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Original Research Article

Cardiac Tolerance of An Ethyl Acetate Extract of *Holarrhena floribunda* (G. Don) Durand and Schinz Leaves in Wistar Rats

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Abstract: *Holarrhena floribunda* is a plant used in traditional medicine for the treatment of various pathologies. However, it could cause toxic effects on the heart of patients. In order to determine its possible cardiotoxic effects, forty rats were randomly grouped into 2 (control group and treated group), combined with 10 rats (control satellite group and satellite treated group). Treated and satellite treated groups received, by gavage, 1000 mg / kg b.w. of the ethyl acetate extract of the leaves of *H. floribunda* (EAHf). Control and satellite control groups were administered orally with distilled water for 90 days. Animals blood samples were taken a day before the beginning of administration of *H. floribunda* and every week during the first month and at the end of every month from the second month until the end of the experiment. Serum was prepared for cardiac biochemical markers assay. The rats of the treated group at the end of the third month and those of the satellite group at the end of the fourth month were euthanized using overdose of ether, the heart of the rats was removed for gross morphology and histopathology analysis. The results of the biochemical parameters showed that this extract induced a significant decrease ($p < 0.05$) in total cholesterol, calcium, potassium and a significant increase ($p < 0.05$) of HDL, sodium levels, and AST activities. On the other hand, ALT, LDH and CPK activities, and serum triglyceride concentrations did not show any significant changes ($p < 0.05$) in treated rats group compared to controls. Microscopic observations of heart tissue sections of rats treated with the ethyl acetate extract of *H. floribunda* showed no lesions, no edema, and no necrosis. These results suggest that *H. floribunda* did not interfere with functioning or altered the integrity of the heart.

Keywords: Cardiac bio-tolerance, *Holarrhena floribunda*, histopathology, wistar rats.

INTRODUCTION

Medicinal plants are recognized to have a very important role on the health of men [1]. WHO [2] has identified more than 22 000 medicinal plants used by traditional medicine? These medicinal plants continue to be, subject to different research work that give preference generally to ethno botanical studies, pharmacological and phytochemical [3]. *Holarrhena floribunda* (Apocynaceae) is a plant used in traditional medicine in Ivory Cost for the treatment of dysentery, diarrhea, colic, infertility and particularly diabetes [4]. This plant has been subject of many previous studies. Some researchers showed its *in vitro* inhibitory effect of the aqueous extract of *H. floribunda* on the growth of non-pathogenic *amoebae* of the species *Amoeba proteus* [5]. Others demonstrated the estrogenic effects in the ovariectomized rat and the antioxidant potential of the methanolic extract of *H. floribunda* leaves [6, 7]. Phytochemical investigations on the plant led to isolation of flavonoids, phytohormones, and alkaloids [8, 9]. More recently, our research team has shown the hypoglycemic activity of the ethyl acetate extract with 1000 mg/kg b.w. as a therapeutic dose [10]. According to Khattabi *et al.;* [11], ignorance of the toxic effects of medicinal plants constitutes a brake on their use. To guarantee the safety use of H floribunda by population, acute and subacute toxicity studies carried out indicate no danger of *H. floribunda* [6, 10]. However, its safety studies on vital organs such as heart, over a long period of use have not been reported in the literature yet.

Considering the heart as the leading engine of blood circulation, this organ could be a potential target of any substance conveyed by the blood. This work was therefore carried out to evaluate the effect of the ethyl acetate extract of *H. floribunda* on wistar rats' heart.

MATERIAL AND METHODS

Plant material

The plant material consists of the leaves of *Holarrhena floribunda*. They were harvested in the department of Agboville (Côte d'Ivoire) from July to September 2013. The plant has been identified at the national floristic center of Felix Houphouët-Boigny University (Côte d'Ivoire) was a voucher specimen was deposited (*Holarrhena floribunda* (G. DON) DURAND AND SCHINZ (Apocynaceae) n ° 13240).

Animal material

Animals were selected as per the Organization of Economic Co-operation and Development (OECD) guidelines no. 408 [12].Healthy young and nulliparous, non-pregnant Wistar rats weighing from 90- 127 mg of 6-8 weeks old. The animals were bred in the animal house of the Center for Ecological Research (CRE) of the University of Nangui Abrogoua (Côte d'Ivoire). The animals are randomly selected, marked to permit individual identification, and kept in plastic cages with wood chips renewed every two days for 5 days prior to dosing to allow for acclimatization of the laboratory conditions (room temperature 25°C (± 3°C), moisture 35 to 60%, light and dark period 12/12 hours, bedding cleaned and sterilized). All animals had a regular supply of clean drinking water and food.

Methods

Preparation of ethanol extract

Holarrhena floribunda leaves were dried out of the sun at room temperature of 27 ± 2 ° C. The dried leaves were pulverized using a mill (RETSCH S M ® 100). The ethanol extract of *H. floribunda* is obtained considering the traditionally method used by traditional healers to treat diabetes. Hundred grams of the previously obtained powder were dissolved in two liters of ethanol 80 %. The solution was homogenized with a magnetic stirrer (STURART SB 162) for 48 hours and filtered on cotton wool and on whatmann n° 1 paper. The residue was re-extracted with ethanol 80 % twice for 6 hours. The solution is also filtered on cotton wool and whatmann n° 1 paper. The filtrates were put together and concentrated using a rotary evaporator (Buchi R110/NKE 6540/2) at reduced pressure at 45 ° C and then dried in an oven (Retch type SM 100, Haan, Germany) at 45° C for 48 hours. This extraction obtained a yield of 13.01%.

Preparation of the ethyl acetate extract

With a separation final, Ten (10) g of the ethanol extract was dissolved into 100 mL of distilled water. 100 mL of hexane was added to the preparation to obtain 200 mL of a solution of water-hexane in the ratio 1:1. After shaking and then decanting the solution, the aqueous layer was collected. The mixture was further successively partitioned (1:1, v/v) by dichloromethane and ethyl acetate. Solvents were evaporated using a rotary evaporator (Buchi Rotavapour) at 45 ° C and extracts were dried using an oven. The powder obtained was the ethyl acetate extract from the leaves of *Holarrhena floribunda*. The yield of the ethyl acetate fraction from the 10 g of ethanol extract was 15.23 %.

Subchronic Toxicity Test

Subchronic toxicity tests were conducted in accordance with OECD Guideline No. 408 [12]. Fifty rats were divided into four groups: control (20 rats), treated (20 rats), control satellite (5) and treated satellite (5). Rats in Control and Control Satellite were administered by gavage with distilled water daily at 2 mL / 100 g b.w. Those of Treated and Treated Satellite groups received orally and daily doses of 1000 mg / kg b.w. of EAHf, the dose which induces the hypoglycaemic effect in hyperglycemic rats according to Gnangoran *et al.;* [10]

Collection of blood samples

Rats were fasted for 24 hours and then anesthetized with ether; 1 ml / 100 g of blood was taken from the retro-orbital sinus. Blood samples were taken a day before the administration of either the extract or distilled water to rats and every week during the first month and every month from the second month until the end of the Experiments. The whole collected blood in dry tubes was centrifuged immediately in a JOUAN BR4i centrifuge (Buckinghamshire, England) at 3000 rpm for 5 min to obtain a serum for the determination of biochemical parameters.

Determination of serum cardiac markers

Cardiac enzymes activities, calcium and serum lipid levels were determined using a Cobas C311® HITACHI biochemistry automaton (Roche Diagnostics, France). The experiments were conducted using commercial kits (Roche Diagnostics, France) based on the manufacturer's instructions, as summarized in Tables 1. Serum sodium, and potassium were determined by direct ion selective electrode methods by using Roche electrolyte analyzer (AVL 9180®, Roche Diagnostics, Germany).

LDL cholesterol and atherogenic index (AI) level were calculated according to Friedwald's formula [13].

LDL-c = Total Cholesterol - HDL-c - TG/5 (g/L)

AI = Total Cholesterol / HDL-c

Table 1: Operating parameters for the quantitative determination of serum cardiac markers

cardiac biochemical markers	Methods (Spectrophotometry)	Wave length (nm)
Alanine aminotransferase (ALT)	Absorption kinetics (Disappearance of NADH)	340
Aspartate aminotransferase (AST)	Absorption kinetics (Disappearance of NADH)	340
Creatine phosphokinase (CPK)	Absorption kinetics (Formation of $NADH/H^+$)	340
Lactate dehydrogenase (LDH)	Absorption kinetics (Disappearance of NADH)	340
Total cholesterol	Colorimetric (formation of phenolic chromogen)	505
HDL-cholesterol	Colorimetric in homogeneous phase	600
Triglycerides	Colorimetric (formation of phenolic chromogen)	505
Calcium	Colorimetric	340

Cardiac tissue sampling and analysis of histological sections

At the end of the third month, the rats of the main test and those of the satellite batches at the end of the fourth month were euthanized by overdose with ether. Their hearts were removed and attached to 10% formalin. On the cores removed from the formalin, longitudinal sections of 5 mm of tissue pieces were taken using scalpel blade and then deposited in cassettes. These cassettes were introduced into an automaton (Technicon® Tissue Tek) where the tissue pieces were respectively subjected to fixing baths with 10% formaldehyde, dehydration baths with 70% and 90% ethanol, Lightening with toluene and impregnation baths with paraffin using microtome (Microme® GmbH (walldorf, Germany), cuts of 3 µm thickness were made. The sections obtained were mounted on slides and then stained with hematoxylin-eosin. Blade readings were made using a binocular optical microscope (Motic®); and the photos were taken using an electronic photography device (Am Scope® FMA050, 8.0 PIXEL) adapted to the objective of the microscope.

Statistical Analysis

The results are expressed as averages followed by the standard error on the mean (M ± ESM). The repeated variance analysis (ANOVA) was used to compare the administered dose (Control/ Test) effects and the treatment time effects. The tests were supplemented by the Bonferroni post-Hoc test. The differences were considered significant for a probability level $p < 0.05$. All these analyzes were carried out using the GraphPad prism Version 5.0 software

RESULTS

Biochemical study

Effect of ethyl acetate extract of leaves of *Holarrhena floribunda* on cardiac enzymes activities of rats

The enzymatic activities of ALT, LDH and CPK of the rats in treated group did not change significantly from those of the rats in control group; But in relation to the values of day0, significant increases were observed in all rats (control and treated group) in the activities of CPK (D14 to D120) and LDH (D28 and 60). The activity of AST, on the other hand, increased significantly in relation to the value of J0 as compared to those of the rats in the control group.

Table 2: Effect of ethyl acetate extract of leaves of *Holarrhena floribunda* on cardiac enzymes activities of rats

Days	Groups	ALT (IU/L)	AST (IU/L)	LDH (IU/L)	CPK (IU/L)
D0	Control	23,26 ± 2,313	39,20 ± 2,970	454,6 ± 27,57	180,2 ± 4,499
	Treated	26,68 ± 2,082	38,00 ± 3,521	445,7 ± 22,51	182,7 ± 4,842
D7	Control	27,10 ± 1,795	38,30 ± 1,913	523,6 ± 28,98	203,0 ± 6,995
	Treated	27,70 ± 2,217	40,10 ± 4,026	605,5 ± 31,41 (a)	177,0 ± 9,897
D14	Control	30,80 ± 3,206	42,00 ± 3,494	531,5 ± 32,07	245,1 ± 8,787 (a)
	Treated	24,95 ± 1,939	47,05 ± 3,541	598,6 ± 31,68	265,1 ± 13,57 (a)
D21	Control	28,10 ± 2,421	45,95 ± 4,595	511,7 ± 24,87	300,1 ± 12,77 (c)
	Treated	28,87 ± 2,443	48,10 ± 4,186	537,5 ± 23,58	303,1 ± 9,688 (c)
D28	Control	27,90 ± 1,764	40,20 ± 1,853	520,6 ± 36,23	325,0 ± 20,14 (c)
	Treated	28,49 ± 2,207	58,00 ± 2,186 (*) (b)	615,1 ± 34,42 (a)	350,0 ±22,99 (c)
D60	Control	25,00 ± 2,513	47,35 ± 3,671	533,3 ± 29,33	380,4 ± 18,76 (c)
	Treated	23,70 ± 2,421	50,65 ± 3,672	605,4 ± 36,05 (a)	360,4 ± 20,07(c)
D90	Control	23,75 ± 2,247	50,45 ± 3,300	525,6 ± 27,04	360,4 ± 20,07(c)
	Treated	26,65 ± 1,643	42,10 ± 3,343	591,5 ± 32,50	373,9 ± 18,98 (c)
D120	Control	31,70 ± 2,563	48,40 ± 3,732	543,5 ± 32,64	396,5 ± 18,03 (c)
	Treated	28,81 ± 2,657	51,40 ± 3,721	496,9 ± 30,46	376,6 ± 20,59 (c)

Values are means ± SEM. The asterisk indicate significant differences in the treated group compared to control group; (*) = P< 0.05; (**) = p< 0.01; (***) = p< 0.001. The letters indicate significant differences for each group according to the time (in relation to day 0); a = p< 0.05; b = p<0.01; c =p<0.001. ALT= alanine aminotransferase; AST= aspartate aminotransferase; LDH= lactate dehydrogenase; CPK= Creatine phosphokinase.

Effect of ethyl acetate extract of leaves of *Holarrhena floribunda* on the serum electrolytes levels of rats.

In the electrolytes, there was a significant decrease in calcium and potassium levels on day 60 and day 90 in rats in the test batch compared with those in the control batch. The level of sodium in the treated rats increased significantly at day 90 compared to the day of D0 and those of the control rats.

Table 3: Effect of ethyl acetate extract of leaves of *Holarrhena floribunda* on the serum electrolytes levels of rats

		Electrolytes parameters		
Days	groups	CALCIUM	POTASSIUM	SODIUM
D0	Control	10,02 ± 0,178	4,005 ± 0,188	141,5 ± 0,642
	Treated	10,01 ± 0,155	3,820 ± 0,146	142,5 ± 0,456
D7	Control	10,60 ± 0,290	4,095 ± 0,172	143,2 ± 0,586
	Treated	9,810 ± 0,182	3,980 ± 0,178	144,2 ± 0,705
D14	Control	10,50 ± 0,250	4,085 ± 0,147	144,1 ± 0,701
	Treated	9,760 ± 0,232	3,865 ± 0,157	145,4 ± 0,595
D21	Control	9,960 ± 0,193	4,145 ± 0,180	143,3 ± 0,746
	Treated	10,02 ± 0,178	4,170 ± 0,180	145,4 ± 0,608
D28	Control	10,65 ± 0,301	4,170 ± 0,180	145,9 ± 0,689 (c)
	Treated	9,960 ± 0,193	3,775 ± 0,156	147,5 ± 0,806 (c)
D60	Control	10,78 ± 0,184	4,190 ± 0,212	145,0 ± 0,811 (c)
	Treated	9,478 ± 0,314 (**)	3,340 ± 0,048 (*)	147,4 ± 0,563 (c)
D90	Control	10,72 ± 0,219	4,170 ± 0,180	142,1 ± 0,770
	Treated	9,535 ± 0,206 (*)	3,320 ± 0,040 (*)	145,7 ± 0,692 (*)
D120	Control	10,39 ± 0,241	4,040 ± 0,171	143,2 ± 1,067
	Treated	10,04 ± 0,257	4,005 ± 0,188	144,2 ± 0,705

Values are means ± SEM. The asterisk indicate significant differences in the treated group compared to control group; (*) = P< 0.05; (**) = p< 0.01; (***) = p< 0.001. The letters indicate significant differences for each group according to the time (in relation to day 0); a = p< 0.05; b = p<0.01; c =p<0.001

Effect of ethyl acetate extract of leaves of *Holarrhena floribunda* on the serum lipids levels of rats.

There were no significant changes in serum triglyceride levels in treated rats compared to control. As for other serum lipids, a significant increase (p <0.05) in the HDL cholesterol level of (+17,41%) at D28 and a significant decrease (p <0.05) on D14, for serum total cholesterol of (-15,87%) and LDL cholesterol of (-17,32%), were observed. As for the atherogenecity index of the treated rats, it decreased with Compared to the rats of the control group during the first two months of the study. This decrease was significant (p <0.05) and reached a rate of (-17.32%) at D28

Table 4: Effect of ethyl acetate extract of leaves of *Holarrhena floribunda* on the serum electrolytes levels of rats

		TC	HDL-C	LDL-C	TG	AI
D0	Control	1,04 ± 0,033	0,540 ± 0,014	0,255 ± 0,009	1,23 ± 0,168	1,95 ± 0,072
	Treated	0,964 ± 0,020	0,538 ± 0,008	0,257 ± 0,009	0,840 ± 0,112	1,79 ± 0,038
D7	Control	1,06 ± 0,035	0,553 ± 0,024	0,249 ± 0,006	1,29 ± 0,159	1,96 ± 0,077
	Treated	0,997 ± 0,034	0,541 ± 0,010	0,249 ± 0,009	1,04 ± 0,190	1,86 ± 0,0749
D14	Control	1,13 ± 0,020	0,582 ± 0,009	0,252 ± 0,006	1,50 ± 0,082	2,00 ± 0,091
	Treated	0,983(*) ± 0,029	0,514 ± 0,003	0,212(*a)±0,000	1,29 ± 0,157	1,92 ± 0,066
D21	Control	1,03 ± 0,027	0,531 ± 0,021	0,253 ± 0,006	1,23 ± 0,147	2,02 ± 0,091
	Treated	1,01 ± 0,027	0,552 ± 0,0181	0,243 ± 0,005	1,06 ± 0,135	1,85 ± 0,068
D28	Control	1,02 ± 0,028	0,517 ± 0,019	0,262 ± 0,009	1,21 ± 0,149	2,02 ± 0,080
	Treated	1,01 ± 0,020	0,607(**) ±0,005	0,248 ± 0,013	0,787± 0,083	1,67 (*)±0,033
D60	Control	0,981 ± 0,031	0,527 ± 0,014	0,263 ± 0,003	0,955 ± 0,157	1,89 ± 0,076
	Treated	0,991 ± 0,033	0,551 ± 0,012	0,248 ± 0,008	0,962 ± 0,136	1,80 ± 0,052
D90	Control	1,02 ± 0,019	0,543 ± 0,015	0,250 ± 0,007	1,12 ± 0,128	1,90 ± 0,064
	Treated	1,05 ± 0,039	0,545 ± 0,010	0,254 ± 0,003	1,25 ± 0,194	1,93 ± 0,076
D120	Control	1,010 ± 0,031	0,539 ± 0,011	0,261 ± 0,004	1,05 ± 0,158	1,88 ± 0,063
	Treated	1,03 ± 0,027	0,515 ± 0,022	0,256 ± 0,003	1,27 ± 0,149	2,06 ± 0,093

Values are mean ± SEM. The asteris indicated significant différences in the Treated group compared to control group; (*) = P< 0.05; (**) = p< 0.01; (***) = p< 0.001. The letters indicate significant differences for each group according to the time (in relation to day 0); a = p< 0.05; b = p<0.01; c =p<0.001. T C= total cholesterol; HDL-C= HDL cholesterol; LDL-C=LDL cholesterol; TG= triglyceride; AI= atherogenic index.

Histological study of rat hearts

The observation of the ventricles portions shows a normal anatomical structure almost identical to the level of all the hearts of the rats. The elongated myocardial fibers with bifurcations at their ends are interconnected (Figures).

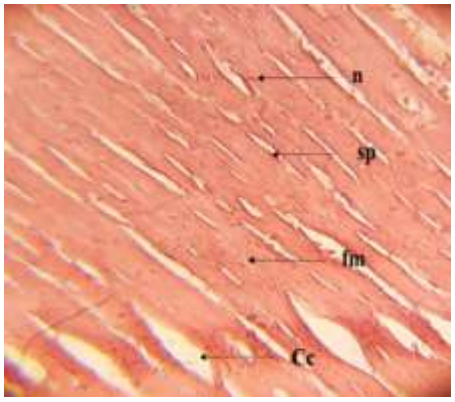

Fig 1a: Photomicrograph of the cardiac tissues of rats of Control group on the 90th day (*H&E* x400).
***sp: sarcoplasm; fm: elongated muscle fibers; n: nucleus; Cc: Cardiac cavity*.**

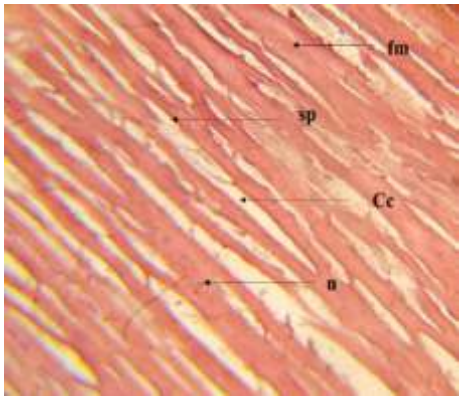

Fig 1b: Photomicrograph of the cardiac tissues of rats of Treated group on the 90th day (*H&E* x400).
***sp: sarcoplasm; fm: elongated muscle fibers; n: nucleus; Cc: Cardiac cavity*.**

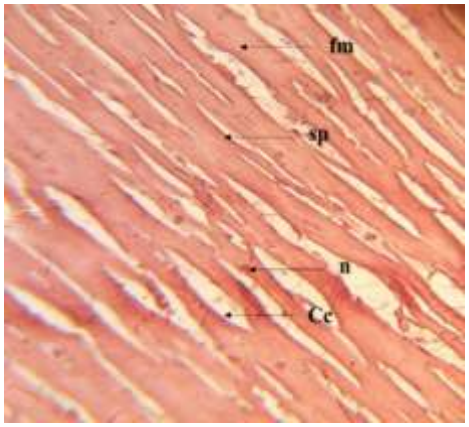

Fig 2a: Photomicrograph of the Heart ventricle portion of rats of control group on the 120th day.(*H&E* x400)
***sp: sarcoplasm; fm: elongated muscle fibers; n: nucleus; Cc: Cardiac cavity*.**

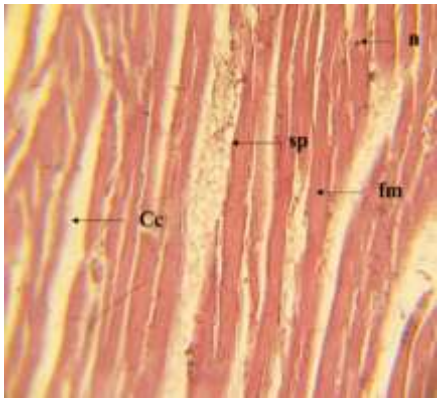

Fig 2b: Photomicrograph of the Heart ventricle portion of rats of treated group on the 120th day.(*H&E* x400)
***sp: sarcoplasm; fm: elongated muscle fibers; n: nucleus; Cc: Cardiac cavity*.**

DISCUSSION

The vast majority of exogenous composites administered to the body are gastrointestinal tract. The composites will therefore be subsequently distributed to the general blood circulation. The heart which is one vital organ undergoes the toxic effect of substances [14]. Changes in cardiac markers have often proved to be an important aid in diagnostic select diseases and determining patients' prognosis [15]. This is why, the effects of *Holarrhena floribunda* on integrity and cardiac function and also on cardiovascular risk factors were evaluate by assaying markers such as: calcium, potassium and sodium (for function); CPK, AST, ALT and LDH (for integrity), total cholesterol, HDL cholesterol and triglycerides for lipid cardiovascular risk factors [16, 17]. The dosage of electrolytes indicated significant reductions in calcium and potassium levels on day 60 and day 90 and a significant increase ($p < 0.01$) of sodium level on day 90 was observed. this study showed that the ethyl acetate extract of the leaves of Holarrhena floribunda caused hypocalcaemia, hypokalaemia and hypernatremia in rats treated with this plant. However, it is widely accepted that decreases in calcium and potassium levels indicate on the one hand, a decrease in the contraction force and, on the other, a slowing of the conduction velocity and the increase of automatism [18, 19]. The high sodium level indicates an increase in excitability [20]. In this regard, our results indicate show the extract could exert negative chronotropic, dromotropic and inotropic effects, and a positive bathmotropic effect. These results show that the action of the extract on cardiac tissue could lead to a decrease in rhythm and to bradycardia, the pathological consequence of which is heart failure [21]. The extract of *Holarrhena floribunda* may therefore be a good candidate for cardiac pathologies associated with tachycardias and arrhythmias [18].

Regarding the effects of *Holarrhena floribunda* on the integrity of cardiac cells, the assay of enzymatic activities showed that the ALT, LDH and

CPK activities did not show any significant changes. However, the serum activity of AST were increased significantly ($p < 0.05$) on Day 28. It is reported that plasma levels of these markers are directly proportional to the degree of necrotic lesions present in the myocardium and thus are markers of myocardial damage [22]. Similar types of results were obtained by experimental animal study, where rats have been induced of myocardial infarction by isoproterenol resulted in elevated SGOT (AST) level. [23]. However, leakage of transaminases in blood stream, particularly that of AST, observed on D28 may result the appearance of a membrane breach due to a temporary disorganization of the sarcolemma [24]. These results suggest that the extract of *Holarrhena floribunda* would have no effect on the heart. This would imply a lack of aggression on the part of the extract on the cardiac tissue.

At the level of the risk factors studied, the serum lipid assay indicates that serum triglyceride levels have not been significantly altered; On the other hand the extract caused significant ($p < 0.05$) decreases in the total cholesterol concentration at D28 and induced significant ($p < 0.05$) increases in HDL cholesterol concentration on Day 14. The effect of our extract, on the lipid balance, could come from the capture and the hepatic purification of the atherogenic cholesterol. This would reflect the ability or power of our extract to contribute to the elimination of atherogenic cholesterol [25]. Based on data from the literature that agrees that estrogen administration results in reduced LDL cholesterol and an increase in HDL [26], we can assume that the effect of the extract on the lipids could come from flavonoids with estrogenic effects and phytohormones whose presence was reported by Paris *et al.;* [27].

CONCLUSION

After oral administration for 90 days at a dose of 1000 mg / kg body weight, the ethyl acetate extract of the leaves of *Holarrhena floribunda* did not result in functional disturbances or cardiac damage in rats. However, biochemical and histological studies of other organs would be necessary in order to provide additional information to those already obtained in this study.

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