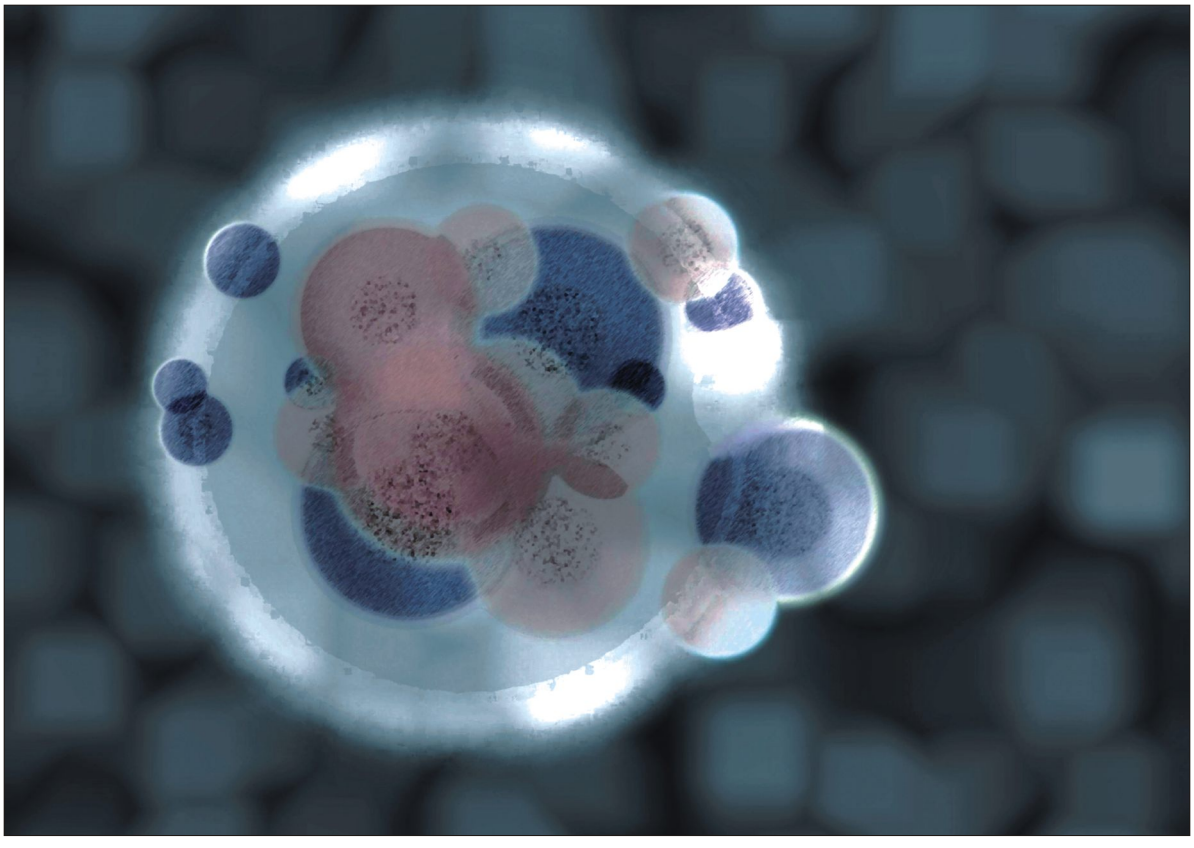

Journal of Biological Sciences

ISSN 1727-3048

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OPEN ACCESS Journal of Biological Sciences

ISSN 1727-3048
DOI: 10.3923/jbs.2021.1.9

Research Article
Insulinomimetic Activity Assessment of Antidiabetic Plants Used in Togolese Pharmacopoeia, *in ovo* and *ex vivo* Study

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Abstract

Background and Objective: *Pterocarpus erinaceus* (Fabaceae), *Amaranthus spinosus* (Amaranthaceae) and *Anogeissus leiocarpus* (Combretaceae) are plants used in the treatment of diabetes many times. The present study was undertaken to observe the insulinomimetic activity of a different type of plants. **Materials and Methods:** The hypoglycemic effect of hydroalcoholic extracts from the stem bark of *P. erinaceus* and the roots of *A. spinosus* and *A. leiocarpus* was evaluated *in ovo* on 11 days-old chicken embryos and *ex vivo* on skeletal muscles isolated from rats. **Results:** All extracts reduced significantly the chicken embryos basal glycemia in the treated groups compared to the control group at the doses of 0.03 and 0.06 mg/100 µL. The extract of *A. leiocarpus* (0.5 and 1 mg mL^{-1}) increased ($p<0.001$ and $p<0.0001$) the absorption of glucose by the muscle in the presence and absence of insulin more than the extracts of *Pterocarpus erinaceus* and *Amaranthus spinosus*. **Conclusion:** Hence, it was concluded that, the three extracts decreased the basal glycemia *in ovo*. Whereas the extract of *A. leiocarpus* has an effect, similar to an insulinomimetic effect.

Key words: *Pterocarpus erinaceus*, *Amaranthus spinosus*, *Anogeissus leiocarpus*, *in ovo*, hypoglycemic activity, absorption of glucose, isolated muscle

Citation: Lawson-Evi Povi, A.E. Motto, K. Atchou, K. Tona, K. Eklu-Gadegbeku and K. Aklikokou, 2021. Insulinomimetic activity assessment of antidiabetic plants used in togolese pharmacopoeia, *in ovo* and *ex vivo* study. J. Biol. Sci., 21: 1-9.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Diabetes is a metabolic disease characterized by chronic hyperglycemia resulting from a relative or absolute defect in the secretion of insulin and/or the action of this hormone at the peripheral tissue[1,2]. It is a major public health problem that is constantly growing. Diabetes can cause multiple complications such as blindness, stroke, kidney failure and even amputation this increases the risk of premature death[3].

Several drugs with different modes of action are now available to manage diabetes, including type 2 which is more common (90-95%)[4]. But at an advanced stage of the disease, the pancreas becomes exhausted and the secretion of insulin becomes scarce, which leads to insulin therapy[5]. Indeed, insulin plays an essential role in the regulation of hyperglycemia. In peripheral tissues such as muscle and adipose tissue, insulin causes glucose uptake by translocation of GLUT4 transporters. In the event of type 2 diabetes, the tissues become resistant to insulin thus preventing the translocation of GLUT4 and the uptake of glucose[6]. Particular attention is therefore paid to the search for new substances with insulin-mimetic action through direct tests on the translocation of GLUT4 or the absorption of glucose by muscle and adipose tissue. In this regard, the insulin-mimetic properties of plant extracts and compounds have been reported by several authors[7]. These compounds, such as the ethanolic extract of Aerva lanata, induce glucose uptake by isolated skeletal muscle in the presence or absence of insulin[8].

Pterocarpus erinaceus, *Amaranthus spinosus* and *Anogeissus leiocarpus* are three plants from the Togolese pharmacopeia traditionally used to treat diabetes. Previous Study had shown a hypoglycemic activity of the methanol extract of *Amaranthus spinosus* comparable to that of glibenclamide (the reference drug). At the dose of 400 mg kg^{-1}, *A. spinosus* extracts reduced significantly the hyperglycemia in oral glucose tolerance test and reduce basal blood glucose comparable to that of the reference drug[9]. This implies the pancreatic action of the extracts of *A. spinosus* by provoking the secretion of insulin, the only hypoglycemic hormone known to date. As for *Anogeissus leiocarpus*, work had shown an antihyperglycemic activity of the extract, comparable to that of metformin. Administered at a dose of 500 mg kg^{-1}, the hydroalcoholic extract of *A. leiocarpus* lowered hyperglycemia in ICR mice with carbohydrate overload but did not show any significant effect on basal glycemia; the same result was obtained with the reference drug metformin used at a dose of 100 mg kg^{-1} [10]. This supposes an extrapancreatic action of this plant. The present study was undertaken in order to evaluate the insulinomimetic action of plants with pancreatic and extrapancreatic activities.

MATERIALS AND METHODS

Study area: The study was carried out from December, 2019 to January, 2020.

Storage and selection of eggs: Healthy Sasso chicken eggs from the Center of Excellence and Research in Avian Science (CERSA, Togo) were incubated at a temperature of 37.7°C with a humidity of 40-60% and a CO_2 saturation of 0.08% for 10 days in a rotary "Pass Reform" brand incubator (North America) whose automatic turning (90°angle) is done every hour. On the 11th day, all the eggs were checked by candling in order to choose those containing viable embryos (fertile eggs).

Animals: Sprague Dawley rats weighing between 160 and 250 g from the animal facility of the Department of Animal Physiology of the Faculty of Science were used. These animals were kept in an environment subject to relative humidity, to a dark/light cycle of 12/12 hrs and at a temperature between 22 and 25°C. The animals were deprived of both food and water during the experiment. Principles of laboratory animal care as described in institutional guidelines and ethics of Laboratory of Physiology/Pharmacology of University of Lome-Togo (ref: 001/2012/ CB-FDS-UL) were followed.

Plant materials: Stem bark of *Pterocarpus erinaceus* Poir., roots of *Amaranthus spinosus* L. and of *Anogeissus leiocarpus* were used as plant material. *Pterocarpus erinaceus* stem barks were collected in Tovegan area, a village located on the road to Kpalime about 80 km from Lome city (Togo); *A. spinosus* roots were collected in Dzagble (Lome) and *A. leiocarpus* roots were collected from Tsévié, Zio (TOGO). The plants have been identified in Botany and Plant Ecology Laboratory of Faculty of Science (University of Lome) where voucher specimen was deposited in the herbarium under the number: 15515 TOGO (*P. erinaceus*),15516 TOGO (*A. spinosus*) and 15483 TOGO (*A. leiocarpus*). The stem bark and the roots were cleaned out with water, cut into small pieces, dried at the laboratory of Animal Physiology at 22°C and then reduced to powder.

Extraction: About 400 g of roots (*A. spinosus*, *A. leiocarpus*) and stem bark (*P. erinaceus*) were extracted in water/ethanol (5:5) for 72 hrs. The crude extract was filtered on Whatman paper and evaporated in vacuum at 45°C using a rotary evaporator (Buchi R120). The resulting dry extracts were collected in a sterile glass vial and placed in a desiccator for 24 hrs and then stored in the refrigerator at a temperature of 4-8°C.

Preparation of solutions: The buffer solutions were prepared as follows:

- For 1 L of PBS (Phosphate Buffer Saline) solution, 8 g of NaCl; 0.2 g KCl; 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4 were put in 800 mL of distilled water. The mixture was adjusted with distilled water to obtain 1000 mL
- In 800 mL of distilled water, 8 g of NaCl; 400 mg KCl; 140 mg $CaCl_2$; 100 mg $MgCl_2$; 100 mg $MgSO_4$; 60 mg Na_2HPO_4, 60 mg KH_2PO_4; 1 g of glucose and 350 mg of $NaHCO_3$ were added to obtain Hank's Balanced Salt Solution (HBSS)
- One liter of KRB (Kreb's-Ringer bicarbonate buffer) was prepared with 118 mM NaCl, 5 mM KCl, 1.28 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$ and 25 mM $NaHCO_3$

Toxicity study of the extracts on the chicken embryos: The toxicity test based on a long-term incubation in the presence of the substance for about 24 hrs, or even until hatching, has been undertaken. The hydroalcoholic extract of *P. erinaceus*, *A. spinosus* and *A. leiocarpus* at the doses of 0.03 and 0.06 mg/100 µL, were previously diluted in HBSS buffer and their injection was made using syringes. Eight groups of 35 fertile 10-day eggs (D10) were made to assess the toxicity of the extracts on chicken embryos:

- Group 1-4: Test groups, received the solutions of extracts of the stem bark of *P. erinaceus* and of the roots of *A. spinosus* at concentrations of 0.03 mg/100 µL and 0.06 mg/100 µL, respectively
- Group 5 and 6: Test groups, received the solutions of extracts of the roots of *A. leiocarpus* at concentrations of 0.03 and 0.06 mg/100 µL, respectively
- Group 7: Control group, received HBSS (Hanks balanced salt solution) which was the vehicle solution extracts
- Group 8: Reference lot, consists of normal eggs (NI) which have not received any injection of substance

Twenty-four hours after injection of the substances (D11), 10 eggs from each group were broken and macroscopic observations were made on the state of the blood vessels of the embryos and on any lesions and vitality. The weight of the albumen, the embryos and their vitality were determined. The 25 eggs of each remaining group were reincubated until hatching. Malformations were looked for in chicks then hatch and death rates were determined as followed[11]:

$$\text{Hatching rate} = \frac{\text{Number of chicks}}{\text{Number of eggs hatched}} \times 100$$

$$\text{Mortality rate} = \frac{\text{Number of deaths}}{\text{Number of eggs hatched}} \times 100$$

Effect of the extracts on blood glucose level of the chicken embryos: The extracts were previously diluted in HBSS buffer and their injection was made using syringes. Eleven groups of 10 eggs were distributed as follows:

- Group 1: Untreated eggs, as the normal control group
- Group 2: Eggs receiving HBSS buffer
- Groups 3 and 4: Eggs receiving respectively 0.03 and 0.06 mg/100 µL of *A. leiocarpus*
- Group 5: Eggs receiving metformin at 0.01 mg/100 µL
- Group 6: Eggs receiving 3.3 U mL^{-1} insulin
- Groups 7 and 8: Eggs receiving respectively 0.03 and 0.06 mg/100 µL of *P. erinaceus*
- Groups 9 and 10: Eggs receiving respectively 0.03 and 0.06 mg/100 µL of *A. spinosus*
- Group 11: Eggs receiving 0.06 µg/100µL of glibenclamide

Blood glucose level were measured according to the method of Haselgrübler[12]. Briefly, the large vessel from the abdomen of the embryo was carefully cut and the blood was measured using an ACCU-CHEK Active (Germany) glucometer after 60, 120 and 180 min of incubation.

Effect of the extracts on uptake of glucose in rat isolated muscle tissue: The previous[13] method with some modifications was adopted. After 18 hrs of fasting, the rats were euthanized by cervical dislocation and the muscles of the femur were exposed, cut into small pieces of 250 mg and pre-incubated in aerated (95% O_2 and 5% CO_2) KRB solution at 37°C for 10 min. At the start of handling, the KRB solution was replaced by KRB containing 11.1 mM of glucose (KRB-G) in all test tubes. Eighteen groups (in triplets) were made up as follows:

- Group 1: Tissue+KRB-G, represent the control group
- Group 2: Tissue+KRB-G+ insulin (100 mU L^{-1})
- Groups 3 and 4: Tissue+KRB-G+ *P. erinaceus* at 0.5 mg mL^{-1} with and without insulin (100 mU L^{-1}), respectively
- Groups 5 and 6: Tissue+KRB-G+ *P. erinaceus* at 1 mg mL^{-1} with and without insulin (100 mU L^{-1}), respectively
- Groups 7 and 8: Tissue+KRB-G+ *A. spinosus* at 0.5 mg mL^{-1} with and without insulin (100 mU L^{-1}), respectively
- Groups 9 and 10: Tissue+KRB-G+ *A. spinosus* at 1 mg mL^{-1} with and without insulin (100 mU L^{-1}), respectively

- Groups 11 and 12: Tissue+KRB-G+glibenclamide at 0.6 µg mL^{-1} with and without insulin (100 mU L^{-1}), respectively
- Groups 13 and 14: Tissue+KRB-G+ *A. leiocarpus* at 0.5 mg mL^{-1} with and without insulin (100 mU L^{-1}), respectively
- Groups 15 and 16: Tissue+KRB-G+ *A. leiocarpus* at 1 mg mL^{-1} with and without insulin (100 mU L^{-1}), respectively
- Groups 17 and 18: Tissue+KRB-G+metformin at 0.1 mg mL^{-1} with and without insulin (100 mU L^{-1}), respectively

The test tubes were incubated in aerated condition for 3 hrs. Aliquots of 10 µL were prelevated from incubation solution at 60, 120 and 180 min and glucose concentration were measured using an enzymatic-colorimetric test, GOD-POD liquid (Cypress diagnostic, Belgium).

The loss of glucose in the incubation solution is assumed to represent the glucose absorbed by the muscle:

$$\text{Glucose absorbed} = \frac{\text{Initial glucose-Final glucose}}{0.25}$$

where, 0.25 represents the fresh weight of the muscle tissue. The glucose absorbed was expressed in mM g^{-1} of fresh tissue.

Statistical analysis: Data were expressed as Mean±SEM (standard error of the Mean) using the GraphPad Prism 6 software. Statistical differences between treated groups and controls were determined by 2-ways ANOVA (analysis of variance) and considered significant for $p<0.05$.

RESULTS

Effect of the extracts on the relative weight of chicken embryos and albumen and on the hatching: Twenty four hours after the injection of the substances, the viability of chicken embryos was 90% for all the groups. Figure 1a showed no significant difference in the relative weight of embryos between the control group and those who received the vehicle (HBSS) and the extract of *Pterocarpus erinaceus*. No significant difference in the weight of the embryos was observed with the extract of *Amaranthus spinosus* (Fig. 1b) and no significant difference in the weight of the embryos was observed with the extract of *Anogeissus leiocarpus* (Fig. 1c).

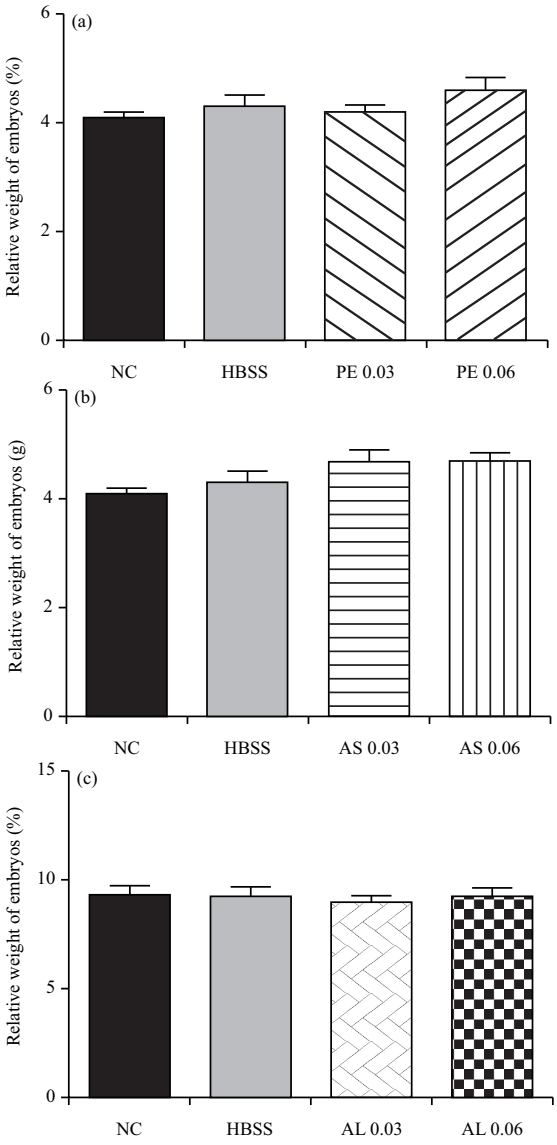

Fig. 1(a-c): Effect of (a) *P. erinaceus*, (b) *A. spinosus* and (c) *A. leocarpus* extract on the relative weight of embryos

The chicken embryos were weighed on day D = 11 after 24 hrs of injection of the test substances. NC: Normal control (without injection), HBSS: Control who received HBSS, PE and AS 0.03 and 0.06: Groups treated respectively with 0.03 and 0.06 mg/100 µL of extracts of *P. erinaceus* and *A. spinosus*. AL 0.03 and 0.06: Groups treated respectively with 0.03 and 0.06 mg/100 µL of extracts of *A. leocarpus*, HBSS: Hanks balanced salt solution. Data were expressed as Mean±SEM, N = 10

Effect of the extracts on the relative weight of chicken albumen: The effect of the different extracts on the relative weight of chicken albumen were represented by Fig. 2. Figure 2a showed no significant difference in the relative weight of albumen between the control groups and groups

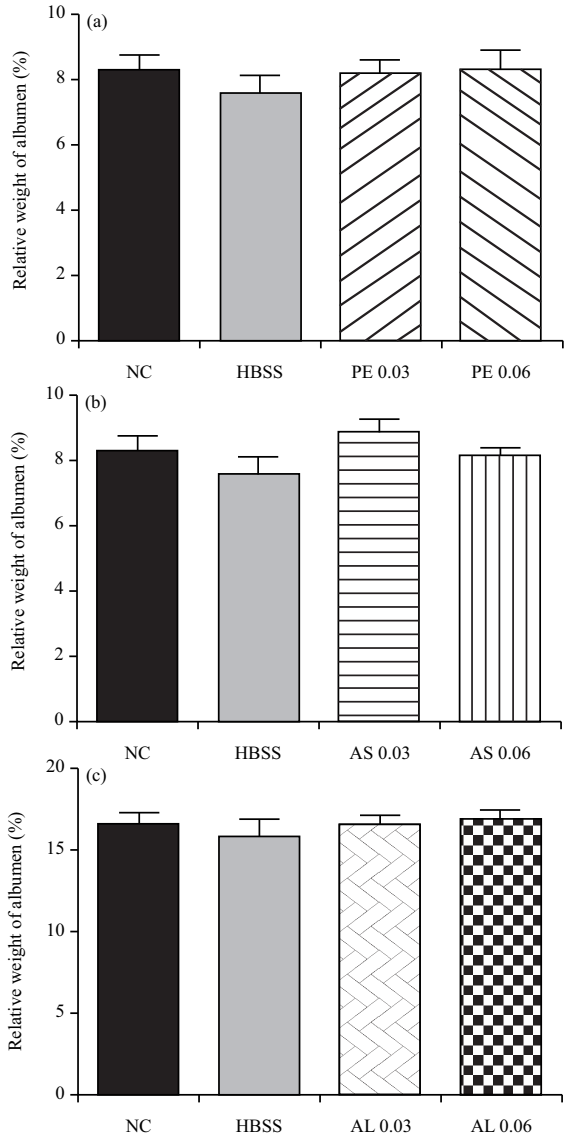

Fig. 2(a-c): Effect of (a) *P. erinaceus*, (b) *A. spinosus* and (c) *A. leocarpus* extract on the relative weight of albumen

Egg albumins were weighed on day D: 11 after 24 hrs of injection of the test substances. NC: Normal control (without injection), HBSS: Control who received HBSS, PE and AS 0.03 and 0.06: Groups treated respectively with 0.03 and 0.06 mg/100 µL of extracts of *P. erinaceus* and *A. spinosus*. AL 0.03 and 0.06: Groups treated respectively with 0.03 and 0.06 mg/100 µL of extracts of *A. leocarpus*, HBSS: Hanks balanced salt solution. Data were expressed as Mean±SEM, N = 10

treated with *Pterocarpus erinaceus*. The extract of *Amaranthus spinosus* had no effect in the relative weight of albumen compared to the control (Fig. 2b) and *Anogeissus leiocarpus* at the doses of 0.03 and 0.06 mg/100 µL had no significant effect either in the relative weight of the albumen compared to the control (Fig. 2c).

Effect of the extracts on the hatching: The effect of the extracts on the hatching rate and the mortality rate was reported in Table 1. Not all of the eggs managed to hatch; the highest hatching rate (100%) was found in the normal control group and the group treated with the extract of *A. leiocarpus* at 0.03 and 0.06 mg/100 µL. No significant difference was recorded between the hatching rate of the eggs having received the extracts compared to the normal control and HBSS control groups.

With regard to mortality, the deaths were recorded in the groups treated with extracts of *P. erinaceus* and *A. spinosus* and that which received HBSS as descripted in Table 1.

Hypoglycemic activity of the extracts *in ovo*: Figure 3 represents the hypoglycemic activity of the extracts tested *in ovo*. The blood glucose level of chicken embryos was measured over 180 min at different time intervals: t60, t120 and t180. The HBSS used as a negative control did not give any significant decrease in blood glucose level over the 180 min.

Figure 3a showed a significant reduction in blood glucose level of chicken embryos with extracts of *A. spinosus* at 0.03 and 0.06 mg μL^{-1} after 180 min of incubation compared to the HBSS control group. Regarding the *P. erinaceus* extract, only the dose of 0.06 mg/100 µL significantly reduced the blood glucose level; an action comparable to that of glibenclamide and insulin, which had induced continuously (over the 180 min) a significant ($p<0.0001$) decrease in the blood glucose level (Fig. 3b).

As shown on Fig. 3c, 60 min after the injection of the *A. leiocarpus* extract (0.03 and 0.06 mg μL^{-1}) into the embryos, there was a significant decrease ($p<0.0001$) in blood glucose level (respectively, 106.00±1.94 and 107.71±1.76 mg dL^{-1} for AL 0.03 and AL 0.06) compared to the HBSS control group (122.00±1.72 mg dL^{-1}). This decrease of the blood glucose level continued at 120 min and returned to normal 180 min after the injection; an effect comparable to that of metformin.

Effect of the extracts on glucose uptake in rat skeletal muscle: Table 2 showed that in the presence of insulin, the glucose absorbed by the muscle tissue increased significantly ($p<0.05$; $p<0.01$) with the extract of *P. erinaceus* (at 1 mg mL^{-1}) after 60 min of incubation (11.40±3.00 mM g^{-1} of fresh tissue) compared to the control group (2.40±0.00 mM g^{-1} of fresh tissue). No significant effect on glucose absorbed was noted with the extract of *A. spinosus* (at 0.5 and 1 mg mL^{-1}) and glibenclamide.

Table 1: Effect of the extracts of *P. erinaceus*, *A. spinosus* and of *A. leiocarpus* on the hatching

	Number of eggs hatched	Number of chicks	Number of deaths	Hatching rate (%)	Mortality rate (%)
NC	25	23	0	92	0
HBSS	25	24	1	96	4
PE 0.03	25	21	2	84	8
PE 0.06	25	20	1	80	4
AS 0.03	25	20	1	80	4
AS 0.06	25	20	2	80	8
AL 0.03	25	25	0	100	0
AL 0.06	25	25	0	100	0

Treated eggs were incubated for 21 days, NC: Normal control group, HBSS: Control with HBSS injection, PE 0.03 and PE 0.06: Groups treated with 0.03 and 0.06 mg/100 µL of *P. erinaceus*, respectively. AS 0.03 and AS 0.06: Groups treated with 0.03 and 0.06 mg/100 µL of *A. spinosus*, respectively. AL 0.03 and AL 0.06: Groups treated with 0.03 and 0.06 mg/100 µL of *A. leiocarpus*, respectively. Hatching rate: (Number of chicks)×100/(Number of eggs hatched) and Mortality rate: (Number of chicks died)×100/(Number of eggs hatched). The results represent the Mean±SEM

Table 2: Effect of total extract of *Pterocarpus erinaceus* and of *Amaranthus spinosus* on the absorption of glucose in the skeletal muscle

	Glucose absorbed by the muscle tissue (mM g^{-1} of fresh tissue)		
Groups	60 min	120 min	180 min
Glucosed medium (GM)	2.40±0.00	9.40±1.00	11.40±1.00
GM+Insulin (100 mU L^{-1})	2.40±0.00	7.40±0.99	13.40±1.00
GM+PE (0.5 mg mL^{-1})	6.40±2.00	7.40±0.99	9.40±1.00
GM+PE (0.5 mg mL^{-1}) + insulin (100 mU L^{-1})	3.40±1.00	8.40±0.00	8.40±2.00
GM+PE (1 mg mL^{-1})	5.40±1.00	12.40±2.00	12.40±2.00
GM+PE (1 mg mL^{-1}) + insulin (100 mU L^{-1})	11.40±3.00**	17.40±3.00*	18.40±2.00*
GM+AS (0.5 mg mL^{-1})	2.40±0.00	5.40±1.00	7.40±0.99
GM+AS (0.5 mg mL^{-1}) + insulin (100 mU L^{-1})	5.40±1.00	7.40±0.99	8.40±2.00
GM+AS (1 mg mL^{-1})	3.40±1.00	10.40±4.00	10.40±0.00
GM+AS (1 mg mL^{-1}) + insulin (100 mU L^{-1})	8.40±2.00	10.40±0.00	11.40 1.00
GM+glib (0.6 µg mL^{-1})	6.40±0.00	13.40±1.00	8.40±2.00
GM+glib (0.6 µg mL^{-1}) + insulin (100 mU L^{-1})	6.40±0.99	15.40±1.00	12.40±4.00

Glucose absorbed: (Gli-Glf)/0.25 g, Gli: Initial glucose, Glf: Final glucose, 0.25 g: Weight of muscle tissue, the skeletal muscle tissue were incubated in a Kreb's-Ringer bicarbonate buffer solution containing 11.1 mM glucose at 37°C. GM: Glucose medium, PE: *Pterocarpus erinaceus,* AS: *Amaranthus spinosus*, Glib: Glibenclamide. The results represent the Mean±SEM, N = 3. *p <0.05, **p<0.01, compared to controls

Table 3: Effect of total extract of *Anogeissus leiocarpus* on glucose absorption in skeletal muscle

	Glucose absorbed by the muscle tissue (mM/g of fresh tissue)		
Groups	60 min	120 min	180 min
Glucosed Medium (GM)	2.40±0.00	9.40±1.00	11.40±1.00
GM+Insulin (100 mU L^{-1})	2.40±000	7.40±0.99	13.40±1.00
GM+AL (0.5 mg mL^{-1})	13.40±1.00***	14.90±1.50	19.40±1.00*
GM+AL (0.5 mg mL^{-1}) + insulin (100 mU L^{-1})	15.40±1.00****	17.40±3.00*	23.40±1.00***
GM+AL (1 mg mL^{-1})	19.40±1.00****	23.40±1.00****	27.40±1.00****
GM+AL (1 mg mL^{-1}) + insulin (100 mU L^{-1})	24.20±1.80****	27.40±1.00****	29.40±1.00****
GM+Met (0.2 mg mL^{-1})	5.40±3.00	10.40±2.00	10.40±0.00
GM+Met (0.2 mg mL^{-1}) + insulin (100 mU L^{-1})	8.40±2.00	12.40±2.00	12.40±4.00

Glucose absorbed: (Gli-Glf)/0.25 g, Gli: Initial glucose, Glf: Final glucose, 0.25 g: Weight of muscle tissue. The skeletal muscle tissue were incubated in a Kreb's-Ringer bicarbonate buffer solution containing 11.1 mM glucose at 37°C. GM: Glucosed medium, AL: *Anogeissus leiocarpus*, Met: Metformin. The results represent the Mean±SEM, N = 3, *p<0.05, ***p<0.001, ****p<0.0001 compared to controls

As shown in Table 3, *A. leiocarpus* extract at 0.5 and 1 mg mL^{-1} induced a significant increase (respectively 13.40±1.00 and 19.40±1.00 mM g^{-1} of fresh tissue) in glucose absorbed compared to the control group (2.40±0.00 mM g^{-1} of fresh tissue) after 60 min of incubation. In the presence of insulin (at 100 mU L^{-1}) the glucose absorbed by the muscle tissue was 15.40±1.00 and 24.20±1.80 mM g^{-1} of fresh tissue respectively, with *A. leiocarpus* extract at 0.5 and 1 mg mL^{-1} compared to the control group (2.40±000 mM g^{-1} of fresh tissue) after 60 min of incubation. Metformin (0.2 mg mL^{-1}) had no significant effect on the absorption of glucose by the muscle tissue.

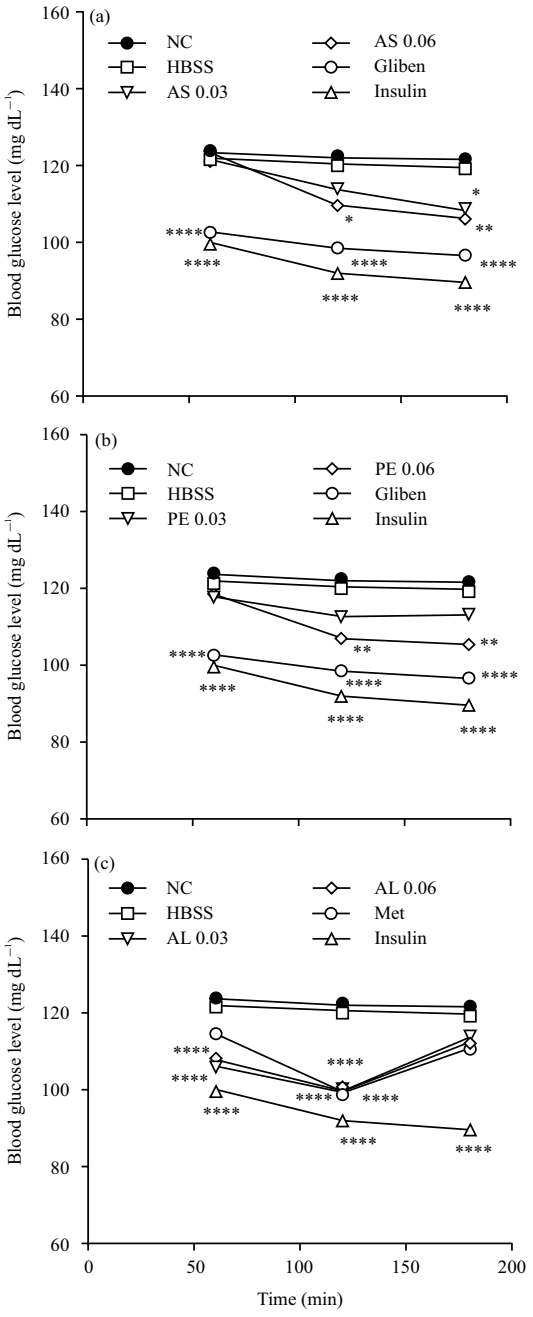

Fig. 3(a-c): Effect of (a) *P. erinaceus*, (b) *A. spinosus* and (c) *A. leocarpus* extract on basal blood glucose level of embryos

The glycaemia was measured from the blood of chicken embryos on day D: 10 at time t = 60, 120 and 180 min after injection of the tested solutions. NT: Control without injection/healthy eggs, HBSS: Witness who received HBSS, PE and AS 0.03 and 0.06: Groups treated respectively with 0.03 and 0.06 mg/100 µL of extracts of *P. erinaceus* and *A. spinosus*, Gliben: Group treated with Glibenclamide 0.06 µg/100 µL, Met: Group treated with metformine, Insulin: Group treated with insulin 3.3 IU mL^{-1}. Data were expressed as average±error. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. The data were compared to the healthy control (NT). N: 10, HBSS: Hanks balanced salt solution

DISCUSSION

The *in ovo* test on chicken embryos was set up[12] and is a modified version of the HET-CAM test (chorioallantoic membrane test of chicken eggs)[14]. *In ovo* studies carried out on chicken embryos have been reported by several authors[15,16]. Indeed, the developing chicken embryo is one of the living systems most used in biological, pharmacological and toxicological research because it constitutes a useful method for studying the toxic potential of a new compound[16]. In addition, the embryo of chicken constitutes a model used in the study of the role of insulin and its growth factor IGF-I (Insulin Growth Factor I) during embryonic development[15,17]. By day 11, serum insulin levels in the embryos are practically nonexistent; however, the embryo at this stage of development is sensitive to the effect of insulin because it has insulin receptors[18,19].

The toxicity test of *Pterocarpus erinaceus*, *Amaranthus spinosus* and *Anogeissus leiocarpus* extracts *in ovo* revealed no morphological change (infirmities, malformations) or gross lesions in the embryos at doses of 0.03 and 0.06 mg/100 µL. No significant difference was observed in the relative weight of embryos and chicken albumen between the control group (HBSS) and those received the extracts. At hatching, no significant difference was recorded between the hatching rate of the eggs having received the extracts compared to the normal control and HBSS control groups. With regard to mortality, the deaths were recorded in the groups treated with extracts of *P. erinaceus* and *A. spinosus* and that which received HBSS. However, the difference between the chick mortality remained insignificant compared to the normal control group. The results obtained showed that the extracts at the doses of 0.03 and 0.06 mg/100 µL had no toxic effect on the embryos from 11 days until the hatching of the eggs.

The hypoglycemic activity of the extracts has been revealed through their effect on the basal blood glucose level of embryos. The results showed that the extract of *P. erinaceus* and *A. spinosus* induced (especially at the dose of 0.06 mg/100 µL) a significant decrease ($p<0.05$; $p<0.01$) in basal glycemia after two hours of administration, compared to the control group. This effect was comparable to that of the reference drug glibenclamide and that of insulin. As for the *A. leiocarpus* extract, a significant decrease ($p<0.0001$) in the basal glycemia was noticed after one hour compared to the HBSS control lot. This decrease in basal glycemia continues at 120 min (respectively, 99.85±3.21 and 99.71±2.30 mg dL^{-1} for AL 0.03 and AL 0.06) and returns to normal 180 min after the injection. The same result was obtained with the reference drug, metformin. The mechanism of action of the two

reference drugs was long time studied. It is well known that glibenclamide, an oral antidiabetic from the sulphonylurea family, has a pancreatic action. It is a hypoglycemic drug that appears to decrease blood glucose acutely by stimulating the release of insulin from the pancreas, an effect dependent on the presence of beta cells in the pancreatic islets[20]. The result obtained supposes that both glibenclamide and the extracts of *P. erinaceus* and *A. spinosus* potentiated the secretion of insulin by the pancreas allowing the glucose used by the organs in formation. The metformin, from the biguanide family, is a normoglycemic drug which has a multifactorial mode of action, by improving insulin sensitivity at the peripheral level (increased glucose use) or by decreasing hepatic glucose production[21]. The activity of the extract of *A. leiocarpus* could be explained by the fact that the extract as well as the metformin have transiently lowered the basal glycemia of the embryo, before the glucagon which maintains in birds a basal blood glucose level high, does not bring this blood glucose back to normal. In this second case, the extract may be like insulin, have allowed the expression of glucose transporters on the surface of cell membranes allowing the glucose uptake by the tissue[21]. This result led us to use an *ex vivo* model expressing insulin-dependant glucose transporters, the rat skeletal muscle.

Skeletal muscle represents a significant total body mass and appears to be one of the most important target tissues for insulin in the absorption of glucose at the peripheral level[22]. In fact, insulin stimulates the transport of glucose in the target tissues by triggering the translocation of the glucose transporter GLUT4, towards the plasma membrane. Insulin resistance, the major abnormality of type 2 diabetes, leads to a decrease in the translocation efficiency of GLUT4. Particular attention is therefore paid to the search for compounds capable of improving this translocation process in the absence of insulin; these are insulin-mimetics compounds. Using fluorescence microscopy (total internal reflection, TIRF) to quantify the translocation of GLUT4 in CHO-K1 cells (cells highly sensitive to insulin), researchers were able to demonstrate the modulating properties of plant extracts on the translocation of GLUT4 in the presence of glucose and thus identified new insulin-mimetic substances[23].

The glucose absorption test in the isolated skeletal muscle of rats was carried out. The effect of the extracts of *P. erinaceus*, *A. spinosus* and of *A. leiocarpus* on glucose absorption was studied by measuring the decrease in the molar concentration of glucose in the incubation medium every hour, for three hours. *Ex vivo* (without any pancreatic activity), only the total extract of *A. leiocarpus* (0.5 and 1 mg mL^{-1}) has been found to significantly increase the absorption of glucose from the muscle, both in the absence and in the presence of insulin; higher absorption in the presence of insulin (90.08, 65.69 and 61.22% at 60, 120 and 180 min, respectively) than without insulin (87.62, 59.82 and 58.39% at 60, 120 and 180 min, respectively). Therefore, the extract of *A. leiocarpus* would act directly alone or in concert with insulin in the absorption of glucose from the muscles. The same observations have been made by authors who have shown that when combined with insulin, the extract significantly increase the absorption of glucose in isolated skeletal muscles[24,25]. *In vivo*, an increase in the plasma insulin concentration leads to the translocation of GLUT4 by the phosphatidylinositol 3-kinase signaling pathway and consequently, the uptake of blood glucose by skeletal muscle. The extract of this fact would firstly improve the sensitivity of the muscle to the action of insulin by stimulating the glucose capture pathways and mechanisms[26] and on the other hand would mobilize the glucose transporters on the surface of the cell membrane in the absence of insulin through insulin-mimetic activity. As for the *P. erinaceus* and *A. spinosus* extracts, their activities on glucose absorption by the muscle were very low. Only the extract of *P. erinaceus* (1 mg kg^{-1}) in the presence of insulin-induced a significant increase ($p<0.05$ and $p<0.01$) in the absorption of glucose by the muscle. This suggests that the extract cannot exert its hypoglycemic activity in the absence of insulin.

The results of this study on the uptake of glucose from skeletal muscles isolated from rats confirm the study *in ovo*.

CONCLUSION

It emerges from this study that the hydroalcoholic extracts of the stem bark of *P. erinaceus* and *A. spinosus* and the roots of *A. leiocarpus* have a hypoglycemic effect *in vivo* and this effect with regard to the extract of *A. leiocarpus* would be probably due to insulin-mimetic action. However, other mechanisms using the fluorescence microscopy to quantify the translocation of GLUT4 or using the inhibitor of GLUT4 like HIV protease inhibitors must be undertaken in order to confirm this action of the plant.

SIGNIFICANCE STATEMENT

This study discovered the insulin-mimetic action of *Pterocarpus erinaceus*, *Amaranthus spinosus* and *Anogeissus leiocarpus* that can be beneficial in the treatment of diabetes. This study will help researchers uncover critical areas of anti-diabetic activity of plant extracts that many researchers were unable to explore. Thus, a new theory on drugs with the insulin-mimetic action not dependent of insulin can be found.

ACKNOWLEDGMENT

The authors thank CERSA (Center of Excellence and Research in Avian Science) for the material support.

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