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Evaluation of the *in vitro* antiplasmodial activity of *Millettia zechiana* and its action on the evolution of anemia in albino rats.

Karamoko Chérif Moustapha[1,2]*, Touré André Offianan[2], Bla Kouakou Brice[1], Tuo Karim[2], N'guessan Yao Honoré[1], Bidie Alain dit Philippe[1]

[1]University of Félix Houphouët Boigny, 22 PO Box 582 Abidjan 22

[2] Institut Pasteur of Côte d'Ivoire, 01 PO Box 490 Abidjan 01

authors' contacts:

cherifkaramoko@gmail.com / (225) 77380309

andre_offianan@yahoo.fr / (225) 05636737

blabrice@yahoo.fr / (225) 07579781

karimtuo@pasteur.ci / (225) 08355392

nyaohonore@gmail.com / (225) 09144337

alphbidie@yahoo.fr / (225) 58766797

*Corresponding author, Tel: (225) 77380309; E-mail : cherifkaramoko@gmail.com

Abstract

Background : Malaria is a parasitic infection that leads to anaemia and death. Unfortunately, the upsurge of chemo-resistance prompted researchers to focus on new antimalarial drugs. **Objectives** : The aim of this work was to evaluate the antiplasmodial and antianemic activity of *Millettia zechiana*. **Methods**: the *In vitro* activity was assessed on clinical isolates and on the standard strain of *Plasmodium falciparum* K1, using the SYBR green I test. Moreover, the antianemic activity was evaluated in phenyl hydrazine induced anemic albino rats.**Results/discussion** : The ethyl acetate and hydroethanolic extract exhibited an antiplasmodial activity with IC_{50s} of 6.14 and 12.14 µg/mL respectively on *Plasmodium falciparum* K1 strain. As for the *in vivo* antianemic activity, the ethyl acetate extract was the most active with better hematological reconstitution percentages. The presence of chemical compounds such as alkaloids, terpenoids and quinonic substances in both extracts, could be responsible for their activities.**Conclusion**: *Millettia zechiana* could be a potential source for novel antimalarial drugs and might be used as an improved traditional medicine on account of its availability.

Keywords: *Millettia zechiana*; chemosensitivity; anemia; medicine, antiplasmodial

1. INTRODUCTION

Malaria is an infectious and life-threatening disease caused by the protozoan parasite *Plasmodium*. This disease is associated with fever, anemia and other diseases **(Chen et *al*., 2016)**. According to the World Health Organization, the prevalence of children suffering from anaemia is higher in malaria endemic areas. Unfortunately, Africa remains the most affected continent with a prevalence estimation ranging from 31 % to 90 % **(WHO, 2008)**. In *falciparum* malaria, anemia can develop rapidly due to profound hemolysis of red blood cells **(Sumbele et *al*., 2016)** and severe malaria may cause subsequent hypoxia and congestive heart failure **(Memendez et *al*., 2000)**. Childrens under 5 years of age account for 70 % of all cases **(WHO, 2017)**. Today, ACTs (Artemisinin-based combination therapies) are the first line treatment against malaria because of its efficacy against *Plasmodium falciparum* **(WHO, 2015)**. However, parasites are developing resistance to each new class of known antimalarial drugs, for instance the upsurge of artemisinin-resistant parasites reported in Cambodia, Thailand, Myanmar, Laos and Vietnam is a real threat to tremendous efforts to control and eventually eradicate malaria **(Cui et *al*., 2015)**. The problem is appalling and new drugs need to be developed **(Nondo et *al*., 2017).** The use of medicinal plants for therapeutic purposes has long been practised **(Ogbonna et *al*., 2008)**; the success of quinine and artemisinin derivatives against resistant strains of *Plasmodium* has prompted researchers to cast a glance on medicinal plants for new antimalarial drugs **(Akuodor et *al*., 2012; Olasehinde et *al*., 2014)**. Thus the main purpose of this research work was to evaluate the antiplasmodial and antianemic potential of different extracts of *Millettia zechiana*.

2. MATERIALS AND METHODS

2.1. Animals

Forty two healthy albino wistar rats of both sexes, weighing between 120 and 210 g were selected for the study. Animals were kept in polypropylene cages with stainless steel covers and were acclimated for one (1) week under hygienic and standard environmental conditions (23°C and 12h light/dark cycle) prior to experimental use. Animals were allowed unrestricted access to water and food pellets (FACI, Abidjan, Côte d'Ivoire). The study protocol was carried out according to the rules and regulations of the Institutional Animal Ethical Committee (IAEC).

2.2 Plant material

Stem barks of *Milletia zechiana* were freshly collected from Saioua in the Western part of Côte d'Ivoire. The sample was identified and authenticated at the National Center of Floristic, University of Felix Houphouët Boigny. Stem barks were air dried at room temperature (25°C) for four weeks and ground using an electrically powered engine (IKAMAG RCT® mill, Staufen, Germany). Powder was stored in a moisture free airtight container for further use.

2.3. Preparation of crude extracts

Five successive extractions using increasing polarity of solvents **(Tuo, 2015)** were carried out. The polarity order of solvents was as followed: Hexane, Ethyl acetate, Ethanol, Methanol and distilled water.

One hundred (100) grams of plant powder was weighed using an electronic weighing balance and macerated in one (1) liter of hexane for 24 hours at room temperature (25°C) using a magnetic stirrer brand (IKAMAG RCT Staufen, Germany). The macerate was filtered twice on hydrophilic cotton

and once on 3mm WHATMANN paper. Filtrate was evaporated to dryness at 40°C using a rotary evaporator (BUCHI 161 Water Bath), the dry powder representing the hexanic extract was stored. After this extraction, the residual pomace was dried. The powder obtained was macerated in one (1) liter of ethylacetate and the extraction was performed according to previous method. Successively, the hydroethanolic, methanolic and then aqueous extraction were carried out according to the same method.

2.4. Yield of crude extract

The yield of the crude extract was calculated according to the following formula **(Koffi et *al*., 2015):**

R (%): Extraction efficiency in %.

We: Weigh of extract after solvent evaporation.

Wv: Weigh of fine powder used for extraction.

2.5. Phytochemical qualitative analysis

Phytochemicals such as steroids, terpenoids, alkaloids, tannins, polyphenols, flavonoids, quinones and saponins were identified in extracts according to the following standard methods **(Mangambu et *al*., 2014)** and summarized in Table I.

Table I: Phytochemical tests

Phytochemicals	Tests	indicators
Alkaloids	Burchard's test	Red-orange precipitate colour
Flavonoids	Cyanidine test	Pink-orange or purplish colouring
Quinones	Borntrager's test	Reddish or purplish Colouring
Polyphenols	Ferric chloride test	Blue-blackish Colouring
terpenoids	Liebermann's test	bleue to green colouring
Saponins	Frothing test	Foaming
Catechic tannins	Ferric chloride test	Green colouring
Gallic tannins	Ferric chloride test	bleue-blackish colouring

2.6. *In vitro* antiplasmodial assay

The *In vitro* susceptibility assay of *Melettia zechiana* was carried out against four (4) clinical isolates (Community Health Centre of Anoukoua-Kouté, Abidjan, Côte d'Ivoire) and resistant *Pf K1* strain of *Plasmodium falciparum* (ATCC MRA-159, MR4, ATCC Manassas, Virginia) synchronized culture at 2 % hematocrit. Resistant *Pf K1* strain of *Plasmodium falciparum* was maintained in culture according to the method described by **Trager and Jensen, (1976)**. Parasite was maintained at 1.5 % hematocrit in human red blood cells (blood type 0+) in medium containing RPMI 1640 (Gibco®, Life Technology, UK), 12.60 mL HEPES buffer (25 mM), 100 mL hypoxanthine, 312.5 µL gentamycin (40 mg/mL) and glucose (20

g/L, Wagtech). Culture was grown at 37 °C in a flask cell culture and confined in a candle chamber saturated with CO_2. Parasite growth was monitored microscopically with a Giemsa stained thin blood smear. Parasite culture was synchronized using 5% sorbitol prior to assays. The standard drug (Chloroquine) with concentrations ranging from 3.125 to 1600 nM and crude extracts of *Melletia zechiana* (at different concentrations from 1.56 to 100 µg / mL) were prepared in a complete culture medium inside the 96 well microplates. Then, the synchronized culture and the clinical isolates were tested in both standard drug and crude extracts and microplates were confined in a candle chamber saturated with CO_2 and incubated at 37°C for 72 hrs. After 72 hrs of incubation, 100 µL from each well was transferred to a new 96-well microplate. 100 µL of mix containing 5 µL of SYBR Green I and 25 mL of lysis buffer were added to each well and incubated at 37°C in darkness for 1 h prior to fluorescence reading using a BIOTEK, FLX 800 plate reader (Excitation/Emission: 485 nm/530 nm). All experiments were carried out in duplicates. The antimalarial activity of the test extract was expressed as IC_{50} (50% inhibitory concentration) was determined from a dose-response curve by non-linear regression analysis using WWARN's IVART (In vitro Analysis and Reporting Tool) software **(Le Nagard et *al.*, 2011)**.

2.7. Anti-anemic activity

Anemia was induced through intra peritoneal injection of phenyl hydrazine at 40 mg/kg for two (2) days as described by **(Yenon *et al.*, 2015)**, after injections, rats were divided into nine groups of six rats each.

Group I-Control rats received 10 mL/kg distilled water from day_2 to day_{22}.

Group II-(negative control) Phenyl hydrazine induced anemic rats received 10 mL/kg of distilled water from day_2 to day_{22}.

Group III-(Positive Control) Phenyl hydrazine induced anemic rats received 1mL/kg of Vitamin B_{12} from day_2 to day_{22}.

Group IV- Phenyl hydrazine induced anemic rats received a daily dose (100 mg/kg) of hexanic extract of *Milletia zechiana* from day_2 to day_{22}.

Group V- Phenyl hydrazine induced anemic rats received a daily dose (100 mg/kg) of acetic extract of *Milletia zechiana* from day_2 to day_{22}.

Group VI- Phenyl hydrazine induced anemic rats received a daily dose (200 mg/kg) of hexanic extract of *Milletia zechiana* from day_2 to day_{22}.

Group VII- Phenyl hydrazine induced anemic rats received a daily dose (200 mg/kg) of acetic extract of *Milletia zechiana* from day_2 to day_{22}.

On completion of the experiment, a blood sample was collected in an EDTA collection tube for each rat to determine biochemical parameters using an automate blood cell counter (Sysmex XN 1000).

2.8. Statistical analysis

All values were analyzed using Graph Pad Prism 4 software and the results were expressed as Means ± standard deviation (SD). One-way analysis of variance (ANOVA) was performed, Significant differences ($P< 0.05$) between means were compared using the Dunnet post-hoc test.

3. RESULTS

3.1. Extraction efficacy

The hydro-ethanolic extract of *Milletia zechiana* had the highest yield recovery of 12.23 while the methanolic extract had the least percentage yield of 0.65% **(Figure 1)**. The extraction efficacy depends on both the nature and polarity of solvents used.

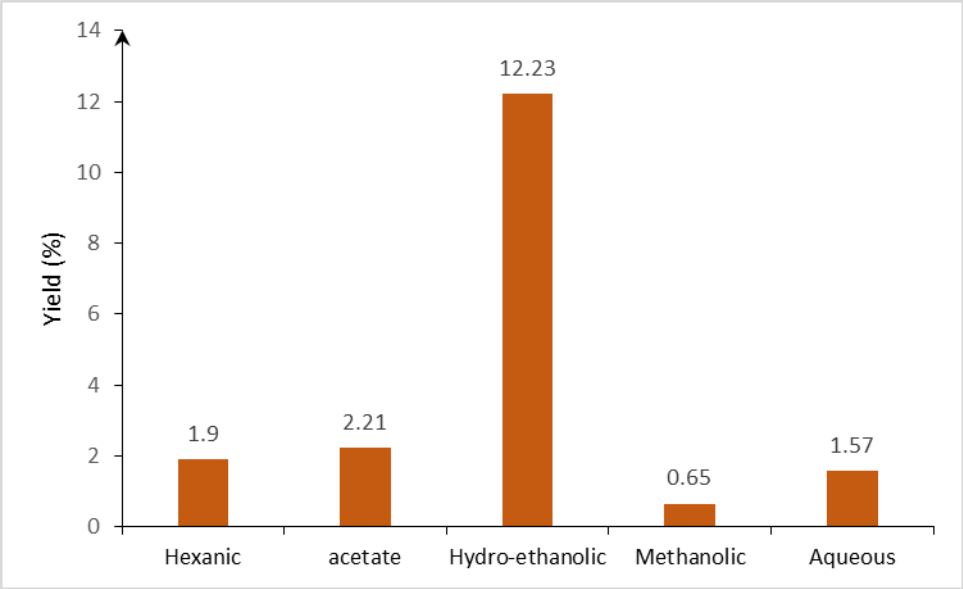

Figure 1: Percentage yield of different extracts of *Millettia zechiana*

3.2. Phytochemical Sreening

Alkaloids, steroids and terpenoids, as well as quinonic substances are the major chemical compounds found in the different extracts of *Millettia zechiana* **(Table II)**.

Table II: Phytochemical screening of different extracts of *Millettia zechiana*

		Steroids and terper-noids	Poly-phenols	Flavo-noids	Tannins		Quinonic compounds	Alkaloids		Saponins
					Gal	Cat		D	B	
Crude extract of *Millettia zechiana*	Hexane	+	-	-	-	-	-	+	+	-
	Ethyl acetate	+	-	-	-	-	-	+	+	-
	Hydro-ethanolic	+	-	-	-	-	+	+	+	-
	Metha-nolic	+	-	-	-	-	-	+	+	-
	Aqueous	+	-	-	-	-	+	+	+	+

Presence of compounds: +

Absence of compounds: -

3.3. *In vitro* antiplasmodial test

The *In vitro* antiplasmodial study showed that the ethyl acetate and hydroethanolic extract of *Milletia zechiana* were the most active on the erythrocytic stage of *Plasmodium falciparum*, with a 50% inhibitory concentration of parasite growth (IC_{50s}) on clinical isolates ranging from 6.07 to 49.45 µg/mL for the ethyl acetate extract and from 6.04 to 46.32 µg/mL for the hydro-ethanolic extract. Furthermore, tested against the Chloroquine resistant K1 strain, both extracts exhibited a promising antiplasmodial activity with IC_{50s} of 6.14 (ethyl acetate extract) and 12.14 µg/mL (hydro-ethanolic extract) **(Table III)**.

Table III: *In vitro* Antiplasmodial Activity of various extracts of *Millettia zechiana* against 4 clinical isolates and Chloroquine resistant K1 strain (*Pf* K1).

	IC_{50}				
Crude extracts (µg/ml)	**Isolate 2**	**Isolate 3**	**Isolate 4**	**Isolate 5**	***Pf* K1 strain**
Mhe	> 50	> 50	> 50	> 50	> 50
Mac	6.07	46.69	49.45	11.79	6.14
MHE	46.32	6.09	12.35	6.04	12.14
Mm	> 50	> 50	> 50	> 50	> 50
Maq	> 50	> 50	> 50	> 50	> 50
CQ (nM)	22.42	25.27	24.38	22.42	819.55

3.4. Antianemic activity

The intraperitoneal administration of Phenylhydrazine to rat significantly reduced (P 0.001) the red blood cell counts (52.34% 1.135), the hemoglobin (34.61% 1.717) and the hematocrit (49.31% 0.528) levels in rats as compared with normal control on day_2 (D_2) (Table IV, V and VI). After ten (10) days of treatment, these hematological parameters significantly increased (P0.001). Moreover, the ethyl acetate extract of *Milletia zechiana* was the most active and even appeared to exhibit a better activity than the standard drug (Vitamin B_{12}).

Table IV: Effect of *Millettia zechiana* extracts on red blood cell counts in phenylhydrazine induced anaemic rats.

Substances	Red blood cell counts (10^6cells/µl)			
	D_0	D_2	D12	D_{22}
Normal control (Dw 10 ml/kg)	9,01 ± 0,26	8,42 ± 0,30	8,91 ± 0,41	8,71 ± 0,30
Negative control (Dw 10 ml/kg)	9,29 ± 0,19	5,22 ± 0,23 - **43,81**^{a***}	5,85 ± 0,20 **+12,07**^{b}	6,23± 0,23 **+19,34** ^{b*}
Positive control (Vit B_{12};1mL/kg/day)	8,18 ± 0,16	4,03 ± 0,37 - **50,70** ^{a***}	6,24 ± 0,20 **+54,72** ^{b***}	7,12 ± 0,15 **+76,47** ^{b***}
MHE (100 mg/kg)	9,56 ± 0,16	4,99 ± 0,80 - **47,78** ^{a***}	6,88 ± 0,23 **+37,79** ^{b***}	7,41 ± 0,26 **+48,50** ^{b***}
Mac (100 mg/kg)	8,10 ± 0,11	3,54 ± 0,79 - **56,24** ^{a***}	6,29 ± 0,18 **+77,62** ^{b**}	6,81 ± 0,13 **+92,21** ^{b**}
MHE (200 mg/kg)	8.13 ± 0,14	4.13 ± 0,77 - **49,20** ^{a***}	5.80 ± 0,89 **+40,41** ^{b***}	6.49 ± 0,15 **+57,14** ^{b***}
Mac (200 mg/kg)	9.14 ± 0,42	4.01 ± 0,11 - **56,13** ^{a***}	7.24 ± 36 **+80,55** ^{b***}	8.81 ± 0,11 **+119,70** ^{b***}

Values are expressed as mean ± SD (standard deviation) with n=6 in each group. ***P<0.001; **P<0.01.
a: mean compared to day 0 (D0) in each group; b: compared to day 2 (D2) in each group. Normal control: rats treated with distilled water (10 ml/kg); negative control: Phenylhydrazine induced anaemic rats treated with distilled water (10 ml/kg); Positive control: phenylhydrazine induced anaemic rats treated with Vitamin B_{12} (1mL/day); MHE: phenylhydrazine induced anaemic rats treated with the Hydro-ethanolic extract of *Millettia zechiana*; Mac: phenylhydrazine induced anaemic rats treated with the Ethyl acetate extract of *Millettia zechiana*, D: Day.
The figures in bold in the table are percentages of evolution of the evaluated parameter within the different groups of rats.

Table V: Effect of *Millettia zechiana* extracts on hemoglobin levels in phenylhydrazine induced anaemic rats.

Substances	Hemoglobin (g/dl)			
	D_0	D_2	D12	D_{22}
Normal Control (Dw 10 ml/kg)	14,80 ± 0,61	14,07 ± 0,41	14,50 ± 0,72	14,27 ± 0,55
Negative control (Dw 10 ml/kg)	15,06 ± 0,55	10,46 ± 0,53 **-23,90** ^{a***}	12,02 ± 0,59 **+22,34** ^{b}	12,9 ± 0,31 **+28,45** ^{b**}
Positive control (Vit B_{12}; 1mL/day)	14,23 ± 0,33	8,3 ± 0,59 **-41,67** ^{a***}	13,70 ± 0,52 **+65,06** ^{b***}	14,27 ± 0,52 **+71,92** ^{b***}
MHE (100 mg/kg)	15,64 ± 0,30	10,58 ± 0,21 **-32,35** ^{a***}	14,60 ± 0,19 **+38** ^{b***}	14,63 ± 0,33 **+38,28** ^{b***}
Mac (100 mg/kg)	14,24 ± 0,42	8,125 ± 1,79 **-42,94** ^{a**}	14,83 ± 0,64 **+82,52** ^{b**}	14,23 ± 0,28 **+83,75** ^{b**}
MHE (200mg/kg)	15.69 ± 0,13	10.18 ± 0,22 **-35,12** ^{a***}	14.52 ± 0,69 **+42,63** ^{b***}	14.74 ± 0,34 **+44,79** ^{b***}
Mac (200mg/kg)	15.08 ± 0,34	8.01 ± 1,02 **-46,87** ^{a***}	14.89 ± 0,45 **+85,85** ^{b***}	15.99 ± 0,52 **+99,58** ^{b***}

Values are expressed as mean ± SD (standard deviation) with n=6 in each group. ***P<0.001; **P<0.01.
a: mean compared to day 0 (D0) in each group; b: compared to day 2 (D2) in each group. Normal control: rats treated with distilled water (10 ml/kg); negative control: Phenylhydrazine induced anaemic rats treated with distilled water (10 ml/kg); Positive control: phenylhydrazine induced anaemic rats treated with Vitamin B_{12} (1mL/day), MHE: phenylhydrazine induced anaemic rats treated with the Hydro-ethanolic extract of *Millettia zechiana* Mac: phenylhydrazine induced anaemic rats treated with the Ethyl acetate extract of *Millettia zechiana*, D: Day.
The figures in bold in the table are percentages of evolution of the evaluated parameter within the different groups of rats.

Table VI: Effect of *Millettia zechiana* extracts on hematocrit levels in phenylhydrazine induced anaemic rats.

Substances	Hematocrit (%)			
	D_0	D_2	D12	D_{22}
Normal Control (Dw 10 ml/kg)	51,70 ± 2,08	47,80 ± 1,65	51,90 ± 2,10	50,83 ± 2,07
Negative control (Dw 10 ml/kg)	52,00 ± 1,56	27,16 ± 1,07 **-43,92** ^{a***}	33,36 ± 2,408 **+14,4** ^{b}	35,06 ± 1,627 **+20,23** ^{b*}
Positive control (Vit B_{12}; 1mL/day)	48,85 ± 0,99	23,13 ± 1,62 **-48,56** ^{a***}	49,47 ± 1,75 **+76,68** ^{b***}	49,48 ± 1,07 **+85,97** ^{b***}
MHE (100mg/kg)	54,66 ± 1,00	25,96 ± 0,61 - **48,85** ^{a***}	49,4 ± 0,59 **+76,68** ^{b***}	52,00 ± 0,93 **+85,98** ^{b***}
Mac (100mg/kg)	48,92 ± 1,20	20 ± 4,68 - **48,90** ^{a***}	47,13 ± 1,70 **+88,52** ^{b***}	51,6 ± 1,04 **+106,4** ^{b***}
MHE (200mg/kg)	54.08 ± 0,78	27,16 ± 0,14 - **49,78** ^{a***}	48,89 ± 0,81 **+80,01** ^{b***}	53,83 ± 0,39 **+98,19** ^{b***}
Mac (200mg/kg)	50.19 ± 0,19	24.89 ± 0,56 - **50,41** ^{a***}	47.89 ± 0,75 **+92,41** ^{b***}	54.55 ± 1,06 **+119,16** ^{b***}

Values are expressed as mean ± SD (standard deviation) with n=6 in each group. ***P<0.001; **P<0.01. a: mean compared to day 0 (D0) in each group; b: compared to day 2 (D2) in each group. Normal control: rats treated with distilled water (10 ml/kg); negative control: Phenylhydrazine induced anaemic rats treated with distilled water (10 ml/kg); Positive control: Phenylhydrazine induced anaemic rats treated with Vitamin B_{12} (1mL/day); MHE: phenylhydrazine induced anaemic rats treated with the Hydro-ethanolic extract of *Millettia zechiana* Mac: phenylhydrazine induced anaemic rats treated with the Ethyl acetate extract of *Millettia zechiana*, D: Day.
The figures in bold in the table are percentages of evolution of the evaluated parameter within the different groups of rats.

4. DISCUSSION

The aim of this work was to evaluate the in vitro antiplasmodial activity of five extracts of *Millettia zechiana and* extracts showing high potential were selected to assesss their anti-anemic activity in phenylhydrazine induced anaemia in rats.

Therefore, extracts were prepared from solvents with increasing polarity. The extraction yield percentage (12.23%) of the hydro-ethanolic extract of *Millettia zechiana* was the highest. This could mean that this extract contains more polar compounds than the others. The phytochemical screening results showed that all extracts contained terpenoids and alkaloids. However, Quinones were only detected in the hydro-ethanolic and aqueous extracts.

The *in vitro* antiplasmodial tests revealed that only two extracts were active on both clinical isolates and on the chloroquine-resistant K1 strain, according to the classification scale of **Jansen et *al*., (2012)**. According to these authors, the hydro-ethanolic and ethyl acetate extracts of *Milletia zechiana* have a promising and moderate activities against clinical isolates of *Plasmodium falciparum*

and chloroquine resistant K1 strain. The studies of **Zihiri et *al*., 2005** and **2010** showed that the ethanolic extract of *Millettia zechiana* had a good antiplasmodial activity (IC_{50s} of 16.1 and 14.1 µg/mL) and these previous results matched with those found with the hydro-ethanolic extract (IC_{50} = 12.14 µg/mL) in this study.

Ethyl acetate and hydro-ethanolic extracts with outstanding antiplasmodial potentials were selected for the anti-anemic activity assay.

Therefore, anemia was induced by intraperitoneal injection of phenylhydrazine (Phz) at a dose of 40 mg/kg for 2 days.

Phenylhydrazine causes hemolytic anemia in rats by decreasing the level of red blood cell counts, hemoglobin and hematocrit **(Yenon et *al*., 2015)**. This anemia characterizes by the early lysis of red blood cells, was reversed 12 days later after administration of the ethyl acetate extract of *Millettia zechiana*. This result could be due to the presence of alkaloids in this extract, since alkaloids and flavonoids are powerful antioxydants which prevent or repair damage done to red blood cells by free radicals or highly reactive oxygen species **(Ogbe and Adoga, 2010)**. **Turaaskar (2013)** reported that most anti-anemic compounds are known for their free radical scavenging activity that reverses anemic conditions. This phytochemical might have contributed to the anti-anemic activity of *Millettia zechiana observed* in the present study by stimulating erythropoiesis in the bone marrow. Thus, the difference between the activities of both extracts could be due to the fact that they do not have similar phytochemical compounds **(Saravanan and Manokaran, 2012)**.

5. CONCLUSION

This study provides evidence that both the ethyl acetate and hydro-ethanolic extracts of *Millettia zechiana* exhibited a good antiplasmodial potential. Furthermore, it appears that the ethyl acetate extract has a very good anti-anemic activity. The results therefore demonstrate that *Millettia zechiana* is a real asset in the search for new antimalarial and anti-anemic drugs. Nevertheless, further studies need to be undertaken to ascertain the *in vivo* toxicity of this plant and identify its active principles.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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