/kg b.w. of HEEAC	0.3 ± 0.003^{c}	0.09 ± 0.01^{b}	0.86 ± 0.03^{a}	369.0 ± 5.86^{b}	91.42 ± 7.7^{c}	89.17 ± 1.32^{a}
Diabetic Rats +10 mg/kg b.w. of atorvastatin	0.5 ± 0.07^{c}	0.015 ± 0.06^{c}	0.66 ± 0.03^{b}	451.5 ± 46.76^{b}	108.82 ± 16.05^{a}	85.71 ± 13.06^{a}

Values are expressed as mean ± standard deviation. Numerical values in the same column assigned by different letters (a, b) are significantly different ($p<0.05$). ASAT: Aspartate amino transferase, ALAT: Alanine Amino Transferase

The blood glucose levels, plasma transaminases levels and oxidative stress markers in liver were assessed (table 5). Blood glucose level was significantly ($p<0.05$) increased and plasma ALAT level was significantly ($p<0.05$) higher in diabetic group compared to control group. In liver of diabetic rats, the MDA levels were significantly increased whereas the activities of catalase and SOD were significantly reduced relative to control (table 5). HEEAC administration resulted in

a significant ($p<0.05$) lowering of ALAT and MDA levels and a significant increase in SOD activity compared to diabetic rats. Blood glucose level was not significantly changed after extract administration.

4. Discussion

The prevention of the development of atherosclerotic disease through lipid level adjustment is a well-known method for reducing cardiovascular mortality [24, 25]. As previously mentioned, DNA methylation is involved in the regulation of blood lipid levels and thus contributes to the cardiovascular risk profile of diabetic patients. The purpose of this study was to evaluate the preventive effect of HEEAC on atherogenic dyslipidemia via its impact on the hepatic methylome in diabetic rats. We analyzed Global DNA methylation in liver tissue (LT) as well as plasma lipid profile and atherogenic risks indices. The results showed significant elevated values of blood TG and LDL-C, significant lower values of HDL-C and a subsequent higher atherogenic risk in diabetic rats compared to control group. Furthermore, global DNA-methylation level in liver was decreased (hypomethylation) in diabetic rats compared to control group. These results are consistent with those reported by Fonkoua et *al*. [26] who found that STZ-induced chronic hyperglycemia was associated with hepatic global DNA-hypomethylation and arthritic dyslipidemias. The majority of DNA-methylation sites modified in liver of diabetic individuals reportedly display hypomethylation. In fact, maintaining glucose homeostasis and preventing the onset of diabetes depends on the liver, a key glucoregulatory organ. In healthy people, insulin inhibits gluconeogenesis while promoting the synthesis of hepatic glycogen and *de novo* lipogenesis. However, insulin does not effectively control hepatic glucose production in diabetic patients, which results in hyperglycemia [5]. In turn, chronic hyperglycemia, promotes gluconeogenesis in the liver which is one-carbon metabolism that uses compounds such as cysteine, methionine, and folate. These metabolites are necessary for S-Adenosyl-methionine (SAM) production and thus required for epigenetic mechanisms [27]. Therefore, the metabolic pathways and enzymes that provide these one-carbon metabolites are fundamental for regulation of the epigenome. Deficit in methionine was associated to the decrease of SAM levels which lead to diminished DNA methylation [28]. Modifications to DNA methylation may have a direct effect on transcription, which may then change a number of pathways involved in metabolic activities like lipid metabolism [3]. These may explain higher cholesterol and triglycerides plasma levels observed in diabetic group compared to control group.

On the other hand, administration of hydroethanolic extract of *A. congolensis* (HEEAC) in diabetic rats for 28 days resulted in a significant decrease of plasma TG and LDL-C, an increase in HDL-C values, and consequently lowered significantly atherogenic risks compared to untreated diabetic rats. The ameliorative effects of HEEAC were significantly higher than those of reference drug atorvastatin. Furthermore, atherogenic dyslipidemia parameters were better in the HEEAC-group than control group. This strong ability of the extract to improve lipid metabolism is in line with the work of Ngoumen et *al*. [13]. Moreover, in diabetic rats treated with HEEAC, global DNA-methylation was significantly increased and was highly correlated with reduction of LDL-C and TC levels. All of these suggest a strong preventive effect of HEEAC on cardiovascular risk factors through its modulation of hepatic global DNA methylation in diabetic rats. In cellular DNA methylation reactions, SAM gives a methyl group and is converted to SAH (S-adenosylhomocysteine) which inhibits methyltransferase activity [6]. As consequence, the ratio SAM to SAH is a key factor for DNA methylation mechanism [6]. Furthermore, it was found that dietary polyphenols may affect the methyl pool by giving a methyl group and thus modulating DNMTs activities [29]. Consequently, that prevents epigenetic alterations of DNA methylation [30]. For this reason, it is possible that HEAAC containing bioactive dietary compounds may restore global DNA methylation patterns by increasing the provision of methyl groups. Therefore, HEAAC may prevent epigenetic alterations of DNA methylation with benefits on blood lipid markers and cardiovascular events. However, there is a need to study its impact in the DNA methylation of genes involved in lipid metabolism such as *ABCG1* and with ABCG1 transcripts.

As weight is a predictive parameter for diabetes [31], we assessed the weight of the rats during the experimental period. We found a significant loss of body weight in diabetic group compared to the control. This weight loss was significantly slowed by HEEAC administration. This may be explained by its impact in several pathways related to lipid metabolism processes such as lipolysis [32].

5. Conclusion

The present results demonstrate that hydroethanolic extract of *A. congolensis* has a strong preventive effect on dyslipidemia and subsequent atherogenic risk. An association was equally found between DNA methylation profile and lipid levels especially on the TC and LDL-C. Therefore, HEEAC supplementation by epigenetically modulation of gene expression involved in lipid metabolism might help to prevent or delay the onset of cardiovascular complications in diabetes.

Abbreviations

TG: triglycerides; TC: total cholesterol; HDL-C: high density lipoprotein-cholesterol; LDL-C: low density lipoprotein-cholesterol; sd-LDL: Small dense low-density lipoprotein; SAM: S-Adenosyl-methionine; DNMT: DNA methyltransferase.

Authors' Contributions

Martin Fonkoua: Conceived and designed the experiments; performed the experiments; wrote the paper. Nafissatou

Maboune and David Goda: Designed and performed the experiments. Dany Joel Ngassa Ngoumen: Analyzed and interpreted the data; wrote the paper. Dupon Ambamba and Maxwell Wandji Nguedjo: performed the experiments and wrote the paper. Jules Kamga: performed the experiments. Jean Paul Chedjou and Javeres Leonel Ntepe Mbah: performed the experiments. Fils Ella: Analyzed and interpreted the data; wrote the paper. Guy Roussel Takuissu Nguemto: Conceived the experiments and wrote the paper. Judith Laure Ngondi: Conceived and designed the experiments and wrote the paper.

Availability of Data and Materials

The data used and/or analyzed in this study are available from the corresponding author on reasonable request.

Declarations

Ethics Approval and Consent

The experimental protocol and the maintenance of the laboratory animals were carried out under the standard ethical guidelines for the use of laboratory animals and care as described in the guidelines of the European Community and the Ethics Committee of the Faculty of Science of the University of Yaounde I.

Competing Interests

The authors declare that they have no competing interests.

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