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ORIGINAL ARTICLE

Hypoglycaemic activity of ethanolic leaf extract and fractions of *Holarrhena floribunda* (Apocynaceae)

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Holarrhena floribunda **is a common plant that has traditionally been used in Africa to treat many diseases such as fever, dysentery, sterility and diabetes. This study was set out to evaluate the hypoglycaemic properties of ethanolic leaf extract of *Holarrhena floribunda* and various fractions of this extract in normal fasted and fed-hyperglycaemic rats. Blood glucose levels (g L^{-1}) were determined at the following times: 1) after a 12 hours period prior to drug administration, 2) An hour after the oral administration of the extract (250-1000 mg kg^{-1}), its fractions (1000 mg kg^{-1}), Glibenclamide (10 mg kg^{-1}) or the vehicle and 3) one and four hours after the oral overload of anhydrous glucose (4 g kg^{-1} body weight). The extract showed a remarkable dose-dependent down-regulation of blood glucose in fasted rats at 1000 mg kg^{-1} ($p<0.05$) and significantly reduced or totally prevented the induction of hyperglycaemia at 500 and 1000 mg kg^{-1} respectively. This Glibenclamide-like hypoglycaemic activity of the extract was found to be present in the dichloromethane and ethyl acetate fractions of the plant. Our results show that the leaves of *H. floribunda* possess hypoglycaemic properties and strongly suggest that its usage in traditional and to a larger extent orthodox medicine may be fully explored.**
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INTRODUCTION

Diabetes mellitus (DM) is a metabolic disorder characterised by hyperglycaemia resulting from a deficit or malfunction in insulin secretion and/or insulin action, both of which cause the impaired metabolism of glucose, lipids and proteins (Gao *et al.,* 2010). The chronic hyperglycaemia of diabetes is associated with the long term damage, dysfunction and failure of various organs (Lyra *et al.,* 2006). It is the leading cause of kidney failure, heart attack, blindness and lower limb amputation and the fourth main cause of death in most developed countries (Eseyin, 2010).

DM, an epidemic occurring in adults throughout the world and affecting more than 4% of the population worldwide (Kim *et al.,* 2006, Eseyin, 2010), is a major public health problem. Its prevalence in Africa was predicted to increase by 93% in the last 15 years (IDF, 2003). There are no recent data on the prevalence of diabetes in Côte d'Ivoire, but the prevalence was estimated to be 3 - 7% in Abidjan, the main city of the country (Lokrou et *al.* 1986; Sobngwi *et al.*, 2002).

One of the strategies in the treatment of DM is based on the use of oral hypoglycaemic agents such as biguanides and sulfonylureas, or insulin to lower blood glucose levels to normal ranges (Teves

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et al., 2004; Kennan *et al.,* 2005). The search for new agents with lower cost and better efficiency has therefore become a matter of major priority. The great number of plants used to manage diabetic patients in Africa might provide a useful source for the discovery of new compounds that can be used as pharmaceutical entities or simple dietary adjuncts to existing therapies (Hostettmann *et al.,* 2000; Tra Bi *et al.,* 2008). *Holarrhena floribunda* (G. Don) Dur. et Schinz (Apocynaceae), commonly called false rubber tree, is a tropical tree that grows to 17 meters high and 1 meter in girth in the deciduous forest and savannah woodland. It is widely distributed in Côte d'Ivoire, Burkina Faso, Mali and some other parts of West Africa. In these regions, the stem-bark and leaves are used to treat various afflictions such as malaria, fever, dysentery, amoebic diseases, diarrhoea, sterility, amenorrhea and diabetes (Arbonnier, 2002; Fotie *et al.,* 2006; Bayala *et al.,* 2006).

To date, there is paucity of data from studies on anti -diabetic properties of *Holarrhena floribunda.* From the World Health Organization recommendation for the evaluation of the real potential of medicinal plants (WHO, 1980) due to their poor scientific scrutiny and justification for traditional use, the present investigation sought to determine the hypoglycaemic activity of crude ethanolic leaf extract of *Holarrhena floribunda* and its various fractions in normoglycaemic rats.

MATERIALS AND METHODS

Plant collection

The leaves of *Holarrhena floribunda* (G. Don) Dur. et Schinz (Apocynaceae) were collected in Abidjan (the southern region of Côte d'Ivoire) in October 2007. The plant was identified and authenticated by Professor Aké-Assi Laurent, National Floristic Centre of the University of Cocody, Abidjan. A voucher specimen (n°13240) of the plant was deposited in the herbarium of the National Floristic Centre of the University of Cocody, Abidjan.

Preparation of extract and fractions

The leaves of *H. floribunda* were cleaned, washed with water, sliced into small pieces, air dried at ambient temperature for two weeks and then ground into powder using a cutting mill (Retsch SM 100-1390 rev/min, Labo and Co, France). The powder (100 g) was extracted with 2 litres of a solution of ethanol (96%)/water (80:20, yielding a final concentration of ethanol of 76.8%) for 24 hours with constant stirring using a shaking water bath (Kottermann, Germany) (this operation was repeated twice). The extract was filtered twice through cotton wool, then through a filter paper (Whatman grade 1, Sigma-Aldrich, France). The filtrate was concentrated using a rotavapor (Flawil, Switzerland) at 45°C, and dried on a water bath (Kottermann, Germany) to obtain 12.86 g of extract, corresponding to a yielded percentage of 12.86% of the starting material. The dried extract (10 g) was suspended in water (100 mL) and the clear supernatant was successively partitioned (1:1, v/v) by hexane, dichloromethane, ethyl acetate and *n*-butanol to obtain fractions of hexane (HF), dichloromethane (DMF), ethyl acetate (EAF), n-butanol (BuF) respectively. The remaining fraction was designated as aqueous fraction (AF). Each fraction was concentrated and freeze-dried, to yield about 640 mg of HF (6.4%), 720 mg of DMF (7.2%), 1800 mg of EAF (18%), 1080 mg of BuF (10%) and 2500 mg of AF (25%).

Animals

Healthy adult albino Wistar rats (age 4 to 5 weeks, weighing 100 to150 g) of both sexes were provided by UFR Biosciences (University of Cocody-Abidjan, Côte d'Ivoire) and were housed in stainless steel cages (34 cm ´ 47 cm ´ 18 cm) with soft wood shavings as bedding, fed with normal commercial pellet diet (Ivograin®, Abidjan, Côte d'Ivoire) and were given water *ad libitum*. They were allowed to acclimate to standard laboratory temperature conditions (temperature 24-28 °C, relative humidity 60-70%, and 12 hour light-dark cycle) for a week before the experiments. They were deprived of food for at least 18 hours prior to experiments but allowed free access to drinking water. The equipment usage and handling of the animals were performed in accordance with the European Council legislation 87/609/EEC for the protection of experimental animals (Mitjans, 2008). The pro-

tocols for the study were approved by the Departmental Ethics Committee.

Phytochemical analysis
The extract of *H. floribunda* and its fractions were screened for the presence of terpenes, flavonoids, sterols, alkaloids, tannins, coumarins and polyphenols. The detection of these constituents was performed according to the methods described in Bekro et al. (2007).

Acute toxicity
Thirty five rats were divided in seven groups of five animals. The ethanolic leaf extract of *Holarrhena floribunda* (HFE), was administrated orally at doses of 250, 500, 1000, 2000, 4000 and 6000 mg kg^{-1} body weight to the animal groups (one dose per group). The control group received distilled water, at 10 ml kg^{-1}. The animals were observed continuously for 2 h under the following profiles (Barik et al., 2008): (I) Behavioural profile (alertness, restlessness, irritability, and fearfulness), (II) Neurological profile (spontaneous activities, reactivity, touch response, pain response and gait, postural abnormalities), and (III) Autonomic profile (defecation and urination). After a period of 1, 3 and 14 days they were observed for any lethality or death.

Experimental design and induction of hyperglycaemia
For the hypoglycaemic studies, rats were randomly divided into five groups of six rats per group. Group 1 received distilled water orally, group 2 received Glibenclamide (10 mg/kg) (Sanofi-Aventis Pharmaceuticals, NJ, USA) and groups 3, 4 and 5 received 250, 500 or 1000 mg of extract/kg body weight, respectively. These groups were named HFE 250, HFE 500, and HFE 1000. The experimental design of the study consisted of a 12 hours fasting period followed by drug pre-treatments and finally the induction of hyperglycaemia by the oral administration of 4 g kg^{-1} of anhydrous glucose (Oral Glucose Tolerance test). Glibenclamide was used in the study as an anti-diabetic reference drug. Blood glucose levels were measured after the fasting period (T_0), an hour after pre-treatment (T_1) and one and four hours after induction of hyperglycaemia (T_2 and T_3, respectively). Blood samples were obtained by nicking the tails with a sharp razor (Aydin et *al.*, 1995), and glucose concentrations were determined using a one-touch glucometer (Accu-Chek Go®, Roche Diagnostics, Mannheim, Germany). The percentage of glycaemic variation was calculated as a time function by applying the following formula:

$$\% \text{ of blood glucose change} = \left(\frac{Gx - G0}{G0}\right) \times 100$$

Where G_0 = initial blood glucose values and G_x = blood glucose values at x hours time interval.

In a subsequent experiment, rats (n=5 in each group) in the same conditions as described above were pre-treated with a single dose (1000 mg kg^{-1}) of the various fractions (HF, DMF, EAF, BuF and AF), distilled water (Vehicle-control) and Glibenclamide (10 mg kg^{-1}, the reference drug). The hypoglycaemic effects were measured at T_0 (during fasting period), T_1 (an hour after glucose load) and T_2 (two hours after glucose load) and the percentage of blood glucose variation calculated as described above.

Data Analysis
Results are expressed as the mean ± SEM. Data were analysed for statistical significance with a one or two-way ANOVA followed by the Fisher-Snedecor test in the SAS statistical program (SAS, 1999) or by a Bonferroni's post hoc test or a Dunnett's Multiple Comparison Test. At a 95% confidence interval, a p value ≤ 0.05 was considered statistically significant. The graphs were plotted using Sigma Plot for Windows Version 11.0 (Systat Software Inc., Germany).

RESULTS

Phytochemical analysis
Screening the *H. floribunda* ethanolic leaf extract for various phytochemical constituents revealed the presence of components such as terpenes, flavonoids, sterols, alkaloids, tannins, coumarins and polyphenols, but the absence of quinones

Acute toxicity study

The behaviour and faeces of the animals were normal. No observations of any other signs of weakness or mortality in rats receiving orally up to 6000 mg kg^{-1} body weight of the extract. This finding suggests that ethanolic leaf extract of *H. floribunda* leaf is safe or non-toxic to rats.

Effects of the ethanolic extract and its fractions on fasted and fed-hyperglycaemic rats

The effects of increasing doses of the extract on blood glucose levels in fasting and fed-hyperglycaemia in normal rats are shown in Table 1 and Figure 1. In fasted rats (T_0), the measures of blood glucose did not show any significant changes between groups, with a mean blood glucose value of 0.93 ± 0.02 g L^{-1}. An hour after administration of extract and the reference drug in fasted rats (T_1), the results showed that blood glucose levels decreased significantly in rats treated with the Glibenclamide (from 0.89 ± 0.05 to 0.62 ± 0.05 g L^{-1}, $p<0.05$; Table 1, Figure 1) and the extract at 1000 mg kg^{-1} (from 1.0 ± 0.06 to 0.78 ± 0.03 g L^{-1}, $p<0.05$), exhibiting blood glucose decreases of 30 and 22%, respectively.

An hour after the administration of anhydrous glucose (T_2), an important rise ($p<0.001$) in blood glucose level was observed in the vehicle-treated group (corresponding to an increase of 66.66%) (Table 1, Figure 1(A)). This significant increase in blood glucose level at T_2 was also observed in the Glibenclamide group, although to a lesser extent (only 3.37% of increase). The fed-hyperglycaemic animals treated with the ethanolic leaf extract of *H. floribunda* also showed a significant increase in blood glucose levels, although this increase was limited to 25% in the HFE 250 group and 20.43% in the HFE 500 group. Results further showed that the 1000 mg kg^{-1} dose of the extract totally prevent the induction of hyperglycaemia in rats (Table 1, Figure 1(A)).

Four hours after the administration of anhydrous glucose (T_3), it was observed that blood glucose levels were maintained at significantly higher levels in the vehicle-treated group ($p<0.005$) and the HFE 250 group ($p<0.05$) when compared to the blood glucose levels obtained at T_0. However, in the Glibenclamide, EHF 500 and HFE 1000 groups, the blood glucose levels at T_3 had returned

Table 1: Effects of the ethanolic leaf extract of *H. floribunda* (HFE) on normal rats (n =6)

Groups	Blood glucose (g/l)			
	T_0 (initial time)	T_1 (1 hour)	T_2 (2 hours)	T_3 (6 hours)
Control	0.81 ± 0.03	0.85 ± 0.01	1.35 ± 0.16 ###§§§	1.18 ± 0.06## §
Glibenclamide	0.89 ± 0.05	0.62 ± 0.05* #	0.92 ± 0.14*** §	0.74 ± 0.09***
HFE 250	1.0 ± 0.03	1.0 ± 0.05††	1.25 ± 0.12† # §	1.26 ± 0.07††† #§
HFE 500	0.93 ± 0.03	0.85 ± 0.05†	1.12 ± 0.15* §	0.94 ± 0.08* £
HFE 1000	1.0 ± 0.06	0.78 ± 0.03$ #	0.97 ± 0.07** $	0.95 ± 0.06 * $

Values are expressed as mean ± SEM (n = 6). Statistical comparison: # T_0 vs (T_1, T_2 or T_3); § T_1 vs (T_2 or T_3); * Control vs (Glibenclamide, HFE250, HFE500 or HFE1000); † Glibenclamide vs (HFE250, HFE500 or HFE1000); £ HFE250 vs HFE500; $ HFE250 vs HFE1000. **For *, #, †, §, £ or $, p<0.05; for **, ##, ††, p<0.01 and for ***, ###, †††, §§§, p<0.001; one-way ANOVA followed by a Fisher-Snedecor multiple comparison test.**

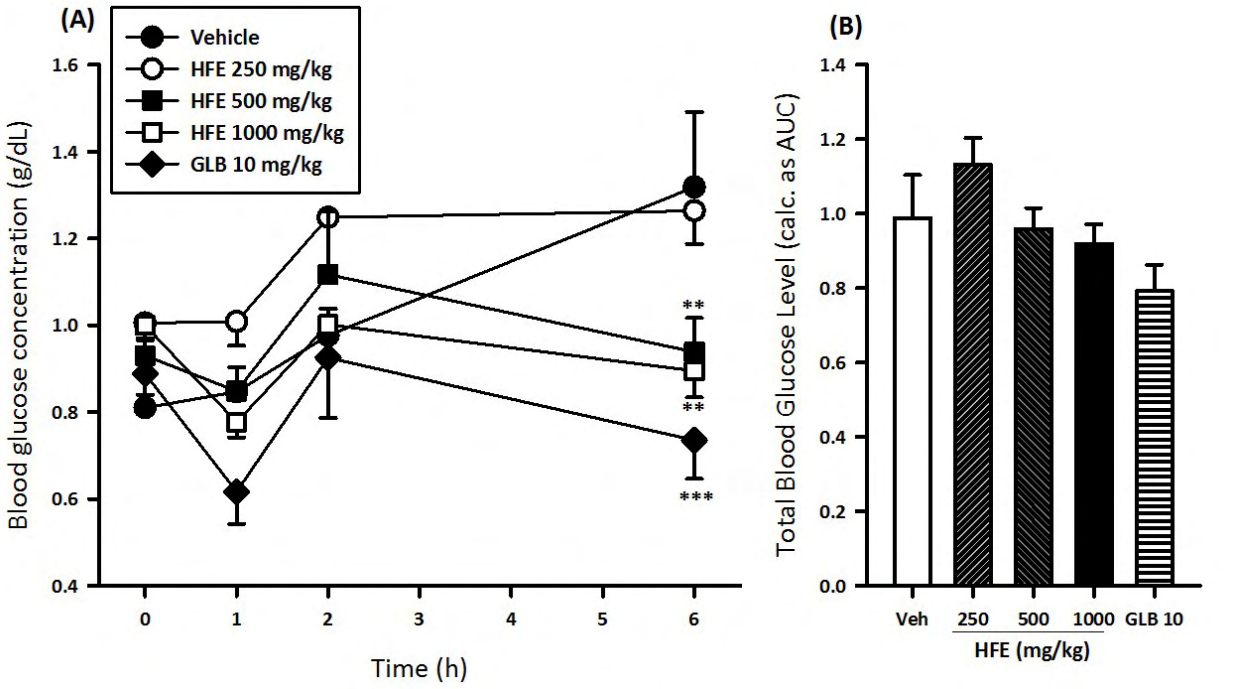

Figure 1: The dose-response effect of HFE 250-1000 mg/kg and glibenclamide (GLB) 10 mg/kg on blood glucose concentration of rats. Left panel (A) show the time-course effects over a six-hour period and the right panel (B) show the total blood glucose level calculated from AUC's over the six-hour period. Data are Means ± SEM (n = 6). $^{**}p < 0.01$, $^{***}p < 0.001$ compared to vehicle treated group (two-way ANOVA followed by a Bonferroni's post hoc test), data in panel (B) were compared to vehicle treated group (Veh) (one-way ANOVA followed by a Dunnett's Multiple Comparison Test).

to their initial values (Table 1, Figure 1(A)). The results of the total blood glucose level (as calculated from the area under the curve, AUC) showed a non-significant dose-dependent decrease in blood glucose concentration by the extract (250-1000 mg kg^{-1}) (Figure 1(B)).

The results obtained for the effect of the various fractions are presented in Figure 2. In the vehicle-treated rats a rise in blood glucose level was observed at one (T_1) and two hours (T_2) after the oral glucose load. In the opposite, Glibenclamide-treated rats showed a significant decrease of blood glucose before at T_0 ($p<0.05$) and after glucose load at T_1 and T_2 ($p<0.001$). Only the dichloromethane and ethyl acetate fractions-treated groups exhibited a significant decrease of blood glucose when compared to vehicle-treated rats. An increase of blood glucose was observed with the rest of the fractions (Figure 2(A)). Among the fractions, though not significant, the total effect on the blood glucose levels were reduced most in the dichloromethane and ethyl acetate fraction-treated groups yet these reductions were inferior to that produced in the Glibenclamide-treated group (Figure 2(B)).

DISCUSSION

The aim of the present study was to evaluate the hypoglycaemic activities of a crude ethanol extract of *Holarrhena floribunda* leaves and the fractions from this extract. This plant was ethno-botanically selected during a 2005 survey in the City of Abidjan

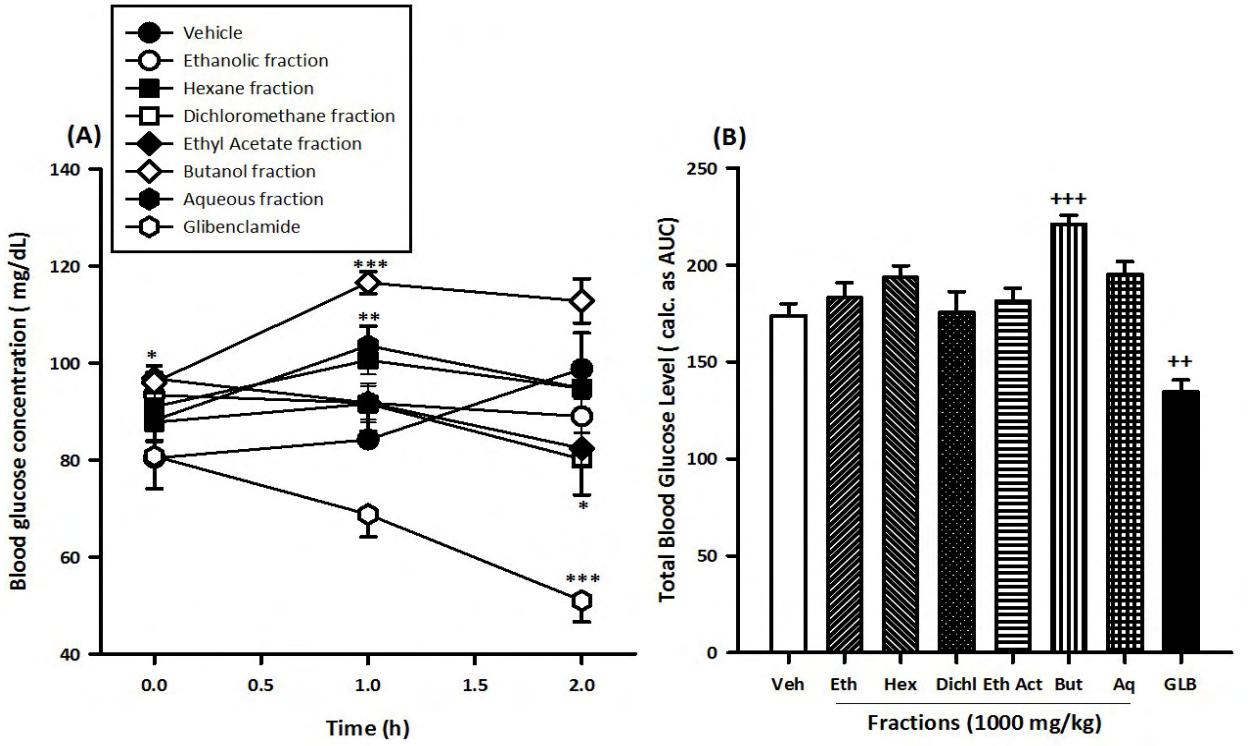

Figure 2: The hypoglycaemic effect of the various fractions of HFE (1000 mg/kg), HFE (1000 mg/kg) and glibenclamide (GLB) 10 mg/kg on blood glucose concentration of rats. Left panel (A) show the time course effects over a two-hour period and the right panel (B) show the total blood glucose level calculated from AUC's over the two-hour period. Data are Means ± SEM (n = 5). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to vehicle treated group (two-way ANOVA followed by a Bonferroni's post hoc test), $^{++}p < 0.01$, $^{+++}p < 0.001$ compared to vehicle treated group (one-way ANOVA followed by a Dunnett's Multiple Comparison Test).

(Côte d'Ivoire) and confirmed by providers of Ivorian traditional medicine, including 9 herbalists and 2 healers. The results of the survey (*data not shown*) have allowed us to collect 21 anti-diabetic plant species, among which *Holarrhena floribunda* was the most frequently quoted. Moreover, to our knowledge this plant has never been studied for its anti-diabetic properties.

The oral glucose tolerance test in normal rats is a simple and appropriate test used by many authors in similar studies and has been used as a routine tool for the preliminary screening of the hypoglycaemic properties of many medicinal plants (Prakasam *et al.,* 2003; Somani *et al.,* 2008; Yasodha *et al.,* 2008). In human, the glucose tolerance test is a standard procedure that is used to diagnose diabetes. One to five percent of people with impaired glucose tolerance (IGT) actually develop diabetes each year. Since impaired oral glucose tolerance (IGT) is indicative of a diabetic predisposition, agents that are capable of bringing blood glucose concentrations within normal limits will help to arrest the progression of impaired glucose tolerance to diabetes (Eseyin, 2010).

The blood glucose values measured in the fasted rats during this study showed that they were normoglycaemic.. These blood glucose values were similar to literature reports describing blood glucose levels in normal rats of equal weight (Dimo *et al.,* 2007; Somani *et al.,* 2008; Yasodha *et al.,* 2008).

The present study shows that the ethanolic leaf extract of *H. floribunda* dose-dependently decreases blood glucose levels in fasted rats and prevents fed-hyperglycaemia induction. In fasted rats, the extract only caused a significant decrease in blood glucose level at the 1000 mg kg^{-1}, which is similar to the hypoglycaemic effect observed with Glibenclamide. The hypoglycaemic effect of Glibenclamide, a second generation sulphonylurea, is explained by both an increase in the endogenous insulin release from pancreatic β-cells and the promotion and facilitation of peripheral glucose uptake and utilisation (Moller, 2001). The *H. floribunda* leaf extract may possibly exert its hypoglycaemic action by similar mechanisms, but further studies should be performed to confirm this hypothesis.

The oral administration of the ethanolic leaf extract of *H. floribunda* also dose-dependently reduced or suppressed the increase in blood glucose level induced by anhydrous glucose, as shown by the limited hyperglycaemic increases of 25% and 20.43% in the HFE 250 and HFE 500 groups, respectively. Moreover, results showed that the 1000 mg kg^{-1} dose of the ethanolic extract totally prevented induction fed-hyperglycaemia in rats. This effect could be explained at least in part by a decrease in intestinal glucose absorption achieved by an extra-pancreatic action that includes the stimulation of peripheral glucose utilization or the enhancement of glycolytic and glycogenic processes with a concomitant decrease in glycogenolysis and gluconeogenesis (Yasodha *et al.,* 2008). Indeed, ethanol extracts from the genus *Holarrhena* have been shown to exert inhibitory activity toward α-glucosidase (Prashanth *et al.,* 2001).

The effect of the various fractions of the ethanolic extract of *H. floribunda* on the blood glucose kinetics in the oral glucose tolerance tests in rats suggest that the hypoglycaemic activity of the ethanolic extract is found in the dichloromethane and ethyl acetate fractions. Thus further work on the effect of these fractions in a diabetic model will produce useful information in the development of a newer therapy for diabetes from this plant source.

The significant increase in the blood glucose level by the butanolic fraction could possibly be due to the presence of an important amount of reducing sugar. This information, if authenticated by a further phytochemical analysis of this fraction, would suggest and recommend to traditional practitioners to avoid preparations from this plant that may be enriched in very polar components in the management of diabetes. The phytochemical screening performed in this study revealed the presence of important components like terpenes, flavonoids, sterols, alkaloids, tannins, coumarins and polyphenols in *H. floribunda* leaves, but also indicated the absence of quinones. This result is in agreement with data reported by other investigators (Hodek *et al.,* 2002; Cemeli *et al.,* 2004; Valachovicova *et al.,* 2004; Zieran *et al.,* 2004; Bogne *et al.,* 2007). The hypoglycaemic property of *H. floribunda* could be mediated by some of these active chemical constituents. Indeed, flavonoids, terpenes, tannins and coumarins have been shown to possess hypoglycaemic activity (Marles and Farnsworth, 1995, Ojewole, 2002). The hypoglycaemic action of flavonoids was reported to be caused by the stimulation of insulin secretion from pancreatic ß-cells or by an insulin-like effect (Marles and Farnsworth, 1995). It has also been shown that alkaloids possess anti-hyperglycaemic activity that is mediated by the inhibition of α-glucosidase (Prashanth *et al.*, 2001).

CONCLUSION

The present study suggests that the leaf extract of *H. floribunda* possesses hypoglycaemic properties in normal rats and this activity lies within the dichloromethane and ethyl acetate fractions of the extract. This study provides some scientific justification for the traditional use of *H. floribunda* in the management of diabetic patients in Côte d'Ivoire.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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