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Research Article

A Study of the Hepatic Tolerance of the Ethyl Acetate Fraction of *Morinda morindoides* (Baker) *Milne-Redhead* (Rubiaceae) Extract in Rabbit

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Abstract

***Purpose**: In this study, the effect of the ethyl acetate fraction of the extract of Morinda morindoides (Baker) Milne-Redhead (Rubiaceae) on the tissue and metabolic integrity of rabbit liver was evaluated.*
***Methods:** Thirty rabbits (divided equally between male and female) were randomly distributed into five (5) groups of six (6) rabbits each. These animals received, by intraperitoneal injection, twice a week the ethyl acetate fraction (F1) of the hydroalcohol extract of Morinda morindoides in doses ranging from 25 to 100 µg/kg body weight for Groups 2 (Gp2) to 5 (Gp5) while Group 1 (Gp1) and control, received 1 ml of MacEwen physiological fluid. Blood sampling was carried out to evaluate various parameters: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyltransferase (γGT), lactate dehydrogenase (LDH), total cholesterol, HDL cholesterol, triglycerides, glucose and total proteins*
***Results:** Analysis of the parameters following administration of the extract fraction showed slight increases in AST and ALT activities as well as the concentrations of total cholesterol and glucose, compared to their initial values. On the other hand, protein, glyceride, total cholesterol and high density lipoprotein cholesterol levels as well as the activities of ALP, γGT and the LDH did not change significantly (p >0.05). Nevertheless, the changes observed during the study were within the limits of the normal values for these various parameters in rabbit.*
***Conclusion:** These results revealed that the ethyl acetate fraction (F1) of the hydroalchol extract of Morinda morindoïdes, when used in the dose range evaluated in this study, may be well tolerated by the liver.*

***Keywords**: Hepatic tolerance, Morinda morindoides, Serum biochemical parameters*

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INTRODUCTION

Morinda morindoïdes (Baker) *Milne-Redhead* (Rubiaceae) is well known in traditional medicine practice in some African countries (Nigeria, Democratic Republic of Congo and Congo-Brazzaville) where its leaves are frequently used for treating malaria, diarrhoea, amoebiasis, haemorrhoids, gonorrheoa and rheumatic pains [1-4]. In Ivory Coast, the leaf of the plant is traditionally used by people in the west central region against diarrhoea [4].

In vitro and in vivo studies on this plant have revealed some interesting results such as antimalarial [2], cardioinhibitory [5], antiamoebic [6], antifungal [7], antibacterial [3], antidiarrheal [4] and antispasmodic [8] activities. Phytochemical investigations on the plant led to the isolation of saponins, flavonoids, terpenes, steroids, tannins, anthraquinones and alkaloids [1,7] while structure elucidation indicated flavonoids [9,10], iridoids [6] and anthraquinones [11].

Studies have previously been performed to improve the antidiarrheoal activity of *Morinda morindoides* and isolate the compounds responsible of this activity. Among the different fractions tested, the ethyl acetate fraction (coded F1) of the hydroalcohol extract of *M. morindoides* offered the most promising biological activity [5,7].

Liver is a target organ and the primary site of detoxification. Being a major site of intense metabolism and, therefore, prone to various disorders as a consequence of exposure to toxins of extrinsic and intrinsic nature, the liver plays an important role in metabolism to maintain energy level and structural stability of the body [12]. It is also a site of biotransformation where toxic compounds are transformed to less harmful substances [12]. However, this function can result in damage to liver cells.

To the best of our knowledge, *in vivo* tolerance studies on *M. morindoides* have not been extensively undertaken. This study was, therefore, conducted to assess the effect of the ethyl acetate fraction (coded F1) of the hydroalcohol extract of the plant on some biochemical markers of the liver since this fraction had previously been demonstrated to exhibit some useful biological activities [5,7].

EXPERIMENTAL

Plant material

The leaves of *Morinda morindoides* (Baker) *Milne-Redhead* (Rubiaceae) were collected in the region of Daloa, west-central Ivory Coast. The plant was authenticated by Professor Ake Assi of the Department of Botany, University of Cocody, Abidjan and a voucher specimen (no. 17710) of the plant was deposited in the herbarium of the National Floristic Center of University of Cocody-Abidjan.

Preparation of *Morinda morindoides* extract

The leaves of *Morinda morindoides* (Baker) *Milne-Redhead* (Rubiaceae) were air-dried at a room temperature (28 ±1 °C) for 7 days and ground into fine powder. The powder was mixed with distilled water (80 g in 2 L of distilled water) for 24 h with constant stirring at 80 °C. The extract was filtered twice through cotton wool, and then through Whatman filter paper no. 1. The filtrate was evaporated to dryness in a rotary evaporator (Buchi) at 60 °C. Twenty five grams of the dry aqueous extract was added to 500 ml of ethanol and water (consisting of 356 ml of ethanol 96 % and 144 ml of distilled water) and after thorough mixing, the supernatant was evaporated in a rotary evaporated following the method of Guede-Guina *et al* [13]. The residue was taken as the hydro-alcohol extract.

Preparation of the chromatographic fraction of the extract

Five grams (5 g) of the hydro-alcohol extract was added to 500 ml of water/ethyl acetate mixture (consisting of 250 ml of distilled water and 250 ml of ethyl acetate) and stirred

continuously for 24 h. After decantation, the supernatant was evaporated in a rotary evaporator. The residue was taken as the ethyl acetate extract of the plant leaf (AcE).

The ethyl acetate extract was subjected to separation on a chromatographic column (2 x 50 cm) with silica gel 60 (Merck, silica gel, 0.063 – 0.200 mm). The extract (0.25 g) was introduced into the column and placed on the silica gel, protected by cotton. Dichloromethane was used as eluent until total infiltration of the extract into the silica gel; thereafter, dichloromethane-methanol (95/5) mixture was used for elution until separation was complete.
The four fractions obtained, based on their colour, were F1 (golden yellow), F2 (dark green), F3 (pale green) and F4 (yellow-orange) [7]. Fraction F1 (AcE F1) was evaporated in a rotary evaporator (at 60 °C) before use in the *in vivo* studies.

Experimental animals

Rabbits, *Oryctolagus cuniculus*, from a rabbit cattle farm in Bingerville, southern Ivory Coast were used in this study. The experimental procedures and protocols used in this study were approved by the Ethical Committee of Health Sciences, University of Cocody-Abidjan. These guidelines were in accordance with the European Council Legislation 87/607/EEC for the protection of experimental animals [14].

Administration of fraction F1

Thirty (30) rabbits (15 males and females each) were acclimatized for a month and half at ambient temperature (28±1 °C) and humidity (70 ± 5%). Their age was approx. 3 months and weighed 1.2 ± 0.2 kg on the average. They were randomly divided into 5 groups of 6 rabbits each (3 males and 3 females). Animals in each group were separated according to their sex in cages (length: 79 cm, width: 50 cm and height: 38 cm). They had free access to both water and food.

Twice a week, the animals received inraperitoneally (ip) 1 ml of an injection according to their group. Group 1 (Gp1), control, received 1ml of MacEwen physiological fluid while Groups 2 (Gp2) to 5 (Gp5) were similarly injected with 30, 60, 60 µg/ml , 90 and 120 µg/ml, respectively, of F1, equivalent to 25, 50, 75 and 100 µg/kg body weight, respectively

Collection of blood

Blood samples were obtained in the morning (from 8 to 11 am) via the marginal ear vein of the animals. Blood sampling was carried out once a week in the two weeks preceding the first application of treatment (S0), during the four weeks of treatment (S1, S2, S3 and S4), and then for two weeks after the treatment period (S5 and S6). These blood samples were collected in tubes (without anticoagulant) and centrifuged at 3000 rpm for 10 min**.** The serum was stored at -20 °C until analysed for enzymatic activities and concentration of biochemical metabolites.

Assay of hepatic parameters in rabbit serum

Hepatic parameters of the serum were measured with an automatic analyzer, Hitachi 902 (Roche), using commercial kits and certified controls (Spinreact S.A., Ctra Santa Coloma, Spain) based on the manufacturer's instructions, as summarized in Tables 1 and 2.

Statistical analysis

The results are presented as mean ± standard deviation (SD). Analysis of variance (ANOVA) with repeated measures was employed to compare the results according to the administered doses and times of treatment. Analysis of variance was considered significant when the level of probability *(p)* was < 0.05. When the value of *p* was significant, a Post-Hoc test of Newman-Keuls was carried out. These analyses were carried out with the software, Statistica 7.1 (Statistica, Statsoft) using a general linear model (GLM)

Table 1: Operating parameters for the quantitative determination of enzymes

Parameter	Enzyme kinetic method	Wavelength (nm)
ALT	Disappearance of NADH	340
AST	Disappearance of NADH	340
ALP	Rate of p-nitrophenol formation	405
γGT	Rate of NAMB	405
LDH	Disappearance of NADPH	340

ALT= alanine aminotransferase; AST= aspartate aminotransferase; ALP=alkaline phosphatase; γGT= gamma-glutamyltransferase; LDH=lactate dehydrogenase; NAMB =2-nitro-5-aminobenzoic acid formation; NADH=nicotinamide adenine dinucleotide; NADPH=nicotinamide adenine dinucleotide phosphate

Table 2: Operating parameters for the quantitative determination of metabolites

Parameter	Colorimetric method	Wavelength (nm)
Total Cholesterol	Cholesterol esterase, oxydase and peroxidase	500
HDL Cholesterol	Cholesterol esterase, oxydase and peroxidase	500
Triglycerides	Lipase, glycerol kinase, oxidase and peroxidase	500
Glucose	Glucose oxydase and peroxidase	500
Total Protein	Copper salts and alkaline medium	550

RESULTS

Effect of AcE F1 on hepatic enzyme activities of rabbit

Table 3 shows the effect of F1 on liver enzyme activities. F1 increased the activity of AST compared to the control group (p = 0.006) but the effect was not dependent on the concentration of the extract fraction. On the other hand, ALT (p = 0.15597), ALP (p = 0.52953), γGT (p = 0.80844) and LDH (p = 0.0845) activities were not influenced by F1 as they were statistically identical to those of the control group.

Effect of AcE F1 on hepatic metabolites of rabbit

Table 4 shows the effect of F1 on serum metabolites. With the exception of the concentration of total cholesterol which decreased compared to that of the control group (p = 0.02207), F1 had no significant effect on the levels of the other metabolites: HDL cholesterol (p = 0.15415), triglycerides (p = 0.67285), glucose (p = 0.10389) and total proteins (p = 0.65492).

Effect of duration of AcE F1 administration on hepatic enzyme activities of rabbit

As Table 5 shows, the activities of the aminotransferases (AST and ALT) increased gradually after weekly administration of F1 until the third week when maximum values of 98.47 ± 6.57 (ALT, p = 0.000) and 88,27 ± 4.42 (AST, p = 0.000) were attained and no further changes were observed. For the other enzymes - ALP (p = 0.2257), γGT (p = 0.0874) and LDH (p = 0.14310) - their initial activities were unchanged with time following weekly administration of the extract fraction.

Table 3: Effect of *M. morindoides* ethyl acetate fraction (AcE F1) on liver enzyme activities of rabbit

Group	Dose (µg/kg)	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	γGT (IU/L)	LDH (IU/L)
GP1	0	65.42 ± 5.28	67.55 ± 4.98[a]	76.55 ± 5.11	20.60 ± 2.59	106.87 ± 4.50
GP2	25	63.83 ± 5.35	70.85 ± 4.37[b]	76.14 ± 4.71	19.95 ± 2.30	106.35 ± 4.19
GP3	50	62.33 ± 3.69	71.99 ± 4.59[b]	74.22 ± 5.61	20.67 ± 2.10	108.67 ± 3.53
GP4	75	58.67 ± 4.90	71.29 ± 4.24[b]	74.87 ± 5.38	20.52 ± 1.99	106.50 ± 4.31
GP5	100	61.17 ± 5.19	70.54 ± 5.31[b]	76.87 ± 5.27	20.35 ± 1.85	107.03 ± 3.93

Values are mean± SD (n = 6); values without common superscript (a or b) in a column differ ($p < 0.05$); *a < b; ALT= alanine aminotransferase; AST= aspartate aminotransferase; ALP=alkaline phosphatase; γGT= gamma-glutamyltransferase; LDH=lactate dehydrogenase*

Effect of duration of AcE F1 administration on hepatic metabolites of rabbit

Table 6 indicates that the concentrations of total cholesterol (p = 0.0001) and glucose (p = 0.000) increased with weekly administration of F1 while the levels of cholesterol HDL (p = 0.2615), triglycerides (p = 0.06311) and total proteins (p = 0.1038) were statistically unchanged.

Table 4: Effect of *M. morindoides* ethyl acetate fraction (AcE F1) on the levels of hepatic metabolites

Group	Dose (µg/kg)	T-C (g/L)	HDL-C (g/L)	Trig (g/L)	Gluc (g/L)	T-Prot (g/L)
GP1	0	0.56 ± 0.05[b]	0.23 ± 0.04	0.76 ± 0.05	1.35 ± 0.06	58.31 ± 3.42
GP2	25	0.53 ± 0.06[a]	0024 ± 0.04	0.74 ± 0.03	1.33 ± 0.04	57.48 ± 3.38
GP3	50	0.53 ± 0.05[a]	0.23 ± 0.03	0.74 ± 0.04	1.33 ± 0.04	57.52 ± 3.53
GP4	75	0.52 ± 0.04[a]	0.23 ± 0.03	0.76 ± 0.04	1.34 ± 0.07	57.69 ± 3.86
GP5	100	0.52 ± 0.05[a]	0.23 ± 0.03	0.74 ± 0.05	1.35 ± 0.06	58.83 ± 2.59

*Values are mean ± SD (n = 6); values without common superscript (a-b)[*] in a column differ (p < 0.05); *a < b; T-C = Total cholesterol; HDL-C = HDL cholesterol; Trig = triglyceride; Glu = glucose; T-Prot = total protein*

Table 5: Hepatic enzyme activities of rabbit following weekly administration of *M. morindoides* ethyl acetate fraction (AcE F1)

Week	ALT (IU/L)	AST (UI/L)	ALP (IU/L)	γGT (IU/L)	LDH (IU/L)
S0	54.96 ± 3.80[a]	60.33 ± 4.18[b]	75.23 ± 7.54	21.10 ± 1.93	106.99 ± 6.29
S1	72.30 ± 5.40[d]	63.17 ± 5.78[b]	75.87 ± 7.30	20.93 ± 2.63	108.43 ± 4.13
S2	95.73 ± 7.09[f]	86.70 ± 8.21[d]	74.90 ± 5.96	20.87 ± 2.79	107.60 ± 4.40
S3	98.47 ± 6.57[g]	88.27 ± 4.42[d]	75.17 ± 5.52	20.20 ± 2.31	106.87 ± 3.85
S4	88.47 ± 5.08[e]	81.67 ± 5.94[c]	74.63 ± 4.15	20.07 ± 1.91	107.57 ± 3.14
S5	66.43 ± 5.33[c]	60.60 ± 4.90[b]	76.63 ± 4.11	19.83 ± 1.95	107.00 ± 4.15
S6	58.1 ± 5.50[b]	52.37 ± 3.86[a]	77.67 ± 3.47	19.93 ± 2.23	105.13 ± 3.51

*Values are mean± SD (n = 6); values without common superscript (a - g) in a column differ (p < 0.05); *a < b < c < d < e < f < g 'ALT= alanine aminotransferase; AST= aspartate aminotransferase; ALP=alkaline phosphatase; γGT= gamma-glutamyltransferase; LDH=lactate dehydrogenase S0 = two weeks preceding the first application of treatment; S1 to S4= weeks of treatment; S5 and S6= weeks after the treatment period*

Table 6: Hepatic metabolite levels (mean ± SD, n = 6) of rabbit following weekly administration of *M. morindoides* ethyl acetate fraction (AcE F1)

Week	Total cholesterol (g/L)	HDL cholesterol (g/L)	Triglyceride (g/L)	Glucose (g/L)	Total protein (g/L)
S0	0.49 ± 0.08[a]	0.22 ± 0.03	0.75 ± 0.06	1.23 ± 0.04[a]	57.47 ± 3.50
S1	0.54 ± 0.07[b]	0.24 ± 0.04	0.74 ± 0.05	1.29 ± 0.06[b]	58.57 ± 4.52
S2	0.53 ± 0.06[b]	0.24 ± 0.04	0.74 ± 0.04	1.40 ± 0.08[b]	58.83 ± 3.03
S3	0.56 ± 0.03[b]	0.23 ± 0.03	0.76 ± 0.05	1.38 ± 0.05[b]	58.73 ± 3.81
S4	0.54 ± 0.05[b]	0.23 ± 0.04	0.76 ± 0.05	1.35 ± 0.06[b]	58.13 ± 3.30
S5	0.53 ± 0.06[b]	0.22 ± 0.03	0.74 ± 0.04	1.36 ± 0.07[b]	57.17 ± 3.39
S6	0.53 ± 0.05[b]	0.23 ± 0.04	0.75 ± 0.05	1.37 ± 0.06[b]	56.87 ± 4.70

*Values without common superscript (a-b) in a column differ (p < 0, 05); *a < b;'ALT= alanine aminotransferase; AST= aspartate aminotransferase; ALP=alkaline phosphatase; γGT= gamma-glutamyltransferase; LDH=lactate ehydrogenase; S0 = two weeks preceding the first application of treatment; S1 to S4= weeks of treatment; S5 and S6= weeks after the treatment period*

DISCUSSION

Influence of ethyl acetate fraction on hepatic tissue integrity

The levels of hepatic enzymes in many disease conditions in humans and other animals have been investigated extensively. Changes in these hepatic parameters have often proved to be an important aid in diagnosing select diseases and determining patients' prognosis [15].

Increase in the activities of ALT, AST, ALP, γ-GT and LDH in plasma is mainly due to the leakage of theses enzymes from the liver cytosol into the blood stream, an occurrence that reflects liver damage and disruption of normal liver function [15]. The initial levels of the various enzymes (ALT, AST, ALP, γ-GT and LDH) found for the control group are in conformity with the respective normal values reported by Medirabbit [16] and Founzegue *et al* [17]. The findings of the present study indicate that although aminotransferase levels increased during the first 3 weeks of treatment they returned to their initial values subsequently with further treatment. This result is better than that obtained for fraction F5 in a previous work where the aminotransferase levels remained high throughout the treatment period [18]. According to the interpretation of Schmidt and Schmidt [15], the initial increase in the enzyme levels found in the present study is moderate and transient and would not cause irreversible damage to the liver. Moreover, the increases observed were within the limits of the standard values of aminotransferases in rabbits [16]. Therefore, fraction F1, if used within the dose range of 25 to 100 µg/kg body weight would not have harmed hepatic cells.

Effect of AcE F1 on hepatic metabolism

The values of the hepatic metabolites (total cholesterol, cholesterol HDL, triglycerides, glucose and total proteins) obtained in this study prior to commencement of F1 treatment are in agreement with those stated by Medirabbit [16] and Founzegue *et al* [17]. According to Xavier [19], liver plays a role in the metabolism and regulation of glucose, lipids and proteins and any damage to the liver would lead to an increase in the levels of these metabolites. However, the present study reveals that only the concentration of total cholesterol and glucose showed a slightly increase; the concentrations of the other metabolites did not change. The slight increase in total cholesterol and glucose levels may be due to the lipid in food [20] and the effect of *Morinda morindoides* on insulin activity or on the metabolism of glycogen [20] rather than damage to hepatic cells. Notably, even these slight changes in these levels were neither dose- nor time-dependent and furthermore, they were within the limits of their standard values in rabbit, thus suggesting that fraction F1, when used in the tested dose range, would not be detrimental to hepatic metabolism.

CONCLUSION

The changes in hepatic aminotransferases and hepatic metabolism following administration of the ethyl acetate fraction of the hydroalchol extract of *Morinda morindoides* are small and transient. Overall, this fraction did not appear to exert any noxious effect on hepatic tissue or metabolism and should thus be well tolerated by the liver. It would, however, be necessary to carry out further studies, including cardiovascular, and renal tolerance studies as well as hematological and histopathological investigations in order to obtain a fuller picture of the safety profile of the extract fraction.

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