



Evaluation of the toxicity of a methanolic total extract of *Mitragyna ciliata* a natural anti-malaric

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ABSTRACT

Mitragyna ciliata (MYTA) is a Rubiaceae used in traditional medicine to treat malaria. Total methanolic extract of MYTA made was used for toxicological studies on laboratory mice and rats. The extract of MYTA was administered intraperitoneally. The doses ranged from 0 to 4000 mg/kg body weight (bw) (i.e., 0, 250, 500, 1000, 2000 and 4000 mg/kg bw) for acute toxicity, and from 0 to 475 mg/kg body weight (bw) (i.e., 0, 125, 250 and 475 mg/kg bw) for subacute toxicity studies. Following administration of high doses, the mice had jerky movements and died lying on their belly. The maximal tolerated dose (MTD) obtained was 500 mg/kg of bw, whereas 50% lethal dose (LD₅₀) was 1412 mg/kg of bw. The study of the effects of MYTA on the complete blood count, blood glucose and the serum markers ((urea, uric acid, creatinine, transaminases (GOT, GPT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), creatinine phosphokinase (CPK)) of noble organs of rats was performed. The results obtained with the serum enzymes (GOT, GPT, ALP, CPK and LDH) showed that no significant increase of activity occurred. This indicated that the heart and liver might not have suffered damages. Besides, significant decreases were observed in the serum activities of GOT, ALP and LDH, suggesting that MYTA could have a potential hepatoprotective effect. Nevertheless, significant increases were observed in the serum concentration of urea, uric acid and creatinine at high doses. This indicates the accumulation of these metabolites in the blood, which could be due to kidney injury, leading to a decrease in renal elimination of waste.

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Keywords: Toxicity, *Mitragyna ciliata*, anti-malaric.

INTRODUCTION

Most African people since the dawn of time, use the rich heritage of Africa for treatment. According to Adjanéhoun and Aké-Assi (1979), about 5000 plant species have been identified in Côte d'Ivoire. Also, Aké-Assi and Sita (1991) identified plants of western Africa used in traditional medicine. Among these plants, *Mitragyna ciliata*, a

Rubiaceae, has been found to possess many virtues. Indeed MYTA has an immunogenic potential, induced a positive inotropic and negative chronotropic effects on the contractile activity of isolated rat heart, had a hypotensive effect on the carotid artery pressure of rabbit, and caused lung hyperventilation and myorelaxation of rabbit's duodenum (Fofana 2004; Bidié, 2005, 2010).

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MYTA would also be cardiotoxic of the digitalic type (Bidié, 2005, 2008, 2010). Furthermore, MYTA is used traditionally to treat malaria (Adjetei, 2007). This anti-malaric potential of MYTA has been scientifically proven by Dje et al. (1997). The author has shown that MYTA inhibits *in vitro* growth of *Plasmodium falciparum* strain chloroquino-résistante FCM 29. It should also be noted that MYTA is administered orally or intravenously and therefore has access to all body functions. Thus, it seemed interesting to conduct studies of toxicity and biotolerance for a more rational use. The determination of toxicological parameters of the methanolic extract of MYTA therefore seems necessary since it must be administered to living organisms.

Since no toxicological study of total methanolic extract had been conducted to date, we have found it necessary to evaluate acute and subacute toxicities of this extract on laboratory animals. Indeed, the lethal dose 50 (LD₅₀), the lethal dose 100 (LD₁₀₀) and the maximal tolerated dose (MTD) of this phytomedicine were determined in laboratory mice, and some haematological and biochemical parameters in rats.

MATERIALS AND METHODS

Plant material and preparation of extract

Mitrgyna ciliata (voucher specimen N° 8888, deposited at the herbarium of the "Centre National Floristique (CNF)" of the University of Cocody-Abidjan, Côte d'Ivoire) is a tropical forest plant found in the Republic of Côte d'Ivoire. The methanolic extract from the bark of *M. ciliata* (MYTA) was prepared by stirring 50 g of finely ground portions in 1,5 L methanol using a magnetic stirrer (IKAMAG RCT) for 48 hrs at room temperature. The methanolic extract was filtered over cotton and Whatman N° 3 filter paper three times. The filtrate was evaporated at 40 °C under reduced pressure with a rotavapor (Buchi 461 Watter Batch). The residue obtained, dissolved in a physiological liquid (Mac-Ewen), was used to prepare MYTA solutions.

Animals

Swiss mice (males and females) aged 6 to 8 weeks and weighing 20 g on average, and rats aged 12 weeks and weighing 150 g

(Desfontis, 2004) on average were collected from the Institut Pasteur Adiopodoumé (Côte d'Ivoire).

Methods

Acute toxicity

The mice used for experimentation were of comparable age (6 to 8 weeks) and were placed in cages containing bedding of wood shavings. This litter was renewed every two days. The experiment consisted of 60 mice divided into 12 batches of 5 mice each, with 6 batches of male mice and 6 batches of female mice and substantially equal weight (20 ± 0.5 g). Intraperitoneally, different concentrations of MYTA were injected into mice using an insulin syringe of 1 mL. These concentrations (0, 250, 500, 1000, 2000 and 4000 mg/kg body weight) were prepared with a physiological solution (Mac-Ewen).

According to Berhens and Karber (1935), the LD₅₀ of a product can be determined using the following formula:

$$LD_{50} = LD_{100} - \frac{(a \times b)}{n}$$

with LD₁₀₀ = dose causing death of all animals in experimentation;

a = average number of animal death between two successive doses.

b = difference between two successive doses (mg/kg body weight).

n = number of animals used per batch or mean animals, with n = 5 mice.

Subacute toxicity

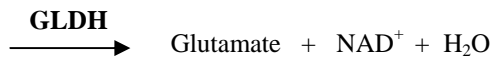
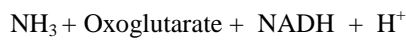
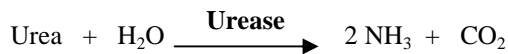
This experiment was conducted in three (03) months to determine the subacute toxicity of the product on animals by injecting the daily dose and repeated concentrations below the LD₅₀ of MYTA. Indeed, 4 batches of 3 rats each, with a control group, were used. For this second part of the study to determine the effect of MYTA on the noble organs (kidney, liver and heart) of animals, varying doses of MYTA were prepared from the MTD (i.e., 0, 125, 250 and 475 mg/kg body weight).

Determination of complete blood count (CBC) and serum markers of kidney, liver and heart

- *Determination of the CBC*: The method used was that described by Yapi et al. (2009).

Indeed, whole blood collected in tubes with anticoagulant (EDTA) was used to determine hemoglobin (Hb) concentration and mean blood cells volume (MBCV) using a semi-automatic blood cell counter.

- *Urea determination*: The enzymatic method used is based on the reaction described by Talke and Schubert (1695). The method has been optimized by Tiffany et al. (1972) who showed that the concentration of urea is proportional to the change in absorbance measured at 340 nm for a given time. The reaction scheme is as follows:



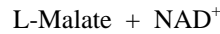
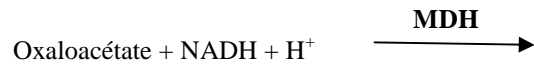
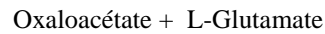
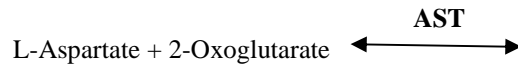
- *Uric acid determination*: According to Fossati et al. (1980) and Tietz (1999), uricase acts on uric acid to produce allantoin, carbon dioxide and hydrogen peroxide. In the presence of peroxidase, hydrogen peroxide reacts with a chromogen (dichlorohydroxybenzene sulfonate and amino-antipyrine) to form a quinonemine which is a red complex. The absorbance measured at 520 nm is proportional to the amount of uric acid.

- *Blood glucose determination*: According to the method of Trinder (1969) and Farrance (1987), glucose is oxidized by glucose oxidase (GOD) to form gluconic acid and H₂O₂. Then peroxidase (POD) reacts with H₂O₂, 4-chlorophenol and the plasma alkaline phosphatase (PAP) to form a red quinonemine. The absorbance of colored complex, proportional to the concentration of glucose in medium, was measured at 500 nm.

- *Determination of creatinine*: This assay is based on colorimetric reaction (Jaffe reaction without pre-stage processing of the specimen) of creatinine with picric acid in alkaline medium. The kinetics of the reaction is measured at 490 nm. This method has been optimized by Fabiny et al. (1971) and Labbe (1996).

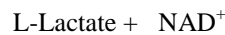
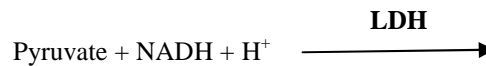
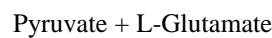
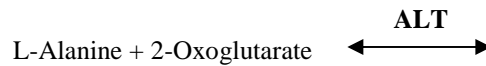
- *Determination of transaminases*: The serum glutamo-oxaloacetic transaminase (GOT, AST) method was developed by Karmen et al.

(1955) and optimized by Henry et al. (1960) (consistent with the recommendations of the IFCC (1986)). The reaction scheme is as follows:



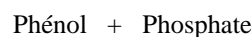
The decrease in absorbance, due to conversion of NADH to NAD⁺ and proportional to the AST activity in the specimen, is measured at 340 nm.

The serum glutamo-pyruvic transaminase (GPT, ALT) method was developed by Wroblewski and La Due, and optimized by Henry (1960) and Bergmeyer (1978) (consistent with the recommendations of the IFCC (1986)). The reaction scheme is as follows:



The decrease in absorbance measured at 340 nm due to conversion of NADH to NAD⁺ is proportional to ALT activity in the specimen.

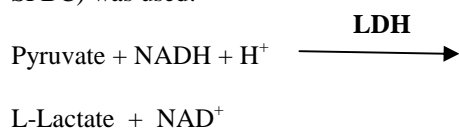
- *Determination of Alkaline Phosphatase (ALP)*: The activity of ALP was determined according to the method of Withold (1996), through the following reaction:



The phenol liberated by hydrolysis of the substrate reacts in the presence of 4-amino-antipyrine and potassium ferricyanide to form a red complex whose absorbance is measured at 510 nm and is directly

proportional to ALP activity in the specimen. Sodium arsenate was used to stop the enzymatic reaction and prevents instability of the color observed with previous methods.

- *Determination of lactate dehydrogenase (LDH)*: The method of Henry et al. (1974) (consistent with the recommendations of SFBC) was used:



The decrease in absorbance (measured at 340 nm) due to conversion of NADH to NAD⁺ was directly proportional to LDH activity in the specimen.

-*Determination of creatinine phosphokinase (CPK)*: The activity of CPK was determined according to the method of Fabiny and Ertingsthausen (1971), optimized by Labbé et al. (1996). It is a colorimetric reaction of creatinine with picric acid under alkaline conditions and the kinetics of the reaction measured at 490 nm.

Statistical analysis

Experiments were run in duplicate, and statistical analyses were performed using the Graph Pad Prism 5.0 software (Microsoft). Analyses of variance (ANOVAs) were conducted and multiple comparisons between means were performed by Tukey and Dunnett procedure. Significance level was defined as $P < 0.05$. Results were given as means \pm standard deviation.

RESULTS

Acute toxicity

Animal behavior

Injection doses of total methanolic extract of MYTA between 250 and 4000 mg/kg bw to the animals showed the following events:

- Regrouping trends followed by immobilization of the mouse was extended over a period of 3 min to 10 hours depending on the doses administered,
- Accelerated respiratory rate (tachypnea)
- Tremor
- Disruption of feeding behavior (loss of appetite)

Immobilization and respiratory disorder are signs preceding the death of mice. These animals died lying on their stomach or side. The deaths were recorded in batches 4, 5 and 6 of male and female mice at doses respectively 1000, 2000, 4000 mg/kg of bw (Table 1). In control batches of mice, no apparent abnormal signs were observed. The mice kept the same aspect, either on behavioral or nutritional.

After 14 days of observation, all mice died in batch 5 (males and females) over a three-day period. It was observed a gradual recovery of mice from other lots, which was illustrated by a return to normalization of respiratory rate and feeding behavior similar to control groups of mice. Total recovery appeared after 3 days.

Subacute toxicity

Behavior of rats after 21 days of observation

This second part of the study was conducted on rats divided into 4 lots of 5 rats each with a control lot. The doses used were based on the MTD. Thus the doses ranged from 0 to 475 mg/kg body weight (0; 125; 250; 475 mg/kg bw).

Observation of rats 21 days after repeated administration of the product showed no mortality in control lot (lot 1) and in lot 2 of rats. However, in the experimental lots (lot 3 and lot 4) of rats, there were deaths. The dose 250 mg/kg bw resulted in death of 1 rat during the first 5 days of repeated treatment. The dose 475 mg/kg bw caused the death of 2 rats on the second day of repeated injection of the product. On the fifth day of treatment, rats from lots 3 and 4 were all weakened. They were motionless with rapid breathing. In addition, there was a change in their nutritional status, which was characterized by a loss of appetite. This change was more pronounced in the lot 4. During the following 14 days, rat that survived, resumed their usual feeding habits as in controls.

Behavior of rats after 3 months of observation

During the 40 days following the first 21 days of observation of animals in control groups and experimental batches (batch 2 and batch 3), no conduct disorder or change in their general appearance and nutritional status

was observed. No mortality was recorded. 30 days (1 month) of observation after these 2 months, no death of any animal throughout the experimental period was recorded. Only some signs of weakness were observed.

Effect on blood cells counts

This study revealed a variation of white blood cells (WBC), red blood cells (RBC), hemoglobin (Hb) and platelets after treatment of animals with MYTA (Table 3). Indeed, the WBC went from 10.47 ± 0.51 to 11.78 ± 0.67 , RBC from 8.33 ± 0.18 to 9.45 ± 0.43 , Hb from 12.52 ± 0.77 to 15.21 ± 0.44 and platelets from 1077 ± 25.51 to 1265 ± 25.41 . For other elements of the CBC except the hematocrit (HCT) and Neutrocytes that increase, a general reduction of all other elements (Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH), Mean corpuscular hemoglobin concentration (MCHC), lymphocytes, monocytes Eosinocytes and Basocytes) was observed (Table 3).

Effect on the kidneys

Biochemical parameters that give an idea of the state of the kidneys or renal activity are: urea, uric acid and creatinine. Generally, increases were observed in the serum concentrations of the three parameters in the experimental groups, compared to the control group. Indeed, for urea, uric acid and creatinine, control values were 0.19 ± 0.008 ;

25.91 ± 0.10 and 7.36 ± 0.23 , respectively. These values were increased to 0.45 ± 0.01 ; 35.55 ± 2.93 and 15.27 ± 0.44 respectively; for the dose of 475 mg/kg body weight of MYTA.

Effect on the heart

In order to get an idea of the condition of the heart of animals subjected to this experiment, the activities of serum transaminases (GOT, GPT) and other enzyme markers of the integrity of the heart (CPK and LDH) were determined. Indeed, the results revealed a change in experimental values from control values (GOT = 191.1 ± 2.93 ; GPT = 63.04 ± 0.4 ; CPK = 101.4 ± 087 and LDH = 242.5 ± 2.92). The experimental values indicate: (GOT = 173.3 ± 5.19 ; GPT = 49.11 ± 18.61 ; CPK = 89.38 ± 9.38 and LDH = 195.4 ± 5.32) after treatment with 475 mg/kg body weight of MYTA.

Effect on the liver

In addition to the activity of serum transaminases, CPK and LDH, blood glucose and serum activity of ALP have been identified. Thus, after treatment with MYTA (475 mg/kg of bw), changes in blood glucose from 1.49 ± 0.008 (control) to 1.12 ± 0.27 and the activity of ALP from 264.6 ± 1.98 to 180.4 ± 11.50 were observed.

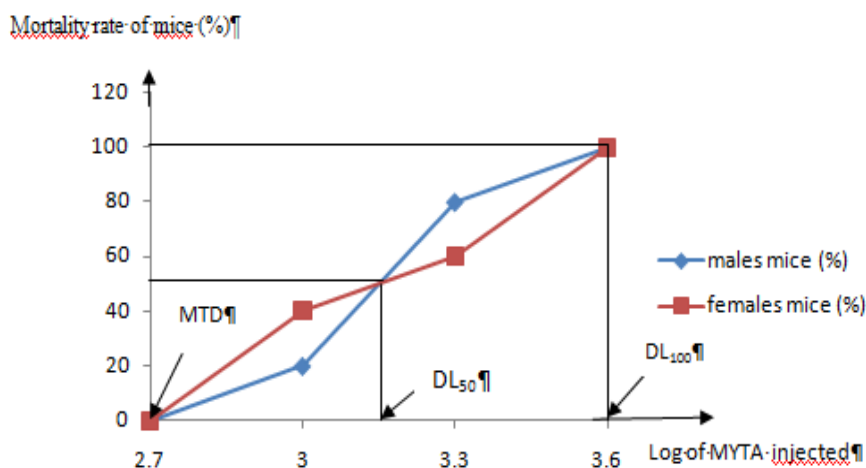


Figure 1: Mortality rate of mice as a function of levels of total methanolic extract of *Mitragyna ciliate*.

MTD: maximal tolerated dose; LD50: lethal dose 50; LD100: lethal dose 100.

Table 1: Mortality of male mice as a function of levels of total methanolic extract of *Mitragyna ciliata*.

Batches Time after injection	Batch 1 (0 mg/kg)		Batch 2 (250 mg/kg)		Batch 3 (500 mg/kg)		Batch 4 (1000 mg/kg)		Batch 5 (2000 mg/kg)		Batch 6 (4000 mg/kg)	
	M	F	M	F	M	F	M	F	M	F	M	F
	5	5	5	5	5	5	5	5	5	5	5	5
15 min	/	/	/	/	/	/	/	/	/	/	1	3
30 min	/	/	/	/	/	/	/	/	1	1	2	3
1 hour	/	/	/	/	/	/	/	1	1	2	2	4
5 hours	/	/	/	/	/	/	/	1	1	2	2	4
10 hours	/	/	/	/	/	/	/	2	1	2	4	5
15 hours	/	/	/	/	/	/	1	2	2	3	5	5
20 hours	/	/	/	/	/	/	1	2	4	3	5	5
24 hours	/	/	/	/	/	/	1	2	4	3	5	5
mortality (%)	/	/	/	/	/	/	20	40	80	60	100	

M: male, F: female

Table 2: Acute toxicity parameters.

Parameters	MTD	LD ₅₀	LD ₁₀₀
Extract level (Total methanolic extract of			
<i>Mitragyna ciliata</i>) (mg/kg)	500	1412	4000

MTD: maximal tolerated dose, LD₅₀: lethal dose 50, LD₁₀₀: lethal dose 100

Table 3: Effect of MYTA on hematological and biochemical parameters of the kidneys, liver and heart.

Samples Parameters	Lot 1 (Control, 0 mg/kg)	Lot 2 (125 mg/kg)	Lot 3 (250 mg/kg)	Lot 4 (475 mg/kg)
	WBC (WBC/ μ L)	10.47 \pm 0.51	6.83 \pm 0.20 *	11.78 \pm 0.67
RBC (RBC/ μ L)	8.33 \pm 0.18	7.03 \pm 0.35	8.62 \pm 0.26	9.45 \pm 0.43
Hb (g/dL)	12.52 \pm 0.77	12.44 \pm 0.36	12.76 \pm 0.58	15.21 \pm 0.44 *
HCT (%)	41.85 \pm 1.31	40.48 \pm 2.20	39.54 \pm 2.23	42.11 \pm 1.68
TCMH (pg)	15.99 \pm 0.13	16.48 \pm 1.18	14.5 \pm 0.46	15.27 \pm 0.40
MCHC (g/dL)	31.20 \pm 0.27	31.28 \pm 0.46	32.06 \pm 0.42	32.18 \pm 0.21
Platelets (Platelets/ μ L)	1077 \pm 25.51	1114 \pm 8.29	1259 \pm 194.3	1265 \pm 25.41
Lymphocytes (%)	63.15 \pm 2.43	61.51 \pm 1.24	57.33 \pm 4.36	56.60 \pm 2.54
Monocytes (%)	6.42 \pm 0.55	5.89 \pm 0.64	4.4 \pm 0.20	4.2 \pm 0.17
Neutrocytes (%)	27.28 \pm 2.94	26.54 \pm 0.75	37.88 \pm 3.68	38.12 \pm 8.32 *
Eosinocytes (%)	2.87 \pm 0.11	2.19 \pm 0.23	1.87 \pm 0.30	1.80 \pm 0.65
Basocytes (%)	00	00	00	00

Urea (g/L)	0.19 ± 0.008	0.22±0.008	0.30±0.005 *	0.45±0.01 *
Uric acid (mg/L)	25.91 ±0.10	26.55±0.38	28.94±1.06 *	29.55±2.93 *
Creatinine (mg/L)	7.36 ±0.23	7.94±0.34	10.10±0.95 *	15.27±0.44 *
Blood glucose (g/L)	1.49 ±0.008	1.25±0.04	1.16±0.25	1.12±0.27
GOT (UI/L)	191.1 ±2.93	187.6±2.62	182.9±5.01	173.3±5.19 *
GPT (UI/L)	63.04 ±0.4	55.87±2.65	52.25±17.6	49.11±18.61
ALP (UI/L)	264.6 ±1.98	209.7±10.05 *	202.7±19.79 *	180.4±11.50 *
CPK (UI/L)	101.4 ±0.87	91.86±3.59	90.12±4.11	89.38±9.38
LDH (UI/L)	242.5 ±2.92	243.5±6.24	220±14.01	195.4±5.32 *

* : significantly different from the control.

WBC : White blood cells; RBC : Red blood cells; Hb : Hemoglobin; HCT : Hematocrit; MCHC : Mean corpuscular hemoglobin concentration; GOT : Glutamo-oxaloacetic Transaminase ; GPT : Glutamo-Pyruvic Transaminase; ALP : Alkaline Phosphatase; CPK : Creatine Phosphokinase; LDH : Lactate dehydrogenase.

DISCUSSION

The results of acute toxicity tests showed that increasing the dose of the total extract of MYTA led to an increase in mortality in mice subjected to the test, and the proportion of affected animals increased with increasing dose. This suggests that there is then a dose-response relationship between the dose of the extract administered and mortality recorded in animals (Fatima et al., 2010).

This study determined the acute toxicity parameters of total methanolic extract after intra-peritoneal injection in mice. These are: the MTD (500 mg/kg of bw), the LD₅₀ (1412 mg/kg of bw) and LD₁₀₀ (4000 mg/kg bw). These parameters indicate that the administration of doses below the MTD induced no mortality, as also shown by Lapointe (2004) and Fatima et al. (2010).

However, doses above the MTD induces deaths for which the rate varies with the dose (Kouassi, 2006). At doses between MTD and the LD₅₀, the mortality rate of female mice is higher than that of males (Laroche et al., 1986). At doses above the LD₅₀, the mortality rate among males is higher (Laroche et al., 1986; Sitwell, 2007). Generally, the LD₅₀ reflects the lethal nature of a substance (Langley et al., 2003).

Moreover, the time of action of the product was faster in female mice than in males. This suggests that female mice are more sensitive to the total methanolic extract of *Mitragyna ciliata* than males. The sensitivity of the animal towards a given drug

may vary according to sex (Laroche et al., 1986; Sitwell, 2007).

In addition, the value of LD₅₀ estimated at 1412 mg/kg bw (3 times that of the MTD) in male and female mice helps to classify the total methanolic extract of MYTA as a moderately toxic substance according to the scale of LD₅₀ classification by Cotonat (1996). The clinical signs of toxicity presented by the mice were:

- Respiratory rate acceleration
- Tremor
- Immobilization of the animal.

According to the table of relationship between clinical signs of intoxication and organs (Laroche et al., 1986) or systems (Hachette, 1989), one might infer that the total methanolic extract of MYTA had an effect on the respiratory system. This finding is in agreement with the work performed by Bidie (2005) on the effect of MYTA on rabbit breathing. According to the author, MYTA induced excessive degradation of lungs. It would also have an action on the neuromuscular system.

The results obtained during the 14 days following the 24 hours of observation showed that the total methanolic extract of MYTA, given as a single dose, had a toxic action that extended over a period of 15 min to 3 days. Beyond 24 hours, mice that survived gradually recovered after 5 days. Given this observation, we can say that the product accumulates in the body and its elimination is retarded, resulting in a longer recovery time.

These results are consistent with the work of Bidie et al. (2010) on the duration of action of MYTA and restoration of carotid blood pressure of rabbits. This suggests that biotransformation of MYTA was slowed, resulting in the accumulation or difficult elimination of metabolites. This could be the basis of damage to organs and tissues.

The study of the subacute toxicity at doses 250 mg/kg bw and 475 mg/kg bw in rats led to the following clinical signs: weakening, immobilization, loss of appetite, increased respiratory rate of rats. These signs of intoxication lasted for 2 to 3 days. This may be due to the accumulation of the MYTA in the body. The return to normal of rats treated after 3 days, compared to controls, is evidence that animals have eliminated the product that had been administered. However, the deaths could be explained by the fact that these animals could not eliminate the product accumulated in the body on time. This suggests that the metabolism of MYTA may be difficult.

Furthermore, the results obtained with the serum enzymes (GOT, GPT, ALP, CPK and LDH) showed that no significant increase of activity occurred. This indicated that the heart and liver might not have suffered damages. Besides, significant decreases were observed in the serum activities of GOT, ALP and LDH, suggesting that MYTA could have a potential hepatoprotective effect. Nevertheless, significant increases were observed in the serum concentration of urea, uric acid and creatinine. This indicates the accumulation of these metabolites in the blood. This suggests a kidney injury, leading to a decrease in renal elimination of waste. The increase observed may also be due to an increased production of these metabolites.

Conclusion

These studies of acute and subacute toxicity of the total methanolic extract of MYTA lead to the determination of the MTD (500 mg/kg of bw), the LD₅₀ (1412 mg/kg of bw) and LD₁₀₀ (4000 mg/kg bw), in addition to some biochemical indices of toxicity. The

results obtained showed the moderate toxic nature of the product, suggesting its safe use at therapeutic dose in humans. The observed toxicity is likely due to difficulties in the elimination of MYTA from the body or its metabolism. However, other tests such as histology of organs and chronic toxicity tests among others are necessary to ensure MYTA moderate toxicity.

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