



RESEARCH PAPER

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Evaluation of immunostimulatory activity of alkaloids *Mitragyna ciliata* in rabbits (*Oryctolagus cuniculus*)

Abba Pacôme Obouayeba^{1*}, Toussaint Latte², Mathurin Okoma³, Sékou Diabaté³, Tanoh Hilaire Kouakou⁴

¹Laboratory of Biochemical Pharmacodynamics, Department of Biosciences, Félix Houphouët Boigny University of Abidjan, Abidjan, Côte d'Ivoire

²Unit of Biochemistry, Central Laboratory, University Hospital of Yopougon, Côte d'Ivoire, Abidjan

³Unit of Physio/Phytopathology, Central Laboratory of Biotechnology, National Agricultural Research Center, Abidjan route Dabou, Côte d'Ivoire

⁴Laboratory of Biology and Crop Improvement, Department of Natural Sciences, Nangui Abrogoua University of Abidjan, Abidjan, Côte d'Ivoire

Key words: *Mitragyna ciliata*, methanolic extracts, total alkaloids extracts, phytochemical analysis, immunostimulatory study.

<http://dx.doi.org/10.12692/ijb/5.8.200-206>

Article published on October 29, 2014

Abstract

Mitragyna ciliata (Rubiaceae) is a plant used in traditional medicine in the treatment of malaria in Côte d'Ivoire. The research for other properties in particular immunostimulatory, of this plant to strengthen malaria treatment is the cause of this study through the determination of phytochemical constituents of the methanolic extracts and the effect of its total alkaloids on cell and humoral markers of immunity of rabbit (*Oryctolagus cuniculus*). The immunostimulatory study was carried out with biological parameters such as white blood cells, gamma globulin, total proteins and albumin/globulin ratio. The qualitative phytochemical analysis showed the richness of this plant in phytochemical compounds. These are alkaloids, anthocyanins, flavonoids, polyphenols, saponins, sterols, terpenes and tannins. Concerning the immunostimulatory study, two major results were obtained. The first element is the fact that within a homogeneous population of rabbits, the rate of biological parameters is not a fixed value. The second element is the fact that the alkaloids *Mitragyna ciliata* not found to have immunostimulatory activity. However, *Mitragyna ciliata* remains an important medicinal plant made many pharmacological proprieties, due to the presence of its phytochemical compounds.

*Corresponding Author: Abba Pacôme Obouayeba ✉ obouapac@yahoo.fr

Introduction

Among the most dangerous to humans diseases are infectious diseases that are a major cause of death worldwide according to the World Health Organization (WHO) (Obouayeba, 2010). The integrity of the organism towards microorganisms is ensured by the mechanical defenses, chemical, and especially by the immune system (Pichard *et al.*, 2002). The strengthening of this system is one of the concerns of the scientific research. Thus, alongside modern medicine, his investigations relevant to substances extracted from medicinal plants used in traditional medicine. The use of plants in therapy is certainly very old but is currently experiencing a resurgence of interest among the public in spite of the advances in modern medicine (Aboughe *et al.*, 2009). In Côte d'Ivoire, this traditional medicine which has about five thousand species of medicinal plants listed by Adjanohoun and Aké (1979) is an advantage for scientific research and real hope for the people. The works on these plants have revealed several pharmacological properties including immunostimulatory activity present in *Aerva lanata* (Kra *et al.*, 2007), *Alternanthera repens* (Zirih *et al.*, 2001), and *Mitragyna ciliata* (Fofana, 2004).

This species is used in traditional medicine in the countries of Gulf of Guinea and has been the subject of several scientific investigations for its many healing properties. These studies have shown that *Mitragyna ciliata* has many pharmacological properties including antimalarial activity (Djè *et al.*, 1997; Traoré *et al.*, 2000; Adjétey *et al.*, 2007), trypanocidal activity (Ogbunugafor *et al.*, 2007), and anti-inflammatory activity (Dongmo *et al.*, 2003). In addition to these therapeutic properties, it is appropriate to examine whether these molecules also have immunostimulatory activity. This would have the advantage of providing an improved traditional medicine consists of a single molecule, in contrast to the one available on the basis of the works of Djè *et al.* (1997) and Fofana (2004). It would help to reduce the cost in the treatment of malaria and would be a distinct advantage for the population. This study falls within the overall framework of the policy of

reevaluation of medicinal plants of the Ivorian pharmacopoeia. So she set the goal to assess the immunostimulatory activity of alkaloids *Mitragyna ciliata* (Rubiaceae) in rabbits. This investigation has two aspects such as phytochemical qualitative analysis of the methanolic extracts of *Mitragyna ciliata* and evaluation of immunostimulatory activity of these plant alkaloids on cell and humoral markers of immunity of rabbit.

Materials and methods

Plant material

The bark of *Mitragyna ciliata* (Rubiaceae) was used as plant material for this study. It was collected in the locality of Jacquville, south-East of Côte d'Ivoire in March 2009. Plant material was washed, cut, dried for several weeks in the laboratory and ground to a fine powder using a mechanical grinder (IKAMAG).

Experimental animal

Male rabbits of the species *Oryctolagus cuniculus* (Leporidae), the mean body weight were 1.2 ± 0.2 kg were used for this study. These animals which came from the animal house of the Pasteur Institute of Adiopodoumé (Abidjan, Côte d'Ivoire) were housed in cages in the animal house of the Biosciences Training and Research Unit, at room temperature. They had free access to food (pellets from Ivograins, Côte d'Ivoire) and water. All the experimental procedures were approved by the Ethical Committee of Health Sciences, Félix Houphouët Boigny University of Abidjan. These guidelines were in accordance with the European Council Legislation 87/607/EEC for the protection of experimental animals.

Chemicals

All chemicals used for analyzes meet the quality standards in accordance with international standards. This is acetic acid, hydrochloric acid, sulfuric acid, methanol, ethanol, dichloromethane, sodium carbonate, sodium hydroxide, magnesium sulphate, glucose, and potassium chloride purchased from Merck Co. (Darmstadt, Germany). Ammonia, calcium chloride, magnesium chloride, sodium dihydrogen phosphate, sodium hydrogen carbonate, ferric

chloride, Dragendorff reagent, the reagent Bouchardat, Stiasny reagent, sodium acetate, formaldehyde and poppy red were obtained from Sigma-Aldrich Co. (Steinheim, Germany).

Preparation of methanolic extracts and total alkaloid extracts of Mitragyna ciliata

Methanolic extracts (ME)

The preparation of this extract was made in accordance with the method described Zirihi *et al.* (2005 a). One hundred grams (100 g) of *Mitragyna ciliata* powder were extracted in 2000 mL of methanol by maceration for 24 hours using a magnetic stirrer (IKAMAG RTC) at room temperature. The macerate was filtered successively on cotton wool and Whatman filter paper (3 mm). The filtrate was concentrated to dryness under reduced pressure at 38°C with BÜCHI rotavapor R-114. The extracts obtained constitute the methanolic extracts (ME) of *Mitragyna ciliata*.

Total alkaloid extracts (TAE)

The preparation of total alkaloid extracts was made using the method described by Zirihi *et al.* (2005 b). Ten grams (10 g) of methanolic extracts were dissolved in 150 mL of dichloromethane, acidified with 40 mL of hydrochloric acid 0.5 N (pH 2) and 180 mL of distilled water. The solution was mixed and allowed to stand. The organic phase was collected, 40 mL of sodium carbonate 0.5 N (pH 10) and two or five drops of sodium hydroxide were added to the aqueous phase after stirring 150 mL of dichloromethane were added to the solution. The solution was again mixed and the organic layer was collected. This operation was repeated three times. Different organic phases were pooled, dried with anhydrous magnesium sulfate and filtered on Whatman filter paper (3 mm). The filtrate was concentrated under reduced pressure at 38°C using a rotavapor R-114. The resultant extracts constitute the total alkaloid extracts (TAE) of *Mitragyna ciliata*.

Preliminary phytochemical analysis

Qualitative phytochemical screening was carried from the methanolic extracts of *Mitragyna ciliata*. The

presence of some phytoconstituents was highlighted by standard procedures used in phytochemistry. Phytochemical analysis alkaloids, anthocyanins, flavonoids, polyphenols, quinones, saponins, sterols, terpenes and tannins were performed following the methods described by Harborne (1998) and Phillipson (2001).

Assessment of immunostimulatory activity

Experimental protocol

The assessment of immunostimulatory activity of the total alkaloid extracts (TAE) of *Mitragyna ciliata* was carried out with 15 rabbits according to the method described by Kra *et al.* (2007). The animals were divided into five experimental groups of three rabbits. The rabbits of group 1 (control) received 1 mL of Mac Ewen (physiological solution). The rabbits of groups 2, 3, 4 and 5 received respectively 10, 20, 25 and 30 mg/kg body weight of total alkaloid extracts (TAE) dissolved in 1 mL of Mac Ewen. The different solutions were administered via intraperitoneal route at single dose. During 14 days of the experimental phase, blood samples were carried out on animals before treatment, the first day (D1). After treatment, blood samples were taken successively on the third day (D3), the seventh day (D7) and the fourteenth day (D14). The blood was collected with sterile needles microlances.

Biological parameters

The choice of the biological parameters has been made to study and Zirihi *et al.* (2003). These include the complete blood count (CBC), total proteins and protein electrophoresis.

Determination of the CBC

The method described by Yapi *et al.* (2009) was used for this analysis. Indeed, whole blood collected in tubes with anticoagulant (EDTA) was used to determine mean of white blood cells volume using a semi-automatic blood cell counter (Sysmex XT - 2000i).

Biochemical parameters

The blood collected into tubes without anticoagulant

(dry tubes) was allowed for 30 min at 37°C for coagulation, and centrifuged at 2500 rpm/min for 10 min. The serum obtained was used to achieve the biochemical analyzes (total proteins and protein electrophoresis). The total proteins were measured using an automatic analyzer (180 HumaStar) of biochemistry according to the biuret reaction. The Helena method of serum protein electrophoresis was used for separation and quantification of serum proteins by electrophoresis on cellulose acetate (Alper, 1974).

Statistical Analysis

Data were processed using Statistica SPSS package version 7.5. Analysis of variance (ANOVA) was performed and means were separated by Newman-Keuls range test at $P < 0.05$. Data are expressed as mean $\pm$ SD, $n = 3$.

Results and discussion

Preliminary phytochemical analysis

The screening of plants for medicinal value has been carried out by numerous researchers with the help of preliminary phytochemical analysis (Phillipson, 2001; Obouayeba *et al.*, 2014). Phytochemical screening test is of paramount importance in identifying new source of therapeutically and industrially valuable compound having medicinal significance, to make the best and judicious use of available natural wealth. The

qualitative phytochemical study of the methanolic extracts of *Mitragyna ciliata* results are presented in table 1. The phytochemical compounds with a positive test are present in the extracts and those with a negative test does not contain. The results of table 1 indicated that the methanolic extracts of *Mitragyna ciliata* contained some phytochemical groups such as alkaloids, anthocyanins, flavonoids, polyphenols, saponins, sterols, terpenes and tannins. These results corroborate those of Bidié *et al.* (2011). The presence of these phytochemical compounds in the methanolic extracts of *M. ciliata* is the source of several pharmacological properties of this plant. Indeed, this has been highlighted by the works of several authors including antimalarial activity (Djé *et al.*, 1997; Traoré *et al.*, 2000; Adjétey *et al.*, 2007), anti-inflammatory activity (Dongmo *et al.*, 2003) and trypanocidal activity (Ogbunugafor *et al.*, 2007). Furthermore, the methanol extracts of this plant showed moderate toxicity through the works of Bidié *et al.* (2010). The presence of anthocyanins and flavonoids known molecules with antioxidant activity (Obouayeba *et al.*, 2014) is evidence that *Mitragyna ciliata* shares this pharmacologic property (Bidié *et al.*, 2011). All these proven therapeutic properties explain the high usage of this plant in traditional medicine by people of African countries in the Gulf of Guinea in general and that particularly in Côte d'Ivoire.

Table 1. Phytochemical constituents of the methanolic extracts (ME) of *Mitragyna ciliata*.

Phytochemical groups	Methanolic extracts
Alkaloids	+
Anthocyanins	+
Flavonoids	+
Polyphenols	+
Quinones	-
Saponins	+
Sterols	+
Tannins	+
Terpenes	+

(+) Present; (-) Absent.

Assessment of immunostimulatory activity

Statistical analysis of the results of the various biological parameters such as white blood cells (table 2), gamma globulins (table 3), albumin/globulin ratio

(table 4) and total proteins (table 5) of immunostimulatory study revealed no statistical difference ($P < 0.05$) in each case between the results of Mac Ewen (control) and those of different doses of

the total alkaloid extracts (TAE) before treatment (D1) and after treatment (D3, D7, D14). It appears that the total alkaloid extracts would not have immunostimulatory activity proved. However, the results of works of Fofana (2004), have shown that proteins of *Mitragyna ciliata* possess immunostimulatory activity. Similarly, the works of Kra *et al.* (2007) showed immunostimulatory activity

in vivo in rabbits by a fraction of glycoproteins isolated from *Aerva lanata*. Knowing that protein structure is much more complex than that of alkaloids therefore a molecular weight of proteins higher than that of alkaloids, one could easily understand that the immunostimulatory power of total alkaloids of *Mitragyna ciliata* was not prevented.

Table 2. White blood cells volume as a function of time for each treatment.

Groups	Treatements	White blood cells volume (μL)			
		D1	D3	D7	D14
Group 1	1 mL of Mac Ewen	3,54 ± 0,70 ^{ab}	3,77 ± 0,44 ^{ab}	3,69 ± 0,80 ^{ab}	3,60 ± 0,40 ^{ab}
Group 2	10 mg/kg bw of TAE	3,70 ± 1,10 ^{ab}	4,02 ± 0,82 ^{ab}	3,80 ± 0,62 ^{ab}	3,50 ± 0,74 ^{ab}
Group 3	20 mg/kg bw of TAE	4,02 ± 0,55 ^{ab}	4,40 ± 0,90 ^a	4,05 ± 0,73 ^{ab}	3,75 ± 0,40 ^{ab}
Group 4	25 mg/kg bw of TAE	3,85 ± 0,34 ^{ab}	5,10 ± 1,30 ^a	4,30 ± 0,90 ^a	3,90 ± 0,70 ^{ab}
Group 5	30 mg/kg bw of TAE	4,20 ± 0,90 ^a	5,75 ± 1,15 ^a	4,80 ± 0,50 ^a	4,60 ± 1,05 ^a

Values are expressed as mean ± SD, n = 3.

Means followed by the same letter are not statistically different (P<0.05).

Table 3. Concentration of gamma globulins as a function of time for each treatment.

Groups	Treatements	Concentration of gamma globulin (g/L)			
		D1	D3	D7	D14
Group 1	1 mL of Mac Ewen	2,07 ± 0,50 ^{ab}	2,20 ± 0,35 ^{ab}	2,15 ± 0,70 ^{ab}	2,09 ± 0,40 ^{ab}
Group 2	10 mg/kg bw of TAE	1,70 ± 0,70 ^{ab}	1,97 ± 0,84 ^{ab}	1,90 ± 0,45 ^{ab}	1,84 ± 0,50 ^{ab}
Group 3	20 mg/kg bw of TAE	2,22 ± 0,60 ^{ab}	2,70 ± 0,80 ^a	2,50 ± 0,50 ^a	2,30 ± 0,60 ^{ab}
Group 4	25 mg/kg bw of TAE	1,85 ± 0,40 ^{ab}	3,05 ± 0,75 ^a	2,70 ± 0,65 ^a	2,50 ± 0,45 ^a
Group 5	30 mg/kg bw of TAE	1,90 ± 0,75 ^{ab}	3,53 ± 0,60 ^a	2,95 ± 0,55 ^a	2,62 ± 0,70 ^a

Values are expressed as mean ± SD, n = 3.

Means followed by the same letter are not statistically different (P<0.05).

Moreover, the works of several authors have shown that *M. ciliata* has many pharmacological properties. Indeed, the results of the works of Djé *et al.* (1997) showed antiplasmodial activity of total alkaloids of *M. ciliata*, and those Adjétey *et al.* (2007) revealed an antimalarial activity of the methanolic extracts of this plant. The anti-inflammatory (Dongmo *et al.*, 2003) and trypanocidal activities (Ogbunugafor *et al.*, 2007) have been put in evidence from studies with extracts of *Mitragyna ciliata*. The biological parameters

(white blood cells, gamma globulin, total protein and albumin/globulin ratio) in the first day (D1) range without being significantly different (P<0.05). These results showed that in a single homogeneous population of rabbits, these biological parameters is not a fixed value, but varies to some margin consistent with normal life rabbits. These results are in according to with those of Kra *et al.* (2007) and Yapi *et al.* (2011).

Table 4. Albumin/globulins ratio as a function of time for each treatment.

Groups	Treatements	Albumin/globulins ratio			
		D1	D3	D7	D14
Group 1	1 mL of Mac Ewen	1,74 ± 0,40 ^a	1,81 ± 0,30 ^a	1,85 ± 0,50 ^a	1,80 ± 0,25 ^a
Group 2	10 mg/kg bw of TAE	1,78 ± 0,60 ^a	1,76 ± 0,75 ^a	1,83 ± 0,62 ^a	1,79 ± 0,55 ^a
Group 3	20 mg/kg bw of TAE	1,75 ± 0,53 ^a	1,70 ± 0,40 ^{ab}	1,88 ± 0,55 ^a	1,82 ± 0,80 ^a
Group 4	25 mg/kg bw of TAE	1,80 ± 0,74 ^a	1,68 ± 0,25 ^{ab}	1,82 ± 0,50 ^a	1,87 ± 0,60 ^a
Group 5	30 mg/kg bw of TAE	1,77 ± 0,30 ^a	1,63 ± 0,50 ^{ab}	1,84 ± 0,60 ^a	1,92 ± 0,45 ^a

Values are expressed as mean ± SD, n = 3.

Means followed by the same letter are not statistically different (P<0.05).

Table 5. Concentration of total proteins as a function of time for each treatment.

Groups	Treatments	Concentration of total proteins (g/L)			
		D1	D3	D7	D14
Group 1	1 mL of Mac Ewen	39,83 ± 2,50 ^{ab}	40,37 ± 3,40 ^{ab}	40,00 ± 2,85 ^{ab}	39,23 ± 2,30 ^{ab}
Group 2	10 mg/kg bw of TAE	40,05 ± 3,20 ^{ab}	41,55 ± 2,75 ^{ab}	39,98 ± 3,50 ^{ab}	38,85 ± 2,70 ^{ab}
Group 3	20 mg/kg bw of TAE	41,77 ± 5,45 ^{ab}	43,30 ± 3,80 ^a	41,50 ± 2,35 ^{ab}	39,80 ± 3,45 ^{ab}
Group 4	25 mg/kg bw of TAE	41,50 ± 3,50 ^{ab}	45,40 ± 4,25 ^a	41,80 ± 4,60 ^{ab}	40,25 ± 2,20 ^{ab}
Group 5	30 mg/kg bw of TAE	41,25 ± 4,20 ^{ab}	46,90 ± 3,50 ^a	43,40 ± 5,50 ^a	41,70 ± 3,60 ^{ab}

Values are expressed as mean ± SD, n = 3.

Means followed by the same letter are not statistically different (P<0.05).

Conclusion

This investigation of *Mitragyna ciliata* revealed the richness of this plant in phytochemical compounds such as alkaloids, anthocyanins, flavonoids, polyphenols, saponins, tannins, terpenes and sterols. Compounds with biological activities have been demonstrated by several authors, in particular the alkaloids. The total alkaloids of *Mitragyna ciliata* whose antimalarial activity has been demonstrated several times have not expressed under our experimental conditions immunostimulatory activity proved. However, *Mitragyna ciliata* is an important medicinal plant made of the many pharmacological properties, due to the presence of its phytochemical constituents.

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