Comparative Hepatoprotective and Antioxidant Activities of Artesunate and Flavonoids extracts from *Artemisia annua* grown in Cameroon

Nkuitchou- Chougouo K. Rosine D.[1], Kouamouo Jonas[1], Titilayo O. Johnson[2], Petga Ange[1], Student of O. John[3] Kouemeni Lysette[1], Chuisseu pascal[1], Lutgen Pierre[4], Tane Pierre[5], Moudipa[6].

[1]*Université des Montagnes, Bangangté Cameroon POBox 208*

[2]*African centre of excellence in phytomedecine research and development, University of Jos Nigeria*

[3]*Department of bioschemistry, University of Jos, Nigeria*

[4]*Iwerliewen Fir Bedreete Volleker (IFBV)-BELHERB, BP 98 Niederanven, L-6905 Luxembourg*

[5]*Laboratory of Natural Products Chemistry, University of Dschang, Cameroon*

[6]*Faculté des Sciences, University of Yaoundé I Cameroon*

Email: nrosinedesiree12@yahoo.com; tel: +237 677468510

Abstract

The hepatoprotective activites of Artesunate, an antimalarial compound derived from artemisinin and the flavonoids extracts of *Artemisia annua* was investigated. The antioxidants activities of artesunate and *Artemisia annua* flavonoids were assessed using 1,1-diphenyl-2- picrylhydrazyl (DPPH) radical scavenging activity and ferric reducing/antioxidant power (FRAP). Hepatoprotective activities were analyzed on two induced hepatitis models. Hepatocyte's viability according to the extract's concentrations was studied by the MTT test, the estimation of ALT activities and the MDA level. Likewise, reduction test showed a poor activity of artesunate. There was no significant difference between poisoned cells and cells treated by artesunate contrary to aqueous extracts and flavonoids extract which showed a significant difference ($p<0.001$). The outcome suggests that artesunate toxicity may possibly cause damages to the hepatocytes and liver function; effect contrary to that of *A annua* flavonoids, which keep cells alive.

Key words: *A annua*, flavonoids, artesunate, *in vitro,* hepatoprotective activity, antioxidants.

Introduction

Artesunate is a semi-synthetic derivative of artemisinin, the active compound of the Chinese herb *Artemisia annua* [1]. This drug, as well as other derivatives of artemisinin (artemether, arteether and dihydroartemisinin) is very effective in the treatment of malaria, especially the chloroquine resistant forms [2, 3]. Malaria is still one of the major causes of mortality and morbidity in developing countries and remains one of the major public health problem in these areas [2]. However, the uncontrolled use of artesunate and other antimalarial drugs is becoming another major concern [4]. Self-medication and self-purchase of antimalarial drugs in endemic areas could result in toxicity and damage to vital body organs [5]. Artesunate has been reported in several studies to show evidence of toxicity to the brain stem [6], the upper colon [7] stomach [7], testicles [8] and the fetus [9]. Some *in vivo* studies have also reported the toxic effect of artesunate on the liver [10], an organ that plays several important roles in the body [11].

Artemisia annua leaf has been known for its antimalarial properties and its richness in chemical compounds such as flavonoids, coumarins, essential oils [12]. Flavonoids have been reported to possess antioxidant and hepatoprotective activities [12]. Kommegne also reported that the extracts of *Artemisia annua* are as effective as Desmodium with an equally high content of flavonoids [13].

The damage caused by artesunate which is in the first line treatment for malaria in Cameroon drives us to seek its effects on the liver compared to the flavonoids in *Artemisia annua* plant to advance in the treatment of malaria. Hence, the objective of this work is to determine the comparative hepatoprotective and antioxidant properties of artesunate and *A. annua* flavonoids on hepatocytes of rats.

Materials and Methods

This study was conducted in the Laboratory of Pharmacognosy and Pharmacology, University of Yaounde I. Cameroon.

The experimental animals

Albino rats of both sexes weighing between 150 and 350 g were used for the study.

Equipments and reagents

The list of equipment used is shown in Annex. Artesunate used has ASE lot numbers of 12011. It was provided by the laboratory Vital Health Care PVT (Kinshasa, Congo). The other reagents used were supplied by PROLABO laboratories (Paris, France) and Sigma (Hamburg, Germany).

Plant material

The dry aerial parts (stems and leaves) of *Artemisia annua* was harvested at the garden of the catholic primary school ‘’Notre Dame’’Bangangte. The voucher number of 65647 / CST which was registered in the National Herbarium of Cameroon (IRAD Yaounde).

Preparation of plant extract

The plant parts were washed, cut, dried under shade to a constant weight. The dried plant materials was pulverised using an electric blender. One gram of the pulverised plant material was infused in 200 ml of distilled water after which the filtrate was oven dried at 40^0C.

Extraction, characterization and assay of flavonoids

Extraction of flavonoids was based on the degree of solubility in polar flavonoids and organic solvents [14]. Four gram of dry matter were soaked in the mixture (ethanol / distilled water) (7/3) with cold renewal for two consecutive times of the solvent and left at 4 ° C for 24 hours at each renewal. This will dissolve the total flavonoids. After filtration, the solvents were evaporated in an oven at 35°C. Each extract was taken up in 25 ml of distilled water and then freed from chlorophyll, lipids, waxes and fats by washing with 25 ml of petroleum ether. The obtained aqueous phases were filtered and evaporated in an oven at 35°C. The operations were repeated three times.

Characterization of flavonoids: reacting Shibata

The presence or absence of flavonoids in a sample was detected by using a simple rapid test of magnesium [14]. Some magnesium fragments and a few drops of HCl were added to 2mls of extract in a test tube. The presence of the flavonoid aglycone in the extracts is indicated by the change of color to orange or red brick.

Determination of total flavonoid content by $AlCl_3$

This assay is based on the formation of a yellow colored complex resulting from the binding between the aluminum trichloride and the lone pairs of the oxygen of the OH groups flavonoids [15]. 1 ml of each extract is added to predissolved 1 ml $AlCl_3$ solution. After 1 h incubation at room temperature, the absorbance is measured at 420 nm.

The flavonoid content is calculated in of mg equivalent of quercetin per g of dry matter, with reference to the standard curve.

Analysis by thin layer chromatography

After the glass cells have been saturated with the elution solvent (toluene / acetic acid 1% / diethyl ether (0.5 / 1 / 1.5) (V / V / V)), TLC plates are prepared to receive samples. Samples and standard solutions are deposited on the deposit line of the TLC plate. Then the plates loaded in the tank were introduced to migrate. Before the end of the migration, the front line is drawn and the plate is dried and revealed under UV at 366 nm, and then sprayed with $AlCl_3$. The frontal ratios are calculated and compared to the standard. Rf = (distance between the origin and the product spot after migration) / (distance between the origin and the solvent front after migration)

***In vitro* evaluation of antioxidant activity of extract of *Artemisia annua*, flavonoids and artesunate.**

Extracts and artesunate were solubilised in DMSO (dimethylsulfoxide) and filtered using cell filter of 0.22 µm to obtain sterile preparations that were used for testing. Aliquots of 1.5 ml were stored in the freezer for later use. Evaluation of antioxidant activity were carried out by various tests:

• The antiradical activity by trapping tests radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) and hydroxyl (HO)

• The reducing power and antioxidant capacity by ions reduction tests and ferric molybdate

The anti-radical activity by trapping tests radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) and hydroxyl (HO)

One thousand nine hundred µl of a methanol solution of DPPH 40 µg / ml and 100 µl of test compounds (artesunate flavonoids extracts, crude extract and vitamin C (positive control)) were introduced into the test tubes. The mixture was kept in the dark for 30 minutes at room temperature, then the absorbance measured at 517 nm. The contribution of the coloration due to

the plant extract was subtracted using in 1900 µl of a solution of methanol added to 100 µl of the compounds.
Different trapping percentages were calculated using the following equation:

$$\%P = \frac{[(DO_{témoin} - (DO_{essai} - DO_{blanc})] \times 100}{DO_{témoin}}$$

Witness DO = Absorbance in the absence of test sample (negative control)
DO test = Absorbance in the presence of test sample
% P = Trapping Percentage
White OD = Absorbance in the presence of test sample without DPPH

Sequestration radical HO

In each test tube are added successively 32 µl of test compounds or vitamin C, 444µl of FeS04 (3 mM), 635µl of H2O2 (1 mM), 635µl of distilled water and sodium salicylate 254µl (10 mM). The mixture is incubated at 37°C for 60 minutes and the absorbance read at 562 nm. The contribution of the coloration due to the plant extract will be subtracted using all reagents except sodium salicylate replaced by its solvent (distilled water). The various trapping percentages are calculated using the above equation.

The reducing power and antioxidant capacity by ions reduction tests and ferric molybdate

Reduction potassium ferricyanide

Is added to each test tube successively 50 µl of test compounds or vitamin C or DMSO (negative control), 1100 µl of phosphate buffer (0.2M; pH 6.6), 1 ml of a solution of ferricyanide potassium 0.25%. The mixture is incubated at 50 ° C for 20 minutes. After cooling in a stream of water, adding 1 ml of trichloroacetic acid (TCA) 10%. The mixture was then centrifuged (3000 rev / min, 10 min, 4°C) and 1 ml of the supernatant will be removed, added to 200 µl of $FeCl_3$ (0.02%) and the absorbance of the green solution was read after 10 minutes at 700 nm. The contribution of the coloration due to the plant extract is subtracted by adding 200 µl phosphate buffer instead of $FeCl_3$ in the tubes used white.

Reduction molybdate

In each test tube were successively added 32 µl of test compounds of vitamin C, then 665 µl of the sulfuric acid solution (0.6 M), 666 µl of the sodium phosphate solution (28 mM) and 666 µl of ammonium molybdate solution (4 mM). The mixture is then incubated at 95 ° C for 90

minutes. After cooling to room temperature, the absorbances were read at 695 nm. The antioxidant capacity is expressed in mg ascorbic acid equivalent per g of extract (mg EAA / g of extract) using the calibration curve of vitamin C.

Cytotoxicity study of extracts on hepatocytes

Nine hundred ninety microliters of cell suspension (500 000 cells / ml) were added to each tube and then treated with 10 .mu.l of substances (artesunate, and flavonoids extracted from Artemisia annua) at final concentrations of 1, 10, 100 to 1000 mcg / ml. Control tubes were incubated with 10 .mu.l of DMSO. The tubes were then homogenized and incubated at 37° C in CO2 atmosphere for 6 h as described previously. After incubation, the tubes were centrifuged (2000 rev / min, 5 min, 4° C). The supernatant was collected for assaying ALT and the pellet washed with 100 .mu.l of PBS pH 7.4, and submitted to the viability MTT assay.

Study of hepatoprotective activity of substances

Hepatoprotectives Properties of substances (artesunate, and flavonoids extracted from Artemisia annua) were studied on models toxic hepatitis induced by CCl_4, and paracetamol. In each case, these properties were evaluated by pre-treatment (10µl chemical moieties plants or silymarin were added to each eppendorf containing 980 .mu.l of cell suspension and the whole was incubated for one hour, then intoxicated by 10µl of paracetamol and CCl4 solution and incubated for 5 hours). [16]

- The hepatocytes were seeded in sterile Eppendorf tubes of 1.5 ml at concentrations of 0.1; 1; 10; 100 to 1000 mcg / ml. . Cell suspensions were dispensed into 1.5 ml Eppendorf tubes at a rate of 980µl / tube and 990µl / tube were then divided into four groups:
- Treatment A or negative control: cells were treated with 10 .mu.l of DMSO
- Treatment B or toxic group: cells were treated with toxic 10µl.
- Treatment C or test group: the cells were treated by addition of 10µl of test substances to a concentration of incubation; 0.1; 1; 10; 100; 1000 mcg / ml dissolved in distilled water.
- Treatment D or positive control group: the hepatocytes were treated with silymarin, under the same conditions of volume and concentration of the extracts.

At the end of the incubation, the tubes will again homogenized and half of the cell suspension will be collected to assess the malondialdehyde. The other remaining half will be centrifuged (2000 rev / min, 5 min, 4 ° C). The collected supernatant used for the assay of ALT and the cell pellet washed with PBS and used for the viability test with MTT.

Viability test

The amount of hepatocytes and viability were assessed by counting using a Malassez cell after trypan blue staining 4%. In an Eppendorf tube, 100 µl of cell suspension, 100µl of trypan blue 0.4% and 800 µl of buffer II were introduced. The mixture was homogenized, and then 25 µl of the suspension were introduced into a Malassez cell (Figure 13) for counting [17]. Viable cells (or brilliant) were counted as dead cells that appear blue (Objective: X10). The percentage (%) of viability was calculated.

$$\% \text{ Viabilité} = \frac{\text{Nombre de cellules viables}}{\text{Nombre de cellules}} \text{ X 100}$$

The cells were counted in ten rectangles, then the average of counted cells was calculated. The concentration of cells / ml is determined using the following formula:

$$N = \frac{n}{np} \times \frac{1}{p} \times \frac{1}{g} \times fd$$

N = number of hepatocytes per ml of medium (cells / ml)
np = number of wells in which the counting is performed
n = number of cells counted using the cell Malassez
p = depth of a well in the Malassez cell (0.200 mm).
df = dilution factor.
surface of the cell (0.0025 mm^2)

Test MTT viability of hepatocytes

The cell pellet from different incentives was taken up in 300µl of a solution of MTT (0.5 mg / ml in PBS), the mixture was incubated at 37 ° C for 1h30min, then centrifuged (2000 trs / min, 5 min , 4 ° C). The supernatant was removed and the drained tubes. Then 500µl of acidified isopropanol was added to each tube to dissolve the formazan crystals formed. Finally, the absorbance of the blue-violet solution was read at 550 nm against the acidified isopropanol solution. The percentage of cell viability calculated by the following formula:

$$\textbf{\% viabilité} = \frac{Absorbance\,échantillon}{Absorbance\,témoin} \times 100$$

- Sample: tubes containing toxic
- Witness: tubes with no toxic

Determination of activity of alanine aminotransferase

In two tubes marked white test and test, we introduced ALT substrate 360µl (0.2M alanine, α-ketoglutarate 2 mM in 0.1 M phosphate buffer, pH 7.4) we have pre incubated for 5 min at 37 ° C water bath. Then 40µl of supernatant culture medium and 40µl were added respectively in the test tubes and white. The whole was homogenized by vortexing and incubated for 30 min at 37 ° C water bath, 200 .mu.l of 2,4-DNPH were added to each tube which were homogenized and incubated at room temperature for 20 min, then 2 ml of 0.4 M NaOH were added to all tubes which were homogenized and incubated at room temperature for 30 min. Finally, the optical density of the maroon solution formed was read against the blank 505nm in a spectrophotometer.

Determination of malondialdehyde

In a test tube, we introduced 500µl of cell suspension and 100 .mu.l Triton X-100 0.2% solution in the PBS. In another tube called white, 500µl of culture medium and 100 .mu.l Triton X-100 were introduced. Then in each of these tubes 125µl of trichloroacetic acid (TCA 20% w / v) and 500µl of thiobarbituric acid (TBA 0.67% w / v) were added. The resulting mixture was homogenized, plugged with glass beads and heated in boiling water for 20 min, then cooled in ice for 5min. The suspension was then centrifuged (3000trs / min, 15min, 4 ° C) and the supernatant collected and the optical density read at 532 nm against the blank. MDA concentrations were calculated by the following formula.

$$[MDA](\mu M/500000\,hépatocytes)=\frac{DO\times V_{ts}\times 10^{6}}{\varepsilon_{MDA}\times V_{sp}\times l}$$

With:

- OD = optical density

- Vts = total volume of supernatant in tubes (.mu.l)

- EMDA = molar extinction coefficient = 1,56x105M MDA-1.cm-1

- Vsp = volume of the supernatant removed for assay (.mu.l)

- L = optical path (1cm)

Statistical analysis of results

The experiments were carried out in triplicate and the results presented as mean ± standard deviations. Normality values inhibitory concentrations, trapping and efficient 50 (IC50; CP50 EC50) calculated using GraphPad Prism 5. The LD50 was also calculated using GraphPad Prism software 5. The simultaneous comparison of groups of data was performed using the test Analysis of variance (ANOVA) followed by Dunnett test. Values were considered statistically different at $p < 0.05$.

RESULTS AND DISCUSSION

Yields of aqueous extracts and extracts flavonoids from Artemisia annua are expressed in the following table:

Table 1: Percentage extracts yield

	total flavonoids extract	aqueous extract
Mass of dry plant used (g)	80	30
Amount of material extracted (g)	11.34	4,07

Extraction yield (%)	14.18	13.57

Table 2: Evaluation of the repeatability of flavonoids assay (average of 3 measurements ± standard deviation).

QUERCETIN CONCENTRATION (mcg / ml)	D.O (420 nm)
5	0.19 ± 0.01
10	0.38 ± 0.01
15	0.58 ± 0.01
20	0.78 ± 0.03
25	0.97 ± 0.02

The dosage of flavonoids in extracts was made through the calibration curve and a quercetin content was obtained (Table 7).

Table 3: flavonoid content (EQ g / kg) of different organic extracts (average of 3 measurements ± standard deviation).

EXTRACT	CONTENT FLAVONOIDS (G EQ / kg of extract)	CONTENT FLAVONOIDS (EQ g / kg of dry leaves)
total flavonoids Bangangté	**99.36 ± 0.51**	**3.45 ± 0.02**
total flavonoids Luxembourg	**98.25 ± 0.38**	**3.17 ± 0.01**

g EQ / kg quercetin equivalent per kilogram.

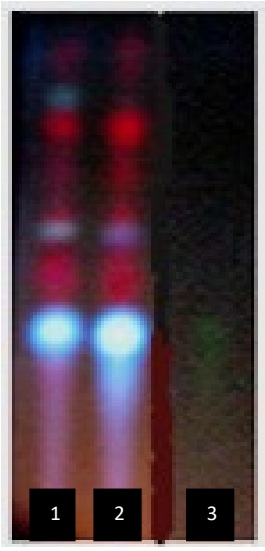


Figure 1a: Revelation 366 nm

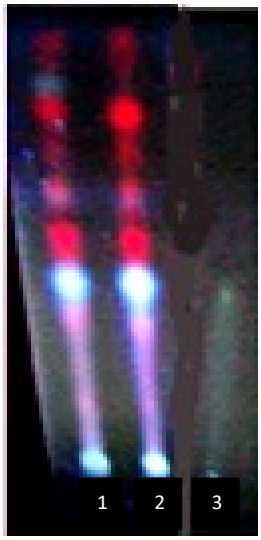


Figure 1b: Revelation 365 nm

after spraying $AlCl_3$. 1: Extract Bangangté 1mg / ml, 2: Extract of Luxembourg 1mg / ml, 3: Quercetin 1mg / ml.

Table 4: Directory TLC on tasks of different extracts before and after spraying AlCl3; eluent: toluene / 10% acetic acid / diethyl ether (0.5 / 1 / 1.5) (V / V / V).

	SPRAYING BEFORE SPRAYING WITH $AlCl_3$		SPRAYING AFTER SPRAYING WITH $AlCl_3$	
	Rf	Color (366 nm)	Rf	366 nm
Quercetin	0.5	Yellow	0.5	Fluorescent
Bangangte	0.93	Red	0.93	Red
Extract	0.84	Yellow	0.84	Yellow
	0.79	Red	0.79	Red
	0.7	Red	0.7	Red
	0.64	Red	0.64	Red
	0.6	Yellow	0.6	Yellow
	0.58	Red	0.58	Red
	0.52	Red	0.52	Red
			0.45	
	0.37	Red	0.37	Red

Luxembourg	0.93	Red	0.93	Red
Extract	0.79	Red	0.79	Red
	0.7	Red	0.7	Red
	0.64	Red	0.64	Red
	0.6	Yellow	0.6	Yellow
			0.59	
	0.58	Red	0.58	Red
	0.52	Red	0.52	Red
			0.45	
	0.37	Red	0.37	Red

These results show that the extraction solvent used is not specific for flavonoids, but also lead coumarins.

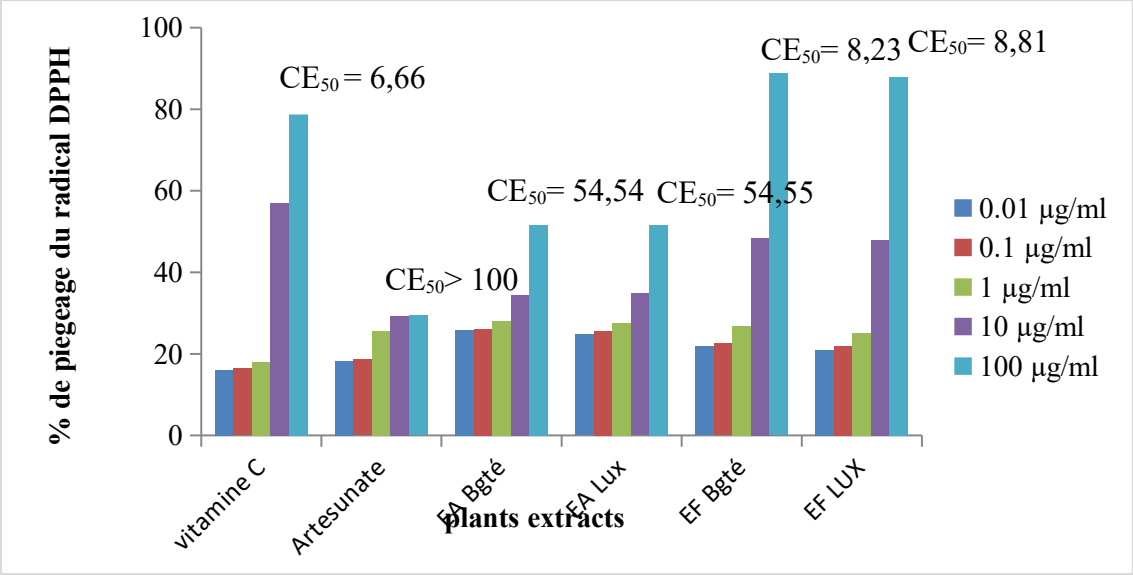


Figure 2: Trapping the radical DPPH ° from plant extracts.

Values are means ± -type deviations of 3 different tests. EA Bgté: aqueous extract of Bangangté EA Lux: aqueous extract of Luxembourg; EF Bgté : total flavonoids extract of Bangangté; EF Lux : extract of total flavonoids of Luxembourg.

It is clear from this figure that the total flavonoids extracts have the best anti-radical activity. With artesunate, we see an increase of up to 10 mcg / ml followed by stagnation at 100 ug / ml.

Moreover, with vitamin C, we note an increase in the concentration-dependent trapping percentage that surpasses our extracts. The EC50 of vitamin C is 6.66 ± 0.74 g / ml while those extracts of total flavonoids from Cameroon, Luxembourg, aqueous extracts of Bangangté, Luxembourg and artesunate are 8, 23 ± 0.56 g / ml, 8.81 ± 0.58 mg / ml, 54.54 ± 0.38 g / ml, 54.55 ± 0.39 g / ml and> 100 mcg / ml.

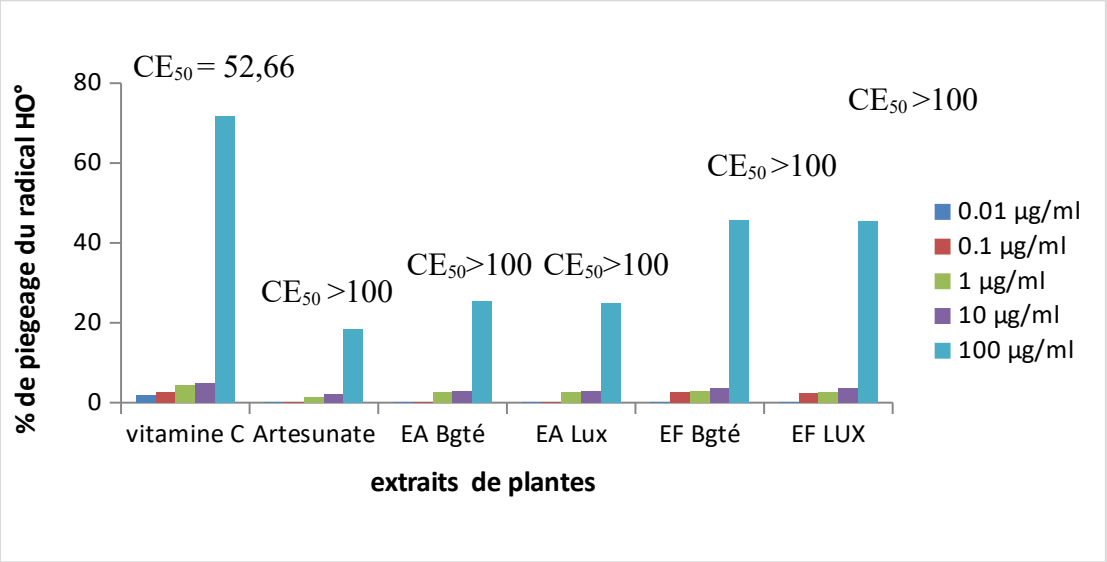


Figure 3: Trapping the radical OH ° from the extracts of plants. Values are means ± -type deviations of 3 different tests in duplicate. EA Bgté: aqueous extract of Bangangté; EA Lux: aqueous extract of Luxembourg; EF Bgté extract of total flavonoids Bangangté; EF Lux extract of total flavonoids of Luxembourg.

The extracts were trapping percentage of the OH radical ° lower than that of vitamin C (52.66 ± 0.11 mcg / ml). The aqueous extracts and flavonoids have a concentration-dependent activity for trapping the OH ° radical. However, to artesunate, the activity is also dependent on concentration but it is lower. Extracts of total flavonoids have a good percentage of entrapment. Nevertheless, our extracts are EC50> 100 ug / ml.

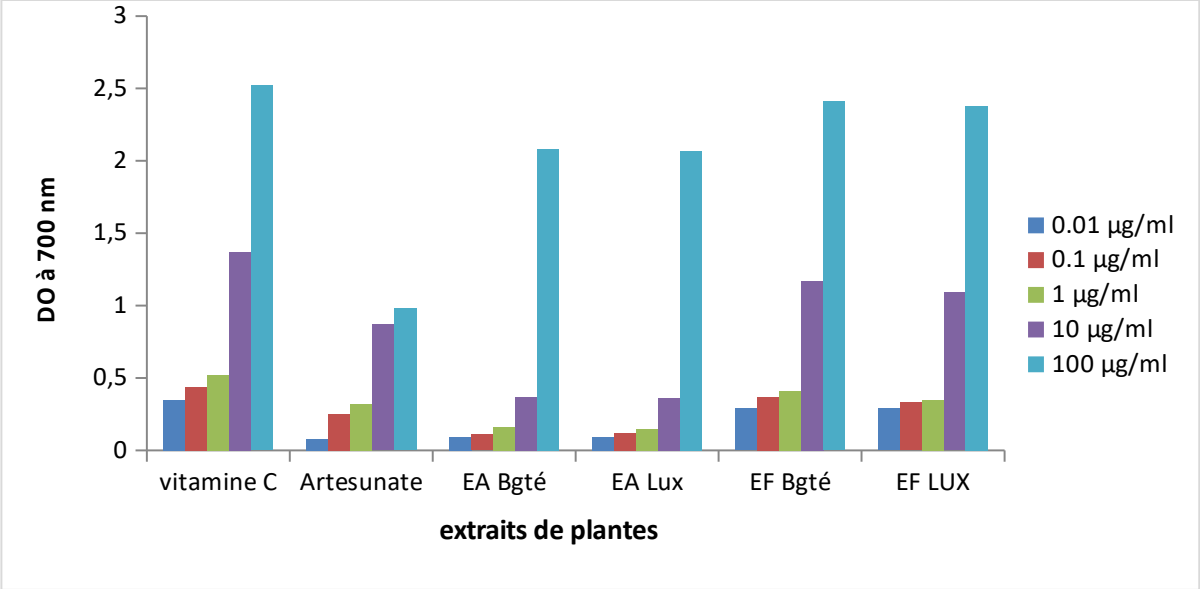


Figure 4: Reduction of ferric iron from plant extracts.

Values are means ± -type deviations of 3 different tests. EA Bgté: aqueous extract of EA Bangangté Lux: aqueous extract of Luxembourg; EF Bgté extract of total flavonoids Bangangté; EF Lux extract of total flavonoids of Luxembourg.

Concentrations of plant extracts and vitamin C increase irreversibly with the decrease of the ferric ion. This increase is also observed with artesunate but the decrease is less than 100 mcg / ml by comparison with other compounds. Extracts flavonoids have the best inhibitory activities ferric iron compared to vitamin

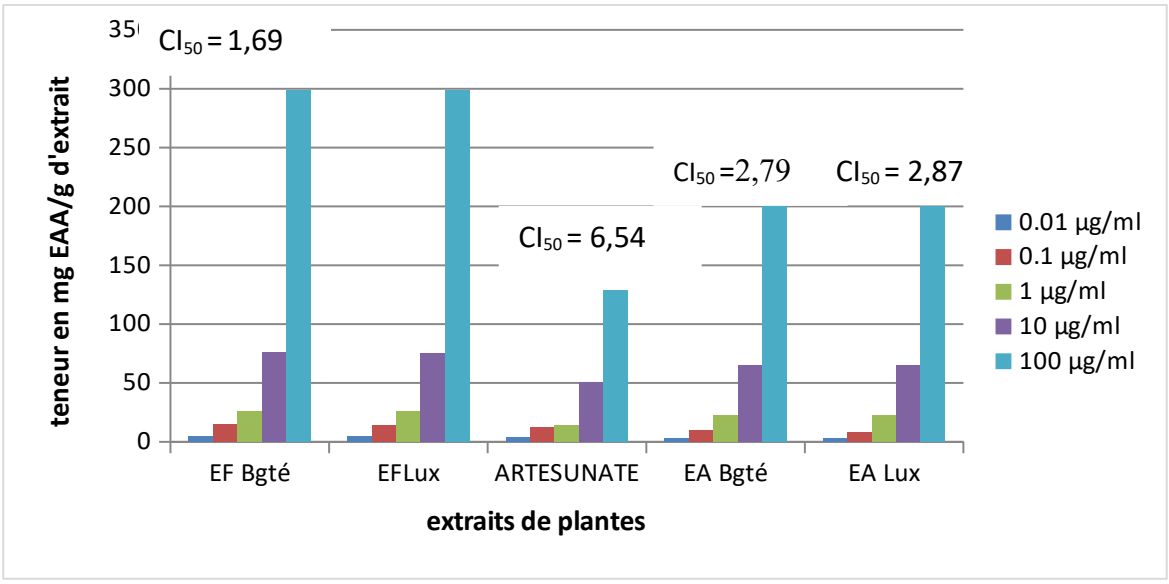


Figure 5: Antioxidant capacity of the plant extracts. Values are means ± -type deviations of 3 different tests. EA Bgté: aqueous extract of EA Bangangté Lux: aqueous extract of Luxembourg; EF Bgté extract of total flavonoids Bangangté; EF Lux extract of total flavonoids of Luxembourg.

IC50 extracts of total flavonoids Bangangté, Luxembourg, aqueous extracts of Bangangté, Luxembourg and artesunate were respectively 1.69 ± 0.21 g / ml, 1.73 ± 0.21 g / ml, of 2.79 ± 0.06 ug / ml, 2.87 ± 0.06 g / ml and 6.54 ± 0.52 g / ml.

Table 4: isolation yields and percentage viability

№ of expérience	Performance (million hepatocytes / g liver)	Cell viability (%)
1	2.37	84.21
2	3.71	80.77
3	2.60	73.08
4	2.25	88.89

In four experiments, we obtained an average yield of isolation of 2.73 ± 0.67 million cells / g of liver to a viable 81.74 ± 6.66%.

Effect of varying concentrations of paracetamol and carbon tetrachloride on the viability of hepatocytes

Hepatocytes suspended in culture medium were incubated in the presence of paracetamol and carbon tetrachloride at various concentrations for 6 hours. The viability of test and measurement of ALT activity was made. Figure 6 below shows the cell viability curves in the middle.

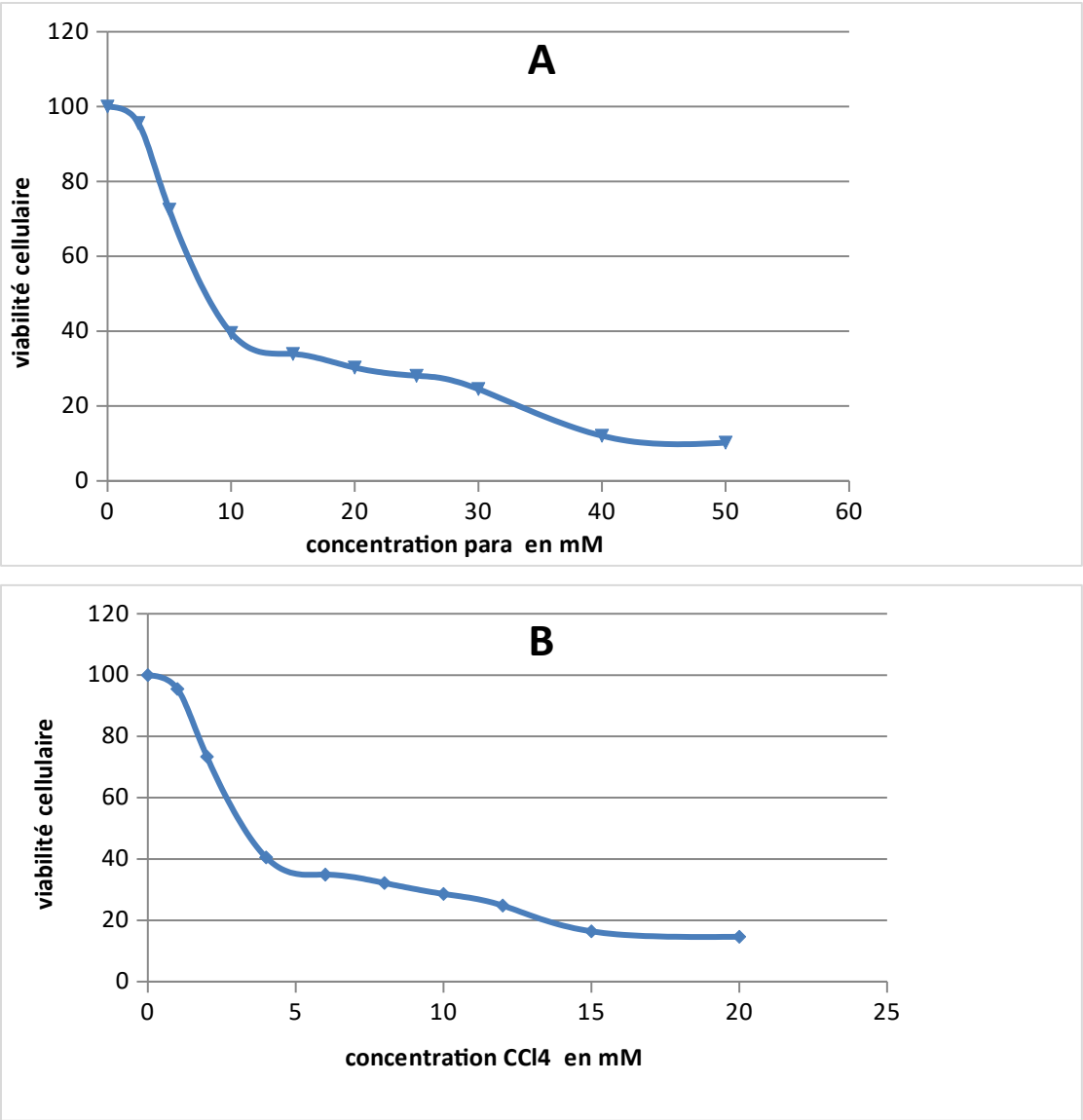
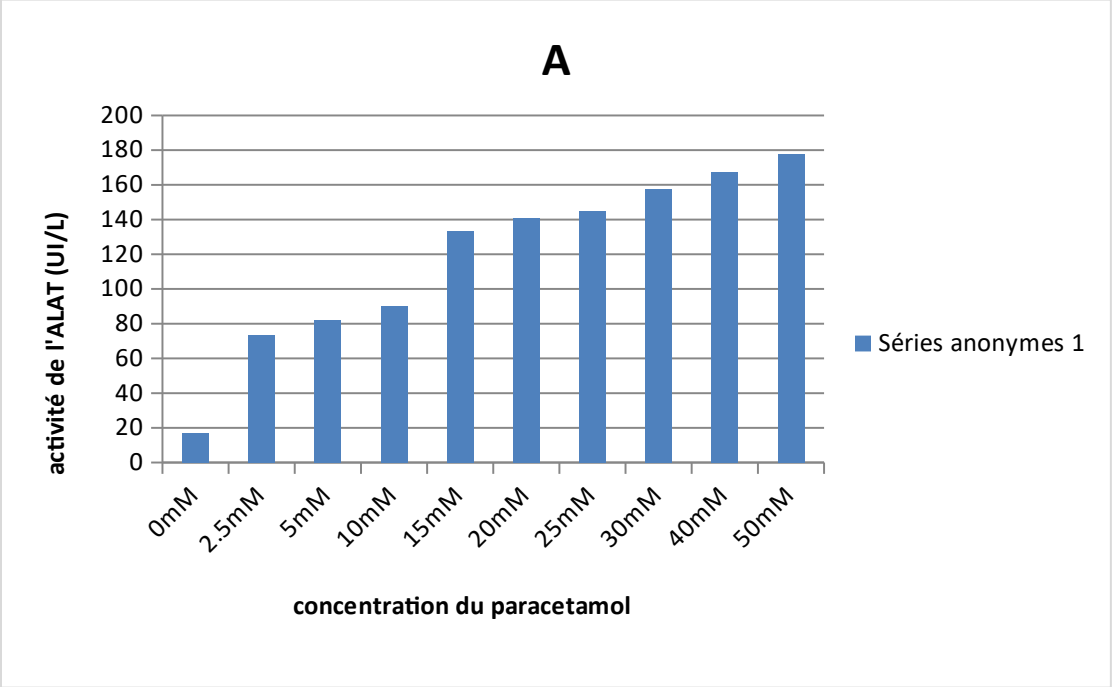


Figure 6: Effect of different concentrations of paracetamol (A) and carbon tetrachloride (B) on the viability of hepatocytes.

Values are means ± -type deviations of 3 different tests. Values statistically different from control (concentration = 0 mm) *** P <0.001.

From these figures, it is clear that the viability of hepatocytes decreases with paracetamol or carbon tetrachloride concentration. Paracetamol LD50 was 11.77 ± 1.54 mM and that of CCl4 of 3.90 ± 1.28 mM.

Figure 7 below shows the curves of ALT activity during paracetamol poisoning and CCl4.



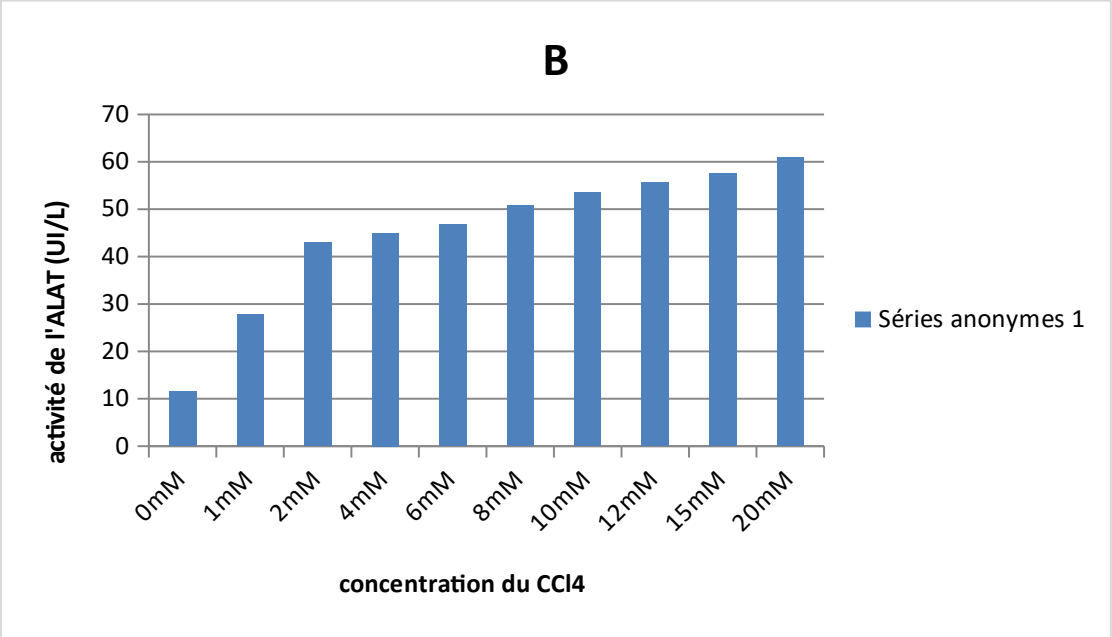


Figure 7: Effect of different concentrations of paracetamol (A) and carbon tetrachloride (B), ALT release in the medium by these cells.

Values are means ± SD of three different tests. Values statistically different from the control (concentration = 0 mm) *** P <0.001. This figure shows that the release of ALT increases in the middle with paracetamol or carbon tetrachlorideconcentration and thus validates the results obtained for cell viability.

The upper limits of the LD50 was used to following our analyzes when cells pretreated poisoning.

III.6. Effect of compounds on the viability of hepatocytes

To anticipate any toxic effect of extracts and artesunate on hepatocytes, the cells were incubated in the presence of increasing concentrations of the extracts from flavonoids and aqueous Bangangté and Luxembourg of Artemisia annua. The viability of hepatocytes by the viability test with MTT / Formazan and ALT activity were assessed (Figure 8 and 9).

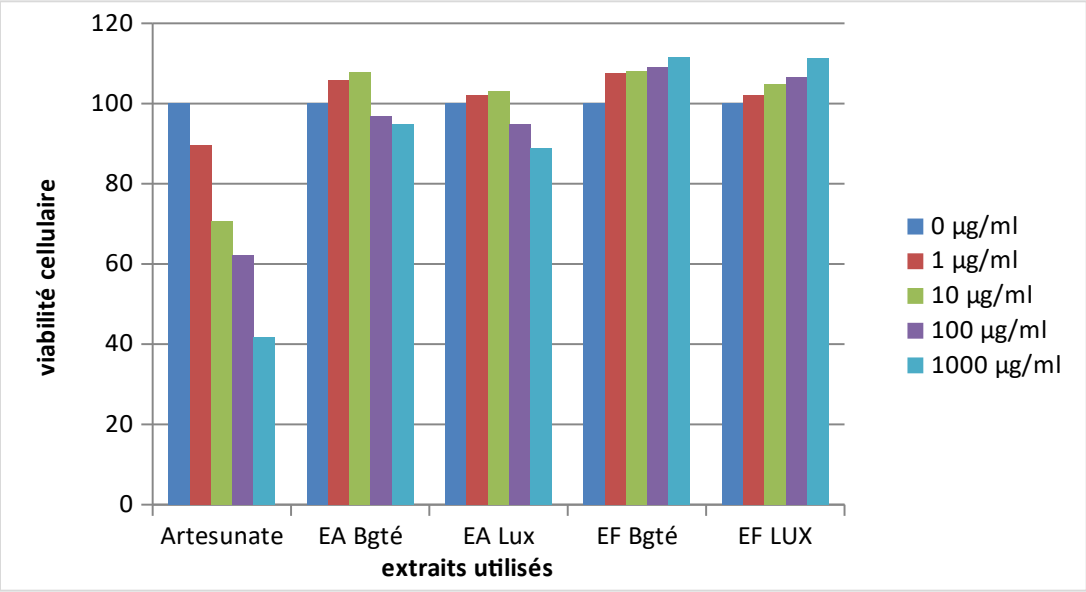


Figure 8: Effect of extracts and artesunate on the viability of hepatocytes.

Values are means ± SD of three different tests. Values statistically different from the control (0 ug / ml) at * p <0.05; ** p <0.01, *** p <0.001. EA Bgté: aqueous extract of EA Bangangté Lux: aqueous extract of Luxembourg; EF Bgté extract of total flavonoids Bangangté; EF Lux extract of total flavonoids of Luxembourg Viability of hepatocytes was not affected by increasing concentrations of flavonoids extracts.

But artesunate significantly reduced the viability of hepatocytes from 10 ug / ml. This drop in viability is observed also when the hepatocytes are incubated in the presence of 1000 mcg / ml of aqueous extract of Artemisia annua Luxembourg**.**

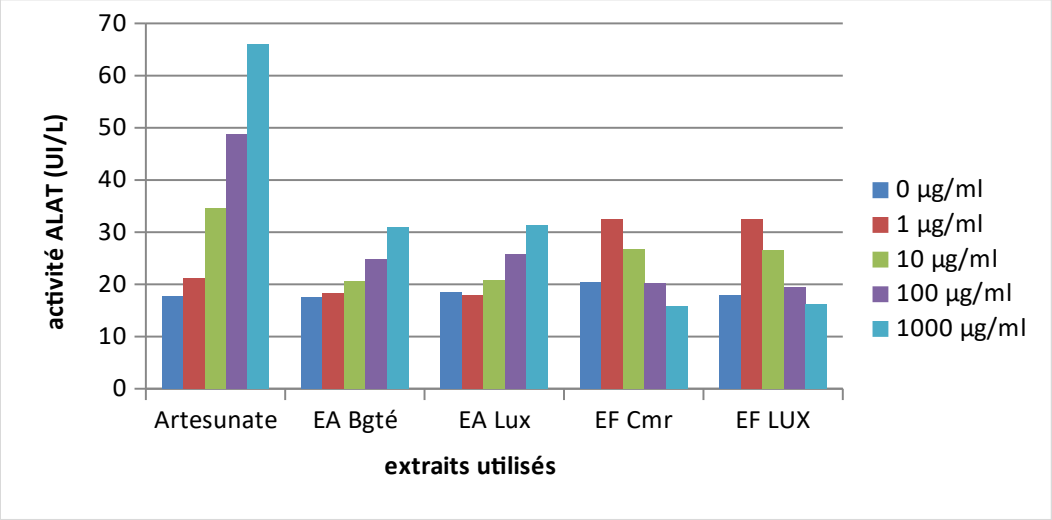


Figure 9: Change in ALT activity based extracts.

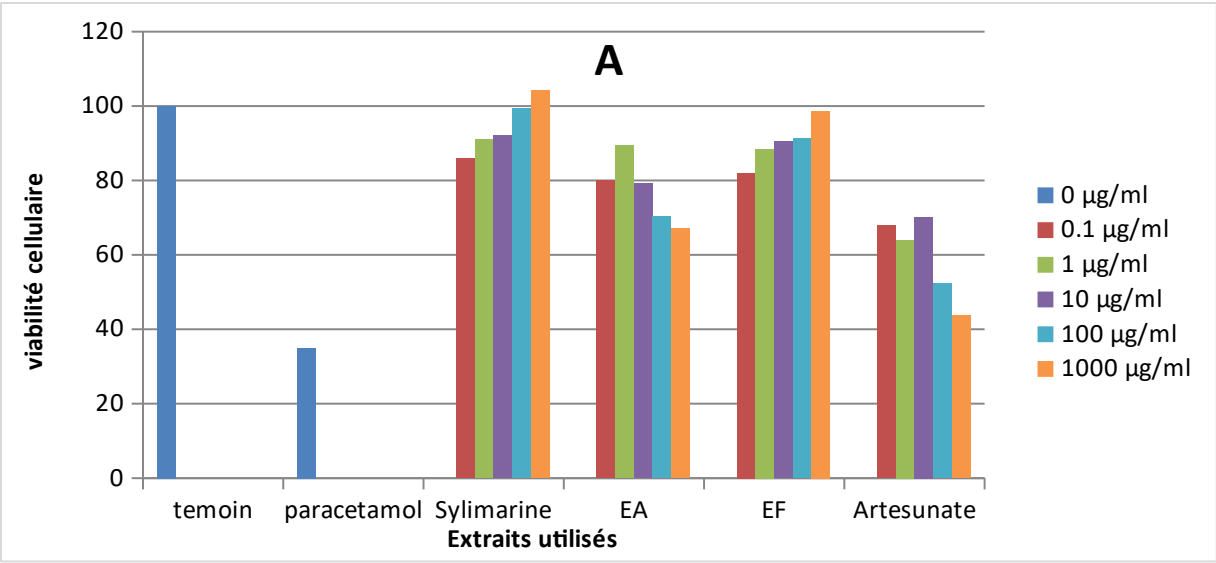
Values are means ± SD of three different tests. Values statistically different from control (0 ug / ml) at ** p <0.01, *** P <0.001. EA Bgté: aqueous extract of EA Bangangté Lux: aqueous extract of Luxembourg; EF Bgté extract of total flavonoids Bangangté; EF Lux extract of total flavonoids of Luxembourg

Concentrations of 10 to 1000 mcg / ml of the extracts of *Artemisia annua* flavonoids decrease the release of ALT in the culture medium. The increasing concentrations of artesunate increase proportionally the release of ALT. As regards the aqueous extracts, it is observed a decrease in

ALT levels at low doses (1 to 10 ug / ml). However, at high concentrations, the aqueous extracts negatively affect the membrane integrity characterized by increased ALT activity. The extracts of *A. annua* Bangangté giving identical results significantly regarding cell viability and release of ALT compared to those of Luxembourg, extracts Bangangté were used to following our analyzes.

III.7.1. Effect on cell viability and measurement of ALT activity

Figures 10 (A) and (B) represent the measurement of cell viability and ALT activity when hepatocytes are pretreated with plant extracts before being poisoned by paracetamol**.**



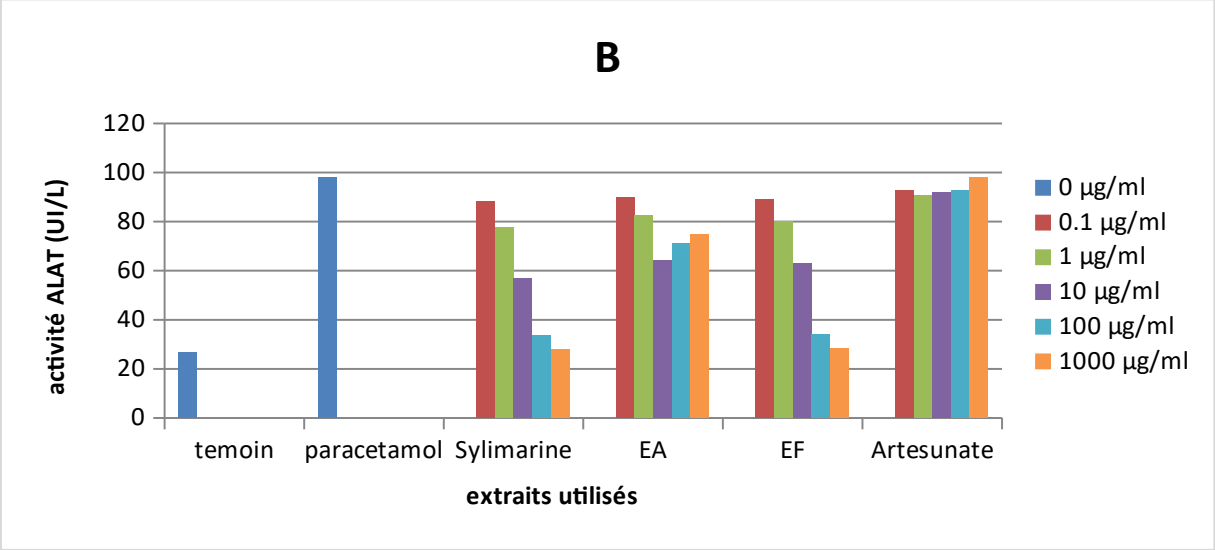
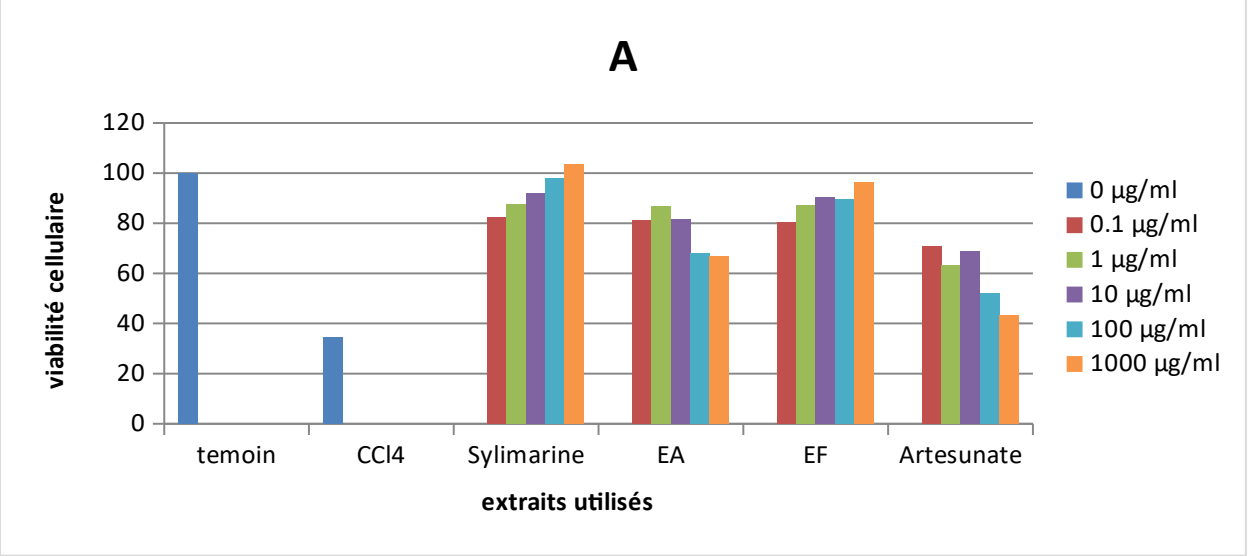


Figure 10: Effect of pretreatment with plant extracts and artesunate on the viability of hepatocytes after poisoning in vitro by 13.31 Mm paracetamol

(A) Test of cell viability by the MTT assay; (B) ALT activity. Values are means ± SD of three different tests. Values statistically different from the control was $p < 0.001$ or negative control b $p < 0.01$, c $p < 0.005$, d $p < 0.001$. EA: aqueous extract, EF: extract of total flavonoids.

Compared to control, paracetamol 13.31 mM decreases the viability of hepatocytes after 6 hours of incubation. Likewise, it increases the release of transaminases in the medium. Silymarin increases the viability from the lowest concentration 0.1 ug / ml and also reduces the release of ALT in the cell medium. Pretreatment of the cells induces a concentration dependent increase in the percentage viability with the extract of total flavonoids. With aqueous extract from A. annua a better viability was found at the concentration of 1 ug / ml and higher activity at 10 mcg / ml. Artesunate to concentration 10 mg / ml, has the best rate and the best business viability. However, concentrations to 1000 mcg / ml, there is a decreased viability and increased the release of ALT.

Figures 11 (A) and (B) represent the measurement of cell viability and ALT activity when hepatocytes are pretreated with plant extracts before being poisoned by CCl4.



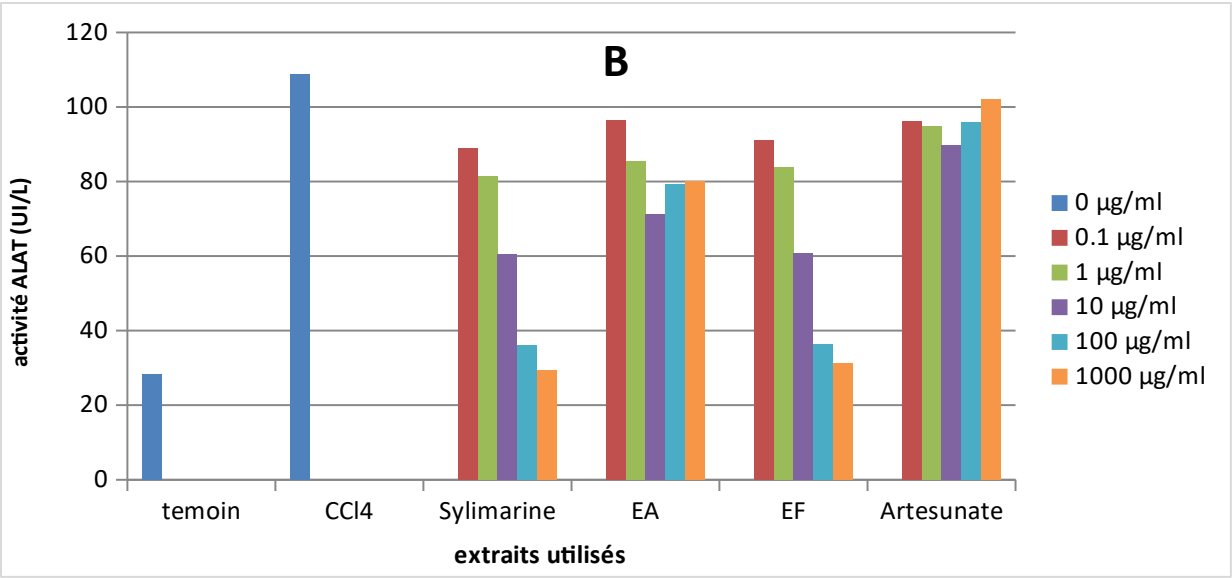
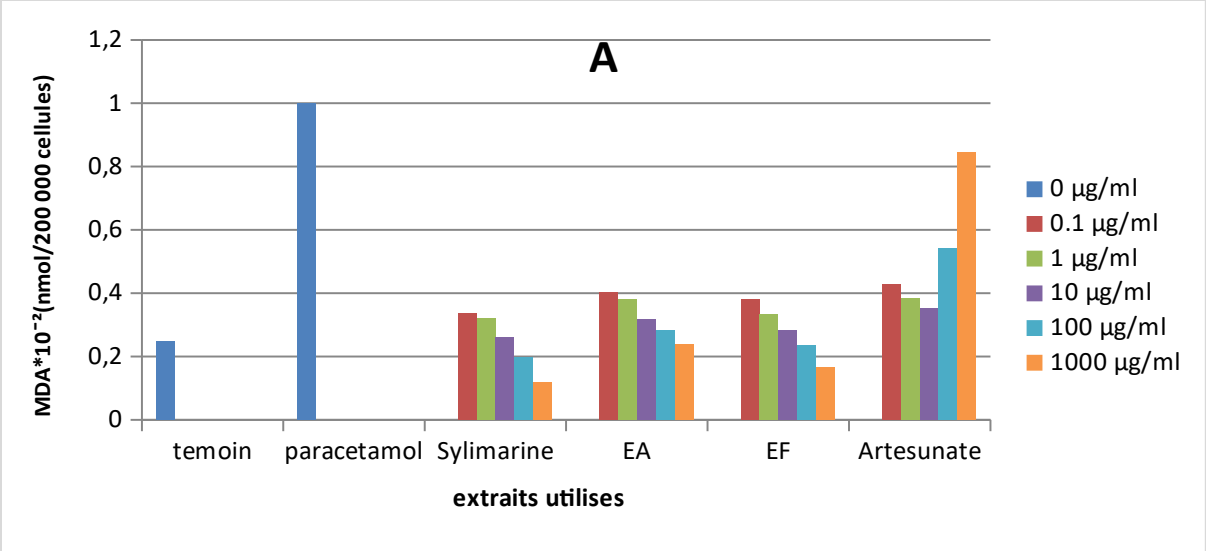


Figure 11: Effect of pre-treatment with plant extracts and artesunate on the viability of hepatocytes after poisoning in vitro by 5.18 Mm CCl4 (A) Test of cell viability by the MTT assay; (B) ALT activity.

Values are means ± SD of three different tests. Values statistically different from the control was p <0.001 or negative control b p <0.01, c p <0.005, d p <0.001. EA: aqueous extract, EF Cmr extract of total flavonoids. Compared to control, the CCl4 5.18 mM decreases the viability of hepatocytes after 6 hours of incubation. Likewise, it increases the release of transaminases in the culture medium. Silymarin increases the viability from the lowest concentration 0.1 mg / ml, in parallel it also reduces the release of ALT in the cell medium. Pretreatment of cells induced a concentration-dependent increase in the percentage of viability with the extract of total flavonoids. With aqueous extract from A. annua a better viability was found at the concentration of 1 ug / ml and higher ALT activity to 10 micrograms / ml. Artesunate to concentration 10 mg / ml, has the best rate and the best business viability. However, concentrations to 1000 mcg / ml, it is observed a decreased viability and increased the release of ALT.

Effect of extracts on the MDA levels

Figures 12 (A) and (B) represent the hepatoprotective effect of plant extracts and artesunate on lipid peroxidation in case of poisoning with paracetamol and CCl4.



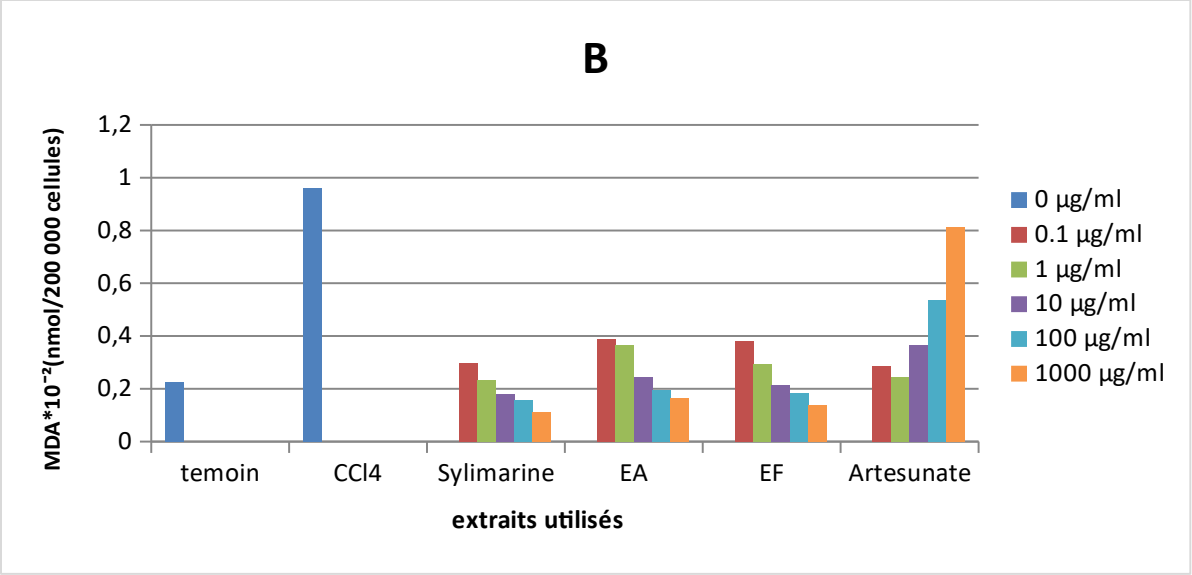


Figure 12: Effect of pre-treatment with plant extracts on rates after Malondialdehyde (A): paracetamol poisoning, (B) poisoning CCl4.

Values are means ± SD of three different tests. Values statistically different from the control was $p < 0.001$ or the negative control of $p < 0.001$. EA: aqueous extract; EF Cmr extract of total flavonoids.

Plant extracts mostly decreased significantly ($p < 0.001$) MDA levels at the concentrations tested and artesunate shows a significant increase in MDA levels to 1000 mcg / ml during paracetamol poisoning and CCl4.

Discussion

Artemisia annua and artesunate are known for their antimalarial properties. Malaria is a public health problem and its treatment often leads to misuse of drugs. Toxic side effects of this abuse are often reported. In our work, we assessed the hepatic properties of these compounds by studying their hepatoprotective and antioxidant properties *in vitro*.

The extraction efficiency of flavonoïds from the plant material was quantified. The dosage of flavonoids by AlCl3 is a universal method which uses quercetin as standard [15]. The ethanol extracts gave an average flavonoid content of 99.56 g / kg extract. In order to separate and identify the flavonoids in *Artemisia annua*, we proceeded by TLC analysis of different extracts. We counted 8 flavonoids in the sample from Bangangté and 7 in the sample from Luxembourg. These results agree with those obtained by Lamero in 2011: an average content of 99,36g / kg extract and a similar number of flavonoids [18].

The evaluation of antioxidant effects was carried out using various methods: the ability to scavenge free radicals DPPH ° and OH ° and the inhibitory effect on oxidation reactions (reduction of ferric ions and of phosphomolybdic acid).

The total extract of flavonoids from Cameroon gives a percentage of trapping of 87.89% when tested at a concentration of 100 µg/ml while the extract of Luxembourg gives a percentage of 88.78% at this same concentration. These results are lower than those for vitamin C which has a percentage of 92.07%, but higher than for artesunate (29.39%). Based on these results, effective concentrations were calculated and a better EC50 (8.23 ± 0.56 g/ml) was obtained for the extract of Cameroon flavonoids, probably due to higher number of flavonoids in this extract than the extract of Luxembourg. A better EC50 also was observed for the aqueous infusion from Cameroon (54.54 ± 0.42 mcg / ml) compared to that of Luxembourg (54.55 ± 0.39 mcg / ml). Artesunate only has an EC50> 100 mg / ml. Evidently, the best EC50 was obtained with vitamin C (6.66 ± 0.74 mg / ml).

These results could be explained by the capability of total extracts of flavonoids to yield protons giving a stable radical. [19] So they would act as first line antioxidants. These results are in agreement with the work of Marc et al in 2004 [19]. The high antiradical activity of the extracts compared to their respective aqueous infusions is probably related to the higher concentration of flavonoids.

The ability to scavenge free OH ° radicals was much lower than for the vitamin C, although however, flavonoids extracts presented an excellent trapping rate. These results are in agreement with the work of Su et al in 2009 [20].

The ability of these extracts to prevent the formation of free radicals was assessed thereafter using reduction assays for ferric ions and molybdate. The reducing power for ferric iron ions was concentration-dependent. Thus, the flavonoid extracts showed reducing powers close to that of vitamin C and a higher value for the Camroon extract. In addition, the aqueous extracts of Artemisia annua have a stronger reducing activity at a concentration of 100 micrograms / ml than artesunate. The reduction of phosphomolybdic acid to molybdenum blue by these compounds was also concentration-dependent, artesunate being weak in this assay. IC50s were calculated and we obtained respectively for total flavonoid extract from Cameroon, Luxembourg, the aqueous extracts of Cameroon, Luxembourg and artesunate 1.69 ± 0.21 g / ml, 1.73 ± 0 21 mcg / ml, 2.79 ± 0.06 g / ml, 2.87 ± 0.06 g / ml and 6.537 ± 0.52 g / ml.

These results confirm previous findings that in addition to scavenging free radicals, these plant extracts contain compounds that inhibit their formation. Nevertheless, artesunate's activities are less, this due to the fact that it is not a flavonoid and does not contain any.

The isolation of hepatocytes allowed us to get a cell yield of 2.73 ± 0.67 million cells / g of liver to an average viability of 81.74 ± 6.66%. This result is lower than that obtained by Kommegne which was 4.16 ± 0.44 million hepatocytes with a viability percentage of 90.5 ± 1.29 [13]. Similarly, our results are lower than those obtained by Xiao et al, 2001 which was 39 ± 12x106 / g, (88 ± 2%). [21] These differences could be explained by differences in the technical isolation and purification of the obtained cells on a Percoll gradient. However, sustainability is satisfactory for the proposed study. That is why we have continued with subsequent studies.

Liver damage caused by paracetamol or carbon tetrachloride, well known hepatotoxic agents in overdose [22], are widely used to evaluate the hepatoprotective activity of drugs and herbal medicine [23]. Toxigenic markers following exposure of cells to a toxic substance are very often the transaminases. An increase in the extracellular medium is a sign of alteration of the plasma membrane; the increase of MDA concentration is a sign of a major membrane lipid peroxidation. [24] In this study, the effects of paracetamol and CCl4 were studied at different concentrations on

the viability of hepatocytes for 6 hours. From this study, the LD50 of paracetamol and CCl4 were respectively 11.28 ± 1.18 mM and 4.52 ± 1,06.mM.

The hepatoprotective properties of our compounds were then evaluated using the values greater than the LD50. The results indicate that exposure of cells to toxic substances induced a significant ($p < 0.001$) increase in MDA levels, the ALT activity in the incubation medium and a significantly lower ($p < 0.001$) cell viability. The pretreatment with the extracts and artesunate showed a significant ($p < 0.001$) decrease in build-up of MDA and ALT activity in the incubation medium and a significant increase ($p < 0.001$) cell viability. However, artesunate showed no significant decrease in the formation of MDA and ALT activity in the incubation medium or significant increase in cell viability in the case of paracetamol poisoning. This might be different in the mechanisms of toxicities of paracetamol and CCl4. We may conclude that the extracts from flavonoids and aqueous infusions are reinforcing membrane integrity and inhibit the membrane lipid peroxidation, essential fort he survival of living cells. Artesunate however has a rather harmful effect on the liver.

These results confirm the work of Nwanjo and Ose which showed that artesunate has a toxicity in liver cells with a considerable increase in enzyme markers (especially transaminases) due to the release of free radicals necessary for the antimalarial activity of this artemisinin derivative.

Toxic concentrations of artesunate start at 100 µg/ml. These results are consistent with the work on the effect of plasma concentrations of artesunate in multiple different administrations as for Miller et al in 2012 25] who found for intravenous administration of doses of 2, 4, and 8 mg / kg artesunate, concentrations up to 28, 411µg / ml, 40, 574 .mu.g / ml and 63, 677µg / ml, Hien et al [26] for intramuscular administration of 2.4 mg / kg found a maximum concentration of 2.195 g / ml and Diem et al [27] who during oral administration of 200 mg for 5 days obtained a maximum concentration of 0.067 ug / ml on day 1 and 0.058 mg / ml on day 5. Thus, normal artesunate concentrations that are 4 mg / ml for intravenous or [25], 2,4mg / kg for intramuscular [26] and 200 mg for oral administration [27] does not cause toxicity to hepatocytes.

Conclusion

The objective of this study was to compare the hepatoprotective activity of flavonoids from *Artemisia annua* versus artesunate, it emerges that

From this study the following conclusions can be drawn:

- The extraction of total flavonoids of *Artemisia annua* was made as they were made gave promising results
- The extracts of total flavonoids have the best antioxidant and hepatoprotective properties; and therefore, the aqueous extracts of *A annua* possess antioxidant properties and are hepatoprotective thanks to the flavonoids they contain.
- Artesunate is toxic to hepatocytes in overdoses.

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