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Acute and subacute toxicity of the methanol extract from *Holarrhena floribunda* G. Don (Apocynaceae)

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ABSTRACT

Holarrhena floribunda is widely used in Cameroonian pharmacopoeia for gastrointestinal disorder. We have now its toxicological effect after acute and subacute administration by oral route in albino Wistar rats. The result of the acute toxicity indicate the medium lethal dose (LD_{50}) values of 6.5 and 7g/kg body weight for male and female rats respectively after 48 hours of treatment. The total lethal dose (DL_{100}) was 8g/kg for both male and female rats. We noted the significant variation ($P<0.05$) of the urea, aspartate aminotransferase (AST) activity in the serum and the 20% homogenate liver samples from the dose of 6g/kg body weight in both male and female rats. These results also indicate significant variation of glutathione ($P<0.05$) in male liver from the dose of 2 to 6g/kg body weight and the alkaline phosphatase activity (AP) of the serum in the female group from the dose of 6g/kg body weight. The results of the subacute toxicity did not showed any significant variation in the body weight, haematological and biochemical parameters studied ($P<0.05$). The results showed that this extract was poorly toxic but consumption of higher dose up to 2g/kg body weight could cause liver injury. Moderate consumption of small doses up to 1g/kg body weight twice a week for five weeks appeared safe.

Keywords: *Holarrhena floribunda*, toxicity, biochemical parameters, acute, subacute

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INTRODUCTION

Holarrhena floribunda, a plant belonging to the Apocynaceae [1], is widely distributed in the centre and west regions of Cameroon. Its stem bark is used in Cameroon to treat various ailments such as abdominal pains, nausea, indigestion, diarrhoea [2]. However, little experimental studies have been done on the biological properties of this plant. The stem bark of *H. floribunda* is febrifuge and could be a quinine substitute, since it showed remarkable inhibitory activity against drug-resistant strains of *Plasmodium falciparum* [3]. It contains conessine that is used for the destruction of amoeba without emetic effect. [2]. This plant has been shown to display a wide spectrum of biological and pharmacological activities such as antibacterial and antiamiboedal [4,5]. *H. floribunda* contains polyphenolic compounds [5] these compounds inhibits the platelet aggregation. However, the toxicity of *H.floribunda* has not intensively studied. Our study was aimed to evaluate the oral acute and subacute toxicity of the methanol extract from the stem bark of *H.floribunda* in rats with the hope that the results would provide information on the safety of this extract prior to the evaluation of its therapeutic efficacy in humans.

MATERIALS AND METHODS

2.1- Plant material

The stem bark of *H. floribunda* was collected in Ndikinimeki, in the centre region of Cameroon, in January 2003. The biological identification of the plant was done by the National Herbarium in Yaoundé, where the voucher specimen is conserved under the reference number, 20147/SFR/CAM.

2.2- Preparation of plant extract

Stem bark of *H. floribunda* was cut into small pieces and air dried at room temperature(30 ± 3°C). The dried materials were ground and macerated in methanol in the ratio 1:5 (w/v) at room temperature for 24 h. The concentrated methanol extract was decanted, filtered with Wathman number 1 filter paper and concentrated in vacuo bellow 40°C using a rotary evaporator to give the crude extract (4.1% w/w) used for the investigations.

2.3- Experimental animals

Sexually mature male and female albino Wistar rats weighing 100-120g was obtained from the animal house of the Biochemistry Department (University of Yaoundé I Cameroon). They were housed under uniform husbandry conditions of 12 h light, 12 h dark cycle and temperature (26 ± 2°C) and allowed free access to drinking water and standard laboratory diet. Rats were deprived of food (16 – 18 h) prior the administration of the extract. The principles of laboratory animal care were followed and the Department's ethical committee approved the use of the animals and the study design.

2.4- Acute toxicity

The bioassays were conducted according to the Word Health Organisation guideline for the evaluation of the safety and efficiency of herbal medicines [6]. For the study, albino Wistar rats were randomly divided into four groups of four animals per sex. The *H. floribunda* extract was dissolved/suspended in distilled water and administrated by gavages at dose of 2; 4; 6 and 8g/kg. The control group receives distilled water *per os* throughout the experiment. The general behaviour of rats was observed continuously for 1h after the treatment and then intermittently for 4 h and there after over the period of 24 h [7]. The rats were further observed for up to 5 days following treatment for any signs of toxicity and deaths and the latency of death. The LD_{100} and LD_{50} values were determined according to the method of [8]. On the day 6, all rats were fasted for 16 – 18 h, and then sacrificed by decapitation. Blood was collected and centrifuged at 3000 g at 4°C for 15 min to obtain the serum, which was stored at -20°C until analysis for biochemical parameters. Liver was carefully dissected out, weighed and homogenized in 10 volumes of tris-Hcl buffer ($tris^0$, Hcl, Kcl) pH 7.4 at 4°C using potter homogenizer. The homogenate was then centrifuged at 6000 g at 4°C for 15 min and supernatants collected were used for biochemical parameters

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2.5- Subacute toxicity

Albino Wistar rats (100-120) were housed four per plastic cage, under the same conditions as described above. The animals were divided into three groups (I – III) of four animals per sex. The *H. floribunda* extract was dissolved in distilled water and administered orally every two days for 35 days to the group I –II at doses of 0.5 and 1 g/kg body weight respectively. The control group receives distilled water. Toxic manifestations and mortality were mentioned daily, and the body weight changes were recorded weekly. At the end of the 35 days period, the animals were sacrificed by decapitation blood was collected in two types of tubes: one with heparin and the other without any additives. The anticoagulated blood (heparin) was analysed immediately for haematological parameters [6]. The second tube was centrifuged at 3000 g at 4°C for 15 min to obtain the serum, with was stored at -20°C until analysis for biochemical parameters. Liver was carefully dissected out weighted and homogenized in 10 volumes of tris – Hcl buffer pH 7.4 at 4°C using potter homogenizer. The homogenate was then centrifuged at 6000 g at 4°C for 30 min and supernatants collected were used for biochemical parameters.

2.6- Biochemical estimations

The haematological parameters: total red blood cells (RBC) white blood cells (WBC) and platelets counts were determined using an autoanalyser (system H1, Bayer Diagnostics) [9]. The biochemical parameters creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), were determined enzymatically by measurement of optical density at the corresponding wave length with spectrophometer (Shimadzu UV-120-01) [10]. Alkaline phosphatase (AP), total and direct bilirubin, urea were evaluated by the method of [10]. Proteins and glutathione were analysed by the methods of [11] and [12] respectively.

Statistical analysis

Values were expressed as mean ± SD. The statistical analyses of variance was done by ANOVA follow by the student-Newman.

RESULTS

3.1- Acute toxicity

Rats treated with the dose of 4 and 6g/kg body weight show some behavioural immediately after oral administration. These changes included high response to external stimuli, increase of mobility, salivation, aggressiveness, while others (asthenia, anorexia and weight loss) were observed later. However, after 24 h, all the changes observed before disappeared. On the contrary, no adverse changes were noted in rats treated with 2g/kg body weight. Dead of rats was noted at 8g/kg. The medium lethal dose (LD_{50}) values for males and females rats were 6.5 and 7g/kg body weight respectively after 48 hours of treatment. The total lethal dose (DL_{100}) was 8g/kg for both male and female rats. The biochemical profiles of the treated and control rats are presented in tables 1 and 2 for the serum and the 20% liver homogenate respectively.

Table 1: Blood biochemical indices of rats of acute toxicity of methanolic extract of *H. Floribunda*

Biochemical indices	Treatment (g/kg)			
Male	**Control (0)**	**2**	**6**	**8**
Urea (mg/L)	1 ± 0.07	1.16 ± 0.12*	1.23 ± 0.08*	2.33 ± 0.11*
Creatinine (mg/L)	8.29 ± 0.28	8.31 ± 0.26	8.36 ± 0.12	9.36 ± 0.19
Protein (mg/mL)	2.63 ± 0.46	2.05 ± 0.23	2.61 ± 0.72	1.91 ± 0.22
AP (UI/L)	11.75 ± 1.84	11.5 ± 1.84	10.88 ± 2.11	8.88 ± 0.47
AST (UI/L)	74.38 ± 6.07	95.31 ± 5	127.19 ± 2.13*	136.38 ± 4.84*
ALT (UI/L)	47 ± 3.91	49 ± 4.89	50 ± 10.90	57 ± 4.69
Total bilirubin (mg/L)	1.85 ± 0.49	1.62 ± 0.75	1.77 ± 0.85	1.47 ± 0.48
Direct bilirubin (mg/L)	0.11 ± 0.09	0.09 ± 0.01	0.08 ± 0.01	0.1 ± 0.01
Female				
Urea (mg/L)	1.48 ± 0.07	1.61 ± 0.08	1.66 ± 0.07	2.12 ± 0.45*
Creatinine (mg/L)	8.05 ± 0.05	8.62 ± 1.13	8.14 ± 0.33	9.68 ± 1.47
Protein (mg/mL)	2.01 ± 0.42	1.99 ± 0.45	2.00 ± 0.30	1.85 ± 0
AP (UI/L)	8.25 ± 0.84	7.5 ± 0.70	9 ± 1.27	12.75 ± 0.95*
AST (UI/L)	69.06 ± 3.94	77.5 ± 8.41	76.25 ± 11.98	100 ± 3.40*
ALT (UI/L)	37.5 ± 3.54	40.5 ± 5.25	46 ± 4.97	44.5 ± 4.34
Total bilirubin (mg/L)	1.04 ± 0.72	0.71 ± 0.19	0.75 ± 0.39	0.71 ± 0.13
Direct bilirubin (mg/L)	0.11 ± 0.1	0.09 ± 0.04	0.09 ± 0.01	0.1 ± 0.03

*Values are expressed as means ±SD * Values significantly different (p<0.05)*

Table 2: Biochemical indices of 20% homogenate liver of rats of acute toxicity of methanolic extract of *H. Floribunda*

Biochemical indices	Treatment (g/kg)			
Male	**Control (0)**	**2**	**6**	**8**
Glutathione (µmole/g of liver)	4.04 ± 1.06	1.72 ± 0.58*	2.4 ± 1.25*	1.15 ± 0.58*
Protein (mg/mL)	0.94 ± 0.14	0.86 ± 0.10	0.84 ± 0.22	0.68 ± 0.05
AP (UI/L)	10.38 ± 1.49	9.38 ± 1.03	9.13 ± 1.49	7.88 ± 0.47
AST (UI/L)	81.25 ± 29.75	104.69 ± 23.57	106.44 ± 11.47	117.81 ± 13.20*
ALT (UI/L)	43 ± 8.65	48 ± 3.82	44 ± 6.35	51.5 ± 4.69
Female				
Glutathione (µmole/g of liver)	2.5 ± 1.32	2.2 ± 0.85	2.18 ± 1.71	1.93 ± 0.39
Protein (mg/mL)	1.29 ± 0.33	0.91 ± 0.11	1.35 ± 0.47	0.7 ± 0.05
AP (UI/L)	8.88 ± 1.93	7.75 ± 0.5	7.88 ± 0.75	7.75 ± 0.95
AST (UI/L)	49.38 ± 3.75	56.88 ± 2.97	49.37 ± 3.75	87.5 ± 7.24*
ALT (UI/L)	33 ± 4.89	27.5 ± 1	34.5 ± 4.12	43.5 ± 4.12

*Values are expressed as means ±SD * Values significantly different (p<0.05)*

Table 3: Haematological values of rats in subacute toxicity after 35 days of administration of methanolic extract of *H. Floribunda*

Treatment (g/kg)	RBC (x 10^5/µL)	WBC (x 10^3/µL)	Platelet (x 10^4/µL)
Male			
Control (0)	53.67 ± 4.06	64.33 ± 8.50	56 ± 5.58
0,5	23 ± 4.58	32 ± 6	27.33 ± 7.21
1	32.33 ± 6.11	35 ± 3.60	31.33 ± 6.50
Female			
Control (0)	81 ± 8.99	84.5 ± 2.39	77 ± 8.23
0,5	39 ± 8.44	41.25 ± 8.5	37 ± 9.69
1	48.5 ± 6.23	52.75 ± 9.10	44.25 ± 5.12

Values are expressed as mean ±SD, RBC: red blood cell, WBC: white blood cell,

3. 2-Subacute toxicity

The methanol extract of *H. floribunda* at dose of 0.5 and 1g/kg given *per os* every 48 h for 35 days did not result in death of the animals. No sign of observable toxicity was detected during the experiment period. The haematological

parameters are presented in table 3. The biochemical profiles of the treated and control rats are presented in table 4 and 5 for the serum and 20% liver homogenate respectively.

Table 4: Blood biochemical indices of rats of subacute toxicity of methanolic extract of *H. Floribunda*

Biochemical indices	Treatment (g/kg)		
Male	**Control (0)**	**0,5**	**1**
Urea (mg/L)	1.24 ± 0.22	1.40 ± 0.17	1.56 ± 0.61
Creatinine (mg/L)	5.12 ± 1.45	4.83 ± 1.63	5.23 ± 1.20
Protein (mg/mL)	4.58 ± 0.39	4.6 ± 0.43	4.1 ± 0.55
AP (UI/L)	9.88 ± 1.43	10.13 ± 2.56	9.75 ± 1.44
AST (UI/L)	63.75 ± 3.44	68.75 ± 2.86	81.18 ± 12.33
ALT (UI/L)	45 ± 3.46	38 ± 5.85	40.5 ± 9.89
Total bilirubin (mg/L)	1.24 ± 0.22	1.21 ± 1.97	1.19 ± 0.96
Direct bilirubin (mg/L)	0.19 ± 0.01	0.16 ± 0.07	0.18 ± 0.03
Female			
Urea (mg/L)	1.35 ± 0.38	1.5 ± 0.1	1.57 ± 0.1
Creatinine (mg/L)	4.23 ± 0.77	4.14 ± 0.29	4.22 ± 0.73
Protein (mg/mL)	4.64 ± 0.12	4.94 ± 0.36	4.48 ± 0.41
AP (UI/L)	8.63 ± 1.18	9 ± 2.08	10.5 ± 2.55
AST (UI/L)	81.25 ± 3.49	88.13 ± 4.46	88.75 ± 5.06
ALT (UI/L)	51 ± 2.97	54.5 ± 1.28	60.5 ± 3.64
Total bilirubin (mg/L)	1.69 ± 0.89	1.68 ± 1.38	1.31 ± 1.38
Direct bilirubin (mg/L)	0.17 ± 0.08	0.21 ± 0.04	0.2 ± 0.01

*Values are expressed as means ±SD * Values signifi cantly different (p<0.05)*

Table 5: Biochemical indices of 20% homogenate liver of rats of acute toxicity of methanolic extract of *H. Floribunda*

Biochemical indices	Treatment (g/kg)		
Male	**Control (0)**	**0,5**	**1**
Glutathione (µmole/g of liver)	2.53 ± 0.31	2.46 ± 0.13	2.23 ± 0.24
Protein (mg/mL)	1.39 ± 0.01	1.58 ± 0.7	1.4 ± 0.41
AP (UI/L)	11 ± 0	11.06 ± 0.96	11.3 ± 0.84
AST (UI/L)	50.5 ± 2.58	64.13 ± 2.39	59.5 ± 1.89
ALT (UI/L)	37.5 ± 13.89	33.5 ± 4.45	30 ± 1.44
Female			
Gluthatione (µmole/g of liver)	2.46 ± 0.72	2.10 ± 0.48	2.15 ± 0.1
Protein (mg/mL)	1.99 ± 0.1	1.56 ± 0.22	1.8 ± 0.22
AP (UI/L)	12.13 ± 1.1	12.81 ± 1.66	11.75 ± 1.33
AST (UI/L)	44.38 ± 2.54	55 ± 2.95	51.44 ± 3.75
ALT (UI/L)	50 ± 4.14	53 ± 8.86	61.5 ± 7.02

*Values are expressed as means ±SD * Values signifi cantly different (p<0.05)*

DISCUSSION

According to [13], substances with DL_{50} values greater than 5g/kg body weight are considered to show low toxicity. Thus the methanol extract of *H. floribunda* can be classified in the category of substances with low toxicity. In acute toxicity, there were no significant changes (P<0.05) in alanine aminotransferase (ALT) activity, creatinine, proteins, total and direct bilirubin in the serum of both sexes. No significant changes in alanine aminotransferase (ALT) activity and proteins were noted in the 20% liver homogenate of both sexes. However, urea and aspartate aminotransferase (AST) activity in the serum and the 20% homogenate liver sample were significantly increased (p<0.05) in both male and female rats as compared to the control group. There was significant decrease in glutathione (p<0.05) only in the males at the 6g/kg body weight. Alkaline phosphatase (AP) activity in the serum was significantly increased (p<0.05) only in female rats. ALT and AST are two liver enzymes that are associated to the hepatocellular damage. Although both AST and ALT are common liver enzymes because of their higher concentrations in hepatocytes, only ALT is remarkably specific for liver function since AST is mostly present in the myocardium, skeletal muscle, brain and kidneys [14,15]. In general, with liver disease, serum levels of AST and ALT rise and fall in the same time [16]. A mild elevation of AST level has been shown to be associated with liver injury or myocardial infarctions [17]. The higher the activity of AST, the larger the infarction size [18,19]. These results indicates that the methanol extract of *H. floribunda* when taken in high dose may cause liver disease.

In subacute toxicity, no significant difference in body weight gain was noted between the control and any of the treated groups at any time period. All parameters (red blood cells, white blood cells and platelets) did not change significantly (P<0.05) as compared to the control group. In general the results showed that the values for RBC, WBC and platelets were slightly decreased in male and female groups compared to the control. Subacute oral administration (up to a dose of 1g/kg body weight) did not cause any significant change (P<0.05) in all biochemical parameters studied as compared to the control group.

CONCLUSION

This study presents strong evidence of the nontoxic effect of the methanol extract of *H. floribunda*. These results showed that the use of the extract of *H. floribunda* is safe and explained the extensive utilisation of the plant in traditional medicine.

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