



Effect of flavonoids of *Gmelina arborea* Roxb. (Verbenaceae) from Côte d'Ivoire on the antioxidant activity and osmotic stability of erythrocytes

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ABSTRACT

Objectives: The excessive production of free radicals in living organisms leads to oxidant stress that would cause numerous pathologies. We determined the antioxidant activity of flavonoids with the intention of contributing to the search of new natural molecules with antioxidant effect.

Methodology and results: The antioxidant activity of flavonoids obtained from the aerial parts of *Gmelina arborea* of Côte d'Ivoire were studied, in addition to investigating the cytotoxicity of the aqueous extract (used in non conventional medicine) and total flavonoids of *G. arborea* on erythrocytes. In vitro assay of the antioxidant potential against the DPPH revealed that fractions of total flavonoids possess radical scavenging capacity. The determined EC₅₀ were 7.362 and 8.072 µg/mL respectively, for ethyl acetate and n-butanol extracts against 6.02 µg/mL for vitamin C. Cytotoxicity assay showed an elevated haemolysis for the aqueous extract (minimum 0.872) against a minimum of 0.295 for total flavonoids.

Conclusion and application of findings: This study showed a good antioxidant activity and good stability from osmotic tests on erythrocytes with the total flavonoids extracts from leaves of *Gmelina arborea*. Flavonoids had excellent anti-oxidants so it play an important role in the cell's defence system. The results showed that flavonoids from leaves of *G. arborea* have low cytotoxicity compared to the aqueous extract. This would explain the use of plant (flavonoids of *Gmelina arborea*) in therapy of traditional medicine.

Key words: *Gmelina arborea*, flavonoids, antioxidant activity, DPPH, EC₅₀, cytotoxicity.

INTRODUCTION

Gmelina arborea Roxb. (Verbenaceae) is native to Asia and known by various names, e.g. Yemane, Gamar, Gumhar and, Sor. This species has been introduced in several countries, particularly in West Africa and especially in Côte d'Ivoire and Nigeria. Ethnobotanical studies report that the species is

widely used to treat many diseases including diarrhoea, hypertension and malaria, among others (Sharma & Balakrishnav, 1993; Arbonnier, 2002; Tiwari et al., 2008). A recent study of the composition of secondary metabolites and antioxidant activity of *G. arborea* showed its

richness in phenolic compounds and natural antioxidant substances that play an important role in pharmacology (N'gaman et al., 2009). The body produces reactive species of oxygen called free radicals (FR) daily (Cadenas & Davies, 2000; Pincemail et al., 2001), which are highly reactive compounds with an unpaired electron and necessary for some vital cellular processes. However, overproduction of FR can be harmful to the organism. They damage diverse cellular components including proteins, lipids or DNA (Boyd et al., 2003; Favier, 2003).

The multiplicity of medical consequences of oxidative stress (related to the excessive production of FR in the body) is related to the diversity of radical species, other variable factors and genetic abnormalities specific to each person (Hadi, 2004). By inducing production of abnormal biological molecules, through over expression of certain genes, oxidative stress is the main cause of several diseases: cancer, cataracts, amyotrophic lateral sclerosis, acute respiratory distress

MATERIALS AND METHODS

Study site and plant species: The taxonomic grouping of *G. arborea* found in Côte d'Ivoire has been identified by Aké Assi (Daloa; 10-02-1965; Aké-Assi; 7769, National Floristic Center of Abidjan-Cocody University, Côte d'Ivoire). The leaves, the bark and the roots of *G. arborea* were harvested within the University of Abobo-Adjame (Abidjan, Côte d'Ivoire). *Gmelina arborea* is an ornamental plant. They were cleaned, dried in a well ventilated room and then ground to fine powder using an electric grinder (RETSCH, type SM 100).

Extraction of total flavonoids (FT): One hundred grams of fine powder of the leaves were macerated in 1 L of EtOH (80%, v/v). After filtration and concentration of the macerate, 10 g of the pellet dissolved in 100 mL of distilled water was treated with 600 mL of hexane. A liquid extraction of the aqueous phase was carried out successively with CHCl₃ (3 × 150 mL), AcOEt (3 × 150 mL) and n-BuOH (3 × 150 mL). Ethyl acetate fractions of yellow color (flavones, flavanones) and n-BuOH red color (anthocyanes) were concentrated using a rotary evaporator (Benkiki, 2006). The inhibition percentages were quantified for concentrations ranging from 0.001 to 2 mg / mL.

syndrome, pulmonary oedema, accelerated aging and, Parkinson's disease (Montaigner et al., 1998; Favier, 2003; Hou et al., 2003; Hadi, 2004).

Much of the interest of current research involves the study of natural antioxidant molecules such as vitamins, carotenoids and polyphenols (tannins, flavonoids, coumarins, alkaloids, terpenes, among others). Most studies published in recent years have shown that hydroxyflavones have antioxidant properties similar to vitamin C (Kokoska & Janovska, 2009). In fact, flavonoids are secondary metabolites known for their excellent antioxidant properties, in addition to other biological properties with which they are endowed (Rao et al., 2008; Kokoska & Janovska, 2009; Wang et al., 2009).

The present study aimed to (i) quantify the antioxidant activity of total flavonoids of *G. arborea* in comparison to that of vitamin C and (ii) test their stability on erythrocyte osmotic stability when formulated as an aqueous extract (recipe commonly used in traditional medicine).

Antioxidant activity: The antioxidant activity of flavonoids was determined in comparison to vitamin C (antioxidant reference) using the stable free radical 1,1-diphenyl-2-picrylhydrazyle (DPPH) (Chen et al., 2004; Ayoola et al., 2006; Singh et al., 2007; Wu et al., 2008; Sun et al., 2009). One mL of a solution of DPPH [concentration of 0.14 mg / mL] prepared in absolute ethanol was added to 2.5 mL of plant extract of variable concentration. The absorbance was read at 517 nm after 30 minutes of reaction with a spectrophotometer (JASCO V500). The percentage of inhibitory activity of flavonoids of DPPH was calculated by the formula $[1 - (A_0/A)] \times 100$, where A₀ and A are the absorbance of plant extract and DPPH respectively. To compare the antioxidant activity of FT with vitamin C, a post ANOVA test (Dunnet test) was performed.

Osmotic stability: Extracts of total flavonoids and aqueous extract of the leaves were used. The extracts were prepared at five different concentrations levels (5, 4, 3, 2 and 1 mg / mL) for volumes ranging from 0.1 to 1 mL for each concentration. The extracts obtained were mixed with saline [NaCl 0 to 1% (p / v)] and brought into contact with aliquots (25 µL) of blood taken from rabbits [*Oryctolagus cuniculus* (Leporidae)]. The rabbits were aged 6 months and

obtained from the experimental farm of the University of Abobo Adjame. The blood was collected in EDTA tubes (after an overnight fast) and then homogenized and incubated for 30 minutes at 37°C. They were then centrifuged (1300 rpm for 10 minutes) with a centrifuge brand Hermile Z 300K. The absorbance of the supernatant was then determined at 540 nm. With the help of the determined values of absorbance, a sigmoidal regression curve given by the Boltzmann formula was plotted for the purpose of calculating the percentage of haemolysis in each extract, according to the following formula (de Freitas et al., 2008).

$$\text{Hemolysis (\%)} = \frac{A1 - A2}{1 + e^{(S-H50)/dS}} + A2$$

Where;

- A1 and A2 are the mean maximal and mean minimal absorbance values of the sigmoid, respectively,
- S is NaCl concentration,
- H50 is the NaCl concentration that causes 50% of hemolysis and

RESULTS

Antioxidant activity of total flavonoids (FT): The mean percentage reduction of DPPH of the vitamin C (83.18 ± 17.79); FT (AcOEt = 68.27 ± 25.36) and n-BuOH = 68.85 ± 21.77) of *G. arborea* was significantly different ($F = 3.58$, $P = 0.03$). The value of Dunnett test (14.33) compared to the difference of means (Table 1) showed that vitamin C had an antioxidant effect higher than that of FT. However the FT had the same antioxidant activity effect ($P > 0.05$). Flavonoids extracts showed a high antioxidant activity (Figure 1). The

- dS is the amplitude of the sigmoidal transition between A1 and A2.

The intensity of the hemolysis was measured by the difference between A1 and A2 (H). The percentage of hemolysis in each test tube was calculated by the equation:

$$\text{Hemolysis (\%)} = \frac{A}{A1} \times 100$$

Statistical Analysis: The percentages of inhibition of different EC50 were analyzed by mean comparisons using analysis of variance, ANOVA (Dagnélie, 1999). The significance of the test was determined by comparing the probability (P) associated with the test statistic of Fischer (F: Fischer-Snedecor statistic) at the threshold $\alpha = 0.05$. For significant differences in threshold, the test of least significant difference (LSD) was conducted (Dunnett test). The averages of the inhibition parameter model were compared with the PROC GLM procedure. Statistical analyses were implemented with the SAS statistical package (SAS, 1999).

inhibition percentages for concentrations ranging from 0.025 to 2 mg / mL were not only high but also close to those of vitamin C. However, the highest antioxidant activity was obtained with concentrations from 0.05 to 2 mg / mL n-BuOH, and 0.05 to 1 mg / mL AcOEt. (Table 2) The evaluation of the 50% effective concentration (EC50) showed that the EC50 extracts assayed are relatively small and therefore effective. However, the AcOEt extract was more effective than the n-BuOH extract.

Table 1: Comparison of the absolute value of the difference in the average total flavonoids and the Dunnett value (Dunnett test).

Difference of means	Value of Dunnett : 14.33
AcOEt – Vitamin C = 68.27-83.19	+
n-BuOH – Vitamin C = 68.85-83.19	+

+: Significant difference between means.

Osmotic stability: The results obtained after evaluating the osmotic stability of erythrocytes showed that the FT had a high percentage of hemolysis when the concentration of the extract is lower (hypotonic solution). When the concentration of FT increased in the reaction medium, there was a decrease in the percentage of hemolysis. This shows that the total flavonoids in the presence of red blood cells didn't

induce complete lysis. It should be noted that all the extracts have an acidic pH (3.44-4.04). The results of the parameters (hemolysis H, H50 and amplitude dS) obtained on curves of aqueous extracts (crude extract) of the ethyl acetate fraction (flavones and flavonols), the fraction of n-BuOH (anthocyanins) and control (saline) are shown in tables 3 and 4. The values of all parameters tested (H50, H and dS) were determined

from the curves and the Boltzmann formula (Figure 2). The comparison between the effect of the tested extracts on osmotic stability (Table 3) showed a significant difference ($P < 0.05$). Compared to the control (NaCl), the values of H were significantly lower with ethyl acetate extracts, n-BuOH, and higher with the aqueous extract. For H50, the ethyl acetate extract had the same effect as NaCl solution. The extracts tested

showed mean values significantly different ($P < 0.05$) between concentrations (Table 4). The values of H50 and H decreased significantly with increasing extracts concentrations ($P < 0.05$). The amplitude values dS were statistically identical for the different concentrations ($P > 0.05$).

Table 2: Analysis of variance of mean percentage inhibition of total flavonoids

Concentrations (mg/ml)	Vit C*	CE50 ($\mu\text{g/ml}$)	n-BuOH*	CE50 ($\mu\text{g/ml}$)	AcOEt*	CE50 ($\mu\text{g/ml}$)
0.001	37.14 \pm 0.59e	6.020	19.66 \pm 6.18d	8.072	10.84 \pm 1.51e	7.362
0.01	88.83 \pm 0.10d		53.23 \pm 10.4c		45.54 \pm 4.10d	
0.025	89.67 \pm 0.09bc		68.73 \pm 2.28b		78.34 \pm 1.87c	
0.05	89.80 \pm 0.09bc		80.47 \pm 2.16a		82.13 \pm 0.59ab	
0.1	90.02 \pm 0.09b		83.03 \pm 1.9a		83.77 \pm 0.82a	
0.5	89.61 \pm 0.09c		83.63 \pm 1.75a		83.98 \pm 1.20a	
1	89.09 \pm 0.10d		82.34 \pm 2.05a		81.79 \pm 2.29abc	
2	91.29 \pm 0.08a		79.7 \pm 2.4a		79.71 \pm 2.06bc	
P	< 0.001		< 0.001		< 0.001	

(*)For each sample, means followed by the same superscript were not significantly different ($P \geq 0.05$), Vit C : Vitamin C, CE50 : Concentration required to reduce radical formation by 50%, n-BuOH : n-butanol, AcOEt : Ethylacetate

Table 3: Analysis of variance to compare the extracts to H, H50, and dS for lysis of erythrocytes

Extracts*	N	H	P(H)	H50	P(H50)	dS	P(dS)
NaCl	15	0.015 \pm 0.004c	< 0.001	0.559 \pm 0.004c	< 0.001	0.075 \pm 0.002b	< 0.04
AcOEt	15	0.420 \pm 0.137b		0.519 \pm 0.094c		0.082 \pm 0.011a	
nBuOH	15	0.483 \pm 0.259b		0.617 \pm 0.107b		0.075 \pm 0.001b	
Aqueux	15	0.991 \pm 0.059a		0.949 \pm 0.041a		0.074 \pm 0.001b	

(*) For each extract, means followed by the same superscript were not significantly different ($P \geq 0.05$), N : Observation, H : The intensity of hemolysis, H50 : NaCl concentration causing 50% hemolysis, dS : Amplitude of the sigmoid transition, NaCl : Sodium chloride, n-BuOH : n-butanol, AcOEt : Ethylacetate

DISCUSSION

This study showed a good antioxidant activity and good stability from osmotic tests on erythrocytes with the FT extracts from leaves of *Gmelina arborea*. Indeed, N'gaman et al. (2009) showed a significant variability in phytochemical composition based on the presence of phenolic flavonoids, coumarins and anthocyanins. Their antioxidant activity would contribute to various therapeutic applications. This observation is supported by the work of Dighe et al. (2007), which showed that the antioxidant activity of the crude extract of leaves of the Indian species of *G. arborea* is related to the presence of phenolic compounds such as flavonoids.

Our investigations of the CE50 revealed that the ethyl acetate extract (EC50= 7,362 $\mu\text{g/mL}$) and n-BuOH (EC50=8,072 $\mu\text{g/mL}$) have a high anti radical activity.

However, the AcOEt extract was more effective than the n-BuOH extract. The results obtained after extraction of total flavonoids of the leaves confirmed that flavonoids of *G. arborea* have an ability to trap DPPH. This property was assessed by the technique of bleaching of DPPH (from purple to pale yellow), which demonstrates the potential of antioxidant flavonoids.

Flavonoids are recognized as excellent anti-oxidants (Bruneton, 1999; Kokoska & Janovska, 2009), that play an important role in the cell's defence system. According to these previous reports, the phenolic compounds are widely distributed in tissues of plants among which we find many different anti-radical and anti oxidant compounds. These secondary metabolites are also known for the diversity of their biological

properties (Drissa et al., 2004). The properties of flavonoids have been widely studied in medicine for their antiviral activity, anti-tumor, anti-inflammatory (Krenn et al., 2009), anti allergic and anti cancer activities (Wang et al., 2009; Yamaguchi et al., 2009). Flavonoids may act differently in the regulatory processes of oxidative stress (Peterson & Dwyer, 1998; Di Carlo et al., 1999; Pietta, 2000; Cotelle, 2001) by

direct capture of reactive oxygen species (ROS), chelation of transition metals such as iron and by inhibiting the activity of certain enzymes responsible for ROS production. Their basic property is based on the fact that they are anti-oxidant and as such, they act at several levels (Hollman & Katan, 1997; Cuyckens & Claeys, 2004).

Table 4: Analysis of variance to compare the extracts to H, H50, and dS for lysis of erythrocytes depending on the concentrations.

Extracts	C*	N	H	P(H)	H50	P(H50)	dS	P(dS)
NaCl	C1	3	0.016±0.001a	>0.05	0.560±0.005a	<0.05	0.075±0.001b	<0.05
	C2	3	0.014±0.005a		0.561±0.002a		0.078±0.002a	
	C3	3	0.016±0.007a		0.563±0.003a		0.075±0.001b	
	C4	3	0.016±0.001a		0.552±0.003b		0.075±0.001b	
	C5	3	0.013±0.004a		0.560±0.005a		0.075±0.001b	
AcOEt	C1	3	0.651±0.029a	<0.05	0.638±0.003a	<0.05	0.075±0.001a	>0.05
	C2	3	0.469±0.015b		0.600±0.005b		0.080±0.007a	
	C3	3	0.375±0.013c		0.517±0.031c		0.083±0.013a	
	C4	3	0.296±0.006d		0.438±0.008d		0.082±0.014a	
	C5	3	0.308±0.016d		0.405±0.013e		0.091±0.014a	
n-BuOH	C1	3	0.824±0.185a	<0.05	0.723±0.003a	<0.05	0.075±0.001a	>0.05
	C2	3	0.703±0.027a		0.703±0.006a		0.074±0.002a	
	C3	3	0.398±0.018b		0.672±0.003b		0.074±0.001a	
	C4	3	0.212±0.035bc		0.507±0.026c		0.075±0.001a	
	C5	3	0.275±0.001c		0.480±0.013d		0.077±0.001a	
Aqueux	C1	3	1.055±0.001a	<0.05	0.987±0.001a	<0.05	0.074±0.001a	>0.05
	C2	3	1.039±0.004a		0.982±0.001a		0.075±0.001a	
	C3	3	0.990±0.036a		0.967±0.006b		0.074±0.001a	
	C4	3	0.967±0.026b		0.093±0.006c		0.074±0.002a	
	C5	3	0.904±0.015c		0.882±0.006d		0.075±0.001a	

(*) For concentrations, means followed by the same superscript are not significantly different ($P \geq 0.05$), C : Concentration (mg/ml), N : Observation, H : The intensity of hemolysis, H50 : NaCl concentration causing 50% hemolysis, dS : Amplitude of the sigmoid transition, NaCl : Sodium chloride, n-BuOH : n-butanol, AcOEt : Ethylacetate, C1 = 1 mg/ml, C2 = 2 mg/ml, C3 = 3 mg/ml, C4 = 4 mg/ml, C5 = 5 mg/ml.

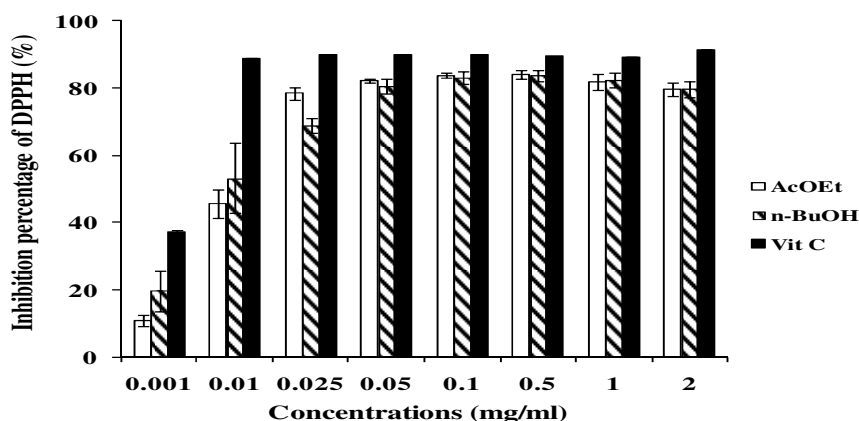
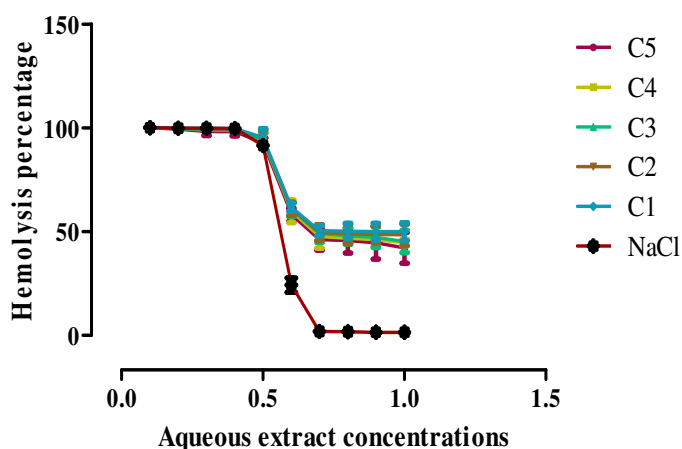


Figure 1: Antioxidant activity of total flavonoids of *G. arborea*.

The results of the effect of flavonoids on osmotic stability of erythrocytes showed differences between the values of parameters obtained in the presence and absence of plant extract. The extracts of *G. arborea* modified the osmotic stability through the disruption of one of the three parameters. A stabilizing effect would be associated with an increase of dS and decreases in H50 and H. A destabilizing effect is indicated by a decrease in dS and an increase in H50, but not in H, since the H value for the control already represents the maximum possible hemolysis.

Changes in values of the calculated parameters could also be due to the acidity of the solution tested. However, studies have shown that between the pH ranges of 3-10, there is no lysis of erythrocytes, indicating that the natural values of the pH of the plant extract did not cause any hemolysis in the presence of the extract (de Freitas et al., 2008). It should be recalled that we used the physiological solution of NaCl, as a buffer, as described by Jain (1973). Significant variations in the percentage of hemolysis that occurred in the presence of the extracts suggested that the aqueous extract and flavonoids at different concentrations had different hemolytic properties. The aqueous extract caused significant hemolysis (99% hemolysis) in the presence of red blood cells, which is two times higher than that of NaCl and flavonoids. It is the same for H50.

Cell membranes must combine the opposite properties of fluidity and stability to function efficiently (Cribier et al., 1993). Changes in spectral properties of hemoglobin (osmotic stability) could be due to a disturbance caused by an increase in the percentage of hemolysis. A degree of fluidity is essential for signals and transport molecules. However, excessive fluidity could affect the stability of these membranes and vice versa. This stability is the ability to maintain the complex biological structure under certain conditions such as oxidative stress, hypotonia, pH extremes, heat and presence of dissolved substances (Van-Ginkel & Sevanian, 1994; Timasheff, 1998). Many secondary metabolites may affect positively (restoration of flow) and / or negatively (excessive fluidity, alteration of membranes) the nature of biological membranes (erythrocytes) (Sharma & Sharma, 2001). Indeed, the aqueous extract is rich in saponins (Im = 1000) (N'gaman et al., 2009) which are secondary metabolites known for their haemolytic property and this could explain the high percentage of hemolysis of extract. The aqueous extract had a significant cytotoxicity. Indeed H50 and dS can assess the effect of the extract on the osmotic stability of erythrocytes (Sharma & Sharma, 2001). The stabilization of hemolysis is caused by a decrease in H50, which reflects the concentration which induces 50% hemolysis of red blood cells followed by an increase in the amplitude dS.



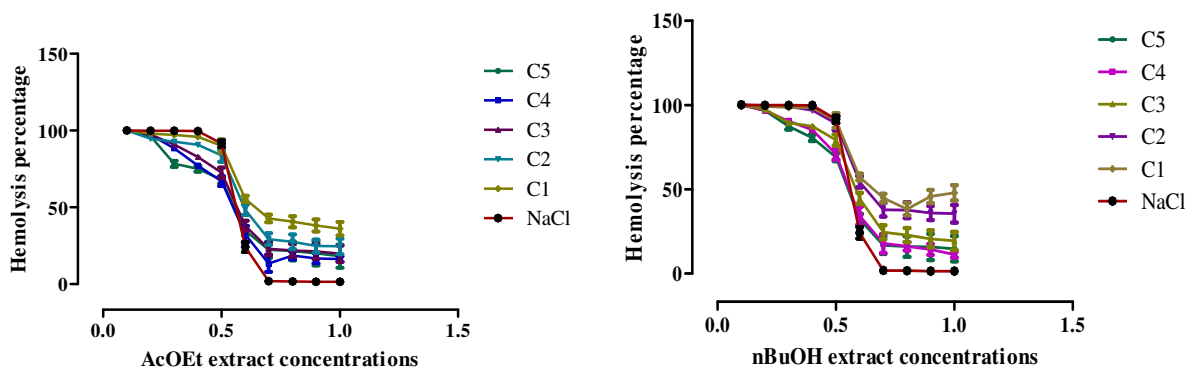


Figure 2 : Flavonoids effects of *G. arborea* on osmotic stability of erythrocytes. C1 = 1 mg/ml, C2 = 2 mg/ml, C3 = 3 mg/ml, C4 = 4 mg/ml, C5 = 5 mg/ml.

The flavonoids presented a lower toxicity and the values of H50 of ethyl acetate extract were statistically equal to that of saline. Thus we deduced that this extract has a lower cytotoxicity which is interesting from an application point of view. Through these results, it would be inadvisable to use the aqueous extract as is

CONCLUSION

This study showed a good antioxidant activity and good stability from osmotic tests on erythrocytes with the total flavonoids extracts from leaves of *Gmelina arborea*. Flavonoids had excellent anti-oxidants so it play an important role in the cell's defence system. The results of this investigation showed that the flavonoids

currently practiced by the rural people without checking its content of saponins. Indeed, while treating the condition for which it is administered, it induces significant erythrocyte lysis that could lead to severe anemia and lead to other diseases.

of the leaves of *Gmelina arborea* especially those extracted with AcOEt had low cytotoxicity compared to the aqueous extract, and therefore can be used in therapy. Further studies are proposed to investigate bioactive molecules of these flavonoids.

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