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## Study of Antisickling and Vasorelaxant Activities and Acute Toxicity Assessment of Crude Extracts of Leaves of *Ficus sycomorus* L. (Moraceae)

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**Abstract:** The leaves of *Ficus sycomorus* are used in Burkina Faso folk medicine for the treatment of sickle cell disease. The present comparative study of crude extracts of leaves (decoction, macerated extract and a 95% ethanol extract) was performed with the aim to assess the efficiency of this traditional use and to determine the most active of the three extracts. Antisickling activity was assessed by the Emmel's test. Vasorelaxant effect on rat aortic rings precontracted by phenylephrine with and without N-nitro-L-arginine methyl ester chloride (L-NAME) was also evaluated. The 95% ethanol extract (20 mg mL<sup>-1</sup>) showed the most antisickling activity on sickle erythrocytes, by inhibiting completely sickling of double heterozygote SC cells in 60 min and that of homozygote SS cells in 90 min. On the aorta this extract exhibited a significant (p<0.05) vasorelaxant activity, better than that of the other extracts, with an IC<sub>50</sub> value of 6.86±0.13 mg mL<sup>-1</sup> against 18.78±0.38 and 28.56±1.27 mg mL<sup>-1</sup>, respectively for the macerated extract and the decoction. When the aortic rings were pretreated with L-NAME, only the ethanolic extract conserved its vasorelaxant activity, up to 73% of relaxation. The acute toxicity of the decoction, assessed by intraperitoneal route and using the Litchfield and Wilcoxon method, led to an LD<sub>50</sub> value of 1553.61 mg kg<sup>-1</sup> b.wt. This places the drug among those with low toxicity according to the WHO scale. These results confirm those previously obtained and provide a scientific basis supporting the use of this plant in folk medicine against sickle cell disease. They indicate the importance of *Ficus sycomorus* in the research of new antisickling molecules.

**Key words:** Sickle cell disease, *Ficus sycomorus*, antisickling, vasorelaxant, acute toxicity

### INTRODUCTION

Sickle Cell Disease (SCD) is a major worldwide health problem. Over 330000 children are born annually with haemoglobin disorders of which 83% are affected by SCD (Modell and Darlison, 2008). Sub-Saharan Africa is the most affected (with about 80% of the global total) (Modell and Darlison, 2008). In this region, 50-80% of the affected children die before the age of five years (Weatherall *et al.*, 2006). The management, and especially the treatment of the disease still remain problematic because the few modern drugs available are expensive in relation to the purchasing power of the majority of African populations. They therefore resort to traditional

phytotherapy whose receipts are sometimes used successfully in the management of the disease. This is the case of *Ficus sycomorus* used in Burkina Faso by traditional healers to alleviate the crisis of drepanocytosis. The morphological characteristics of *Ficus* (a genus from Moraceae botanical family) vary considerably (Arbonnier, 2000). *Ficus sycomorus* is a tree growing in tropical and subtropical regions of the world and is generally 10-30 m tall, with about 3 m wide. Its bark is smooth or rough, gray, pink or red. It produces a white latex.

A preliminary study showed that *Ficus sycomorus* was rich in phenolic compounds and had radical scavenging and antibacterial properties

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(Ramde-Tiendrebeogo *et al.*, 2012) supporting its use for the treatment of some troubles associated to sickle cell disease. Indeed the production of highly reactive oxygen radicals generates a state of oxidative stress which causes chronic hemolysis (Sess *et al.*, 1998). Similarly, infectious complications are a major cause of mortality in children with sickle cell anemia (Diagne *et al.*, 2003). In order to understand the pharmacology of extracts of this plant, the present comparative study of crude extracts of leaves of *Ficus sycomorus* was performed by evaluating their *in vitro* and *ex-vivo* activities against sickling and vasoconstriction of rat aorta. The acute toxicity of the aqueous decoction (traditional form of use) was also evaluated.

## MATERIALS AND METHODS

**Drugs and chemicals:** Hydrea 500 mg and Torental LP 400 mg were purchased in officine (Ouagadougou, Burkina Faso). All other drugs and chemicals used in this study were purchased from manufacturers via their local resellers: Citrate phosphate dextrose adenine solution USP was purchased from Poly Medicure (Faridabad, India); NaCl, NaHCO<sub>3</sub>, KCl, KH<sub>2</sub>PO<sub>4</sub>, MgSO<sub>4</sub>, CaCl<sub>2</sub>, NaH<sub>2</sub>PO<sub>4</sub>, D-(+) Glucose and phenylephrine were from Sigma (Grenoble, France); Sodium Metabisulfite from Jiading Malu (Shanghai, China); N-nitro-L-arginine methyl ester chloride (L-NAME) was obtained from Sigma (Darmstadt, Germany).

**Plant material:** The leafy stems of *Ficus sycomorus* were harvested at the edge of the dam N°2 of Tanghin (Ouagadougou), in July 2009. After identification by Professor Millogo J., botanist, a specimen voucher was deposited in the herbarium of the University of Ouagadougou under registration number 01/2009. Plant material was dried in a ventilated room, away from the sun for two weeks and then finely ground.

**Extraction and preparation of test solution:** The aqueous decoction (10%) was prepared by reflux of 25 g of the leaves powder in 250 mL of distilled water for 30 min. After cooling, extract was filtered and centrifuged for 10 min at 2000 g (ALC 4217 Centrifuge) then the obtained supernatant was frozen and lyophilized.

The ethanolic extract was obtained by adding 250 mL of 95% ethanol to 50 g of the extract. The mixture was left to stand for 24 h at room temperature. After filtration, a small quantity of water was added in the extract and then the ethanol was thoroughly evaporated using a rotary evaporator (Büchi RE 111). The resulting solution was frozen and lyophilized. For testing, the decoction and the

ethanolic extract were prepared each at 1 and 2%, in saline (0.9% NaCl). In addition, an aqueous macerated was prepared by mixing 1 g or 2 g of powder in 100 mL of saline (0.9% NaCl) and by allowing to stand at room temperature for 12 h. After filtration through Whatman paper, test solutions of 0.83 and 1.66%, respectively were obtained.

**Antisickling activity:** Blood samples were obtained from voluntary sickle cell patients (or with parental consent for minors) followed by a pediatrician at the clinic "Les Tisserins" (Ouagadougou). These patients were homozygous SS aged from 32 months to 16 years, or double heterozygous SC with ages between 8 and 26 years. Blood was withdrawn by venipuncture in 5 mL EDTA tubes. One milliliter of a preservative solution (Dextrose Adenine Solution USP) was added to allow blood storage for at least 3 weeks in the refrigerator. Antisickling activity was performed according to Emmel's test as described by Mpiana *et al.* (2007) with minor modifications. In different test tubes from donors, were placed 0.5 mL of blood and 0.5 mL of extract. Blank sample was prepared by replacing the extract by 0.5 mL of saline (PBS, pH 7). After 30, 60, 90, 120 or 150 min of contact, a drop of the mixture was deposited, by means of a micropipette, on a glass slide containing a drop of saline (PBS, pH 7). Then a drop of a 2% sodium metabisulfite solution (rapid sickling factor) was added. After homogenization, a coverslip was placed on the glass slide and the preparation was luted with nail polish. The slides were read after 15 min, by using an optical microscope (Carl Zeiss AxioLab) (X40). All slides were carefully referenced by identifiers of blood donors, the extract studied and the contact duration. A digital camera (Canon PowerShot A490) was used to digitize the micrographs. The percentage of sickled cells on each slide was determined. Hydrea (10 mg mL<sup>-1</sup>) and Torental (12 mg mL<sup>-1</sup>) were used as positive controls.

**Vasorelaxation test:** Wistar rats from Institute for Research in Health Sciences (IRSS) weighing 150-37 g, were used. Rats were kept under a well-ventilated room at 25±2°C, with a relative humidity of 44-56% and light and dark cycles of 12 h, respectively. They had free access to standard dry pellet diet (protein 29%) from the center of promotion of poultry farming (CPAVI, Bobo-Dioulasso) and to water from the tap *ad libitum*. The vasorelaxation test was performed as described by Andriambelison *et al.* (1997), with slight modifications

**Preparation and assembly of aortae:** Rats were anesthetized intraperitoneally using a 40% solution of urethane and then placed in supine position on a

dissecting board. The thoracic aortae was rapidly and carefully dissected and placed into Krebs solution (pH 7.4) containing 118 mmol L<sup>-1</sup> NaCl, 4.7 mmol L<sup>-1</sup> of KCl, 1.1 mmol L<sup>-1</sup> of MgSO<sub>4</sub>, 1.2 mmol L<sup>-1</sup> of KH<sub>2</sub>PO<sub>4</sub>, 1.5 mmol L<sup>-1</sup> of CaCl<sub>2</sub>, 25 mmol L<sup>-1</sup> of NaHCO<sub>3</sub> and 10 mmol L<sup>-1</sup> of glucose. The aortae were removed free of the connective tissue and the fat and then cut into rings of approximately 5 mm length. All dissecting procedures were done with extreme care to protect the endothelium from inadvertent damage. The aortic rings were suspended in a tissue bath containing Krebs solution (pH 7.4) at 37°C, while being continuously bubbled with a gaseous mixture of 95%O<sub>2</sub>-5%CO<sub>2</sub>. Resting tension was adjusted to 2 g. After an equilibration period of 60 min, the vessels were maximally contracted with phenylephrine (1 µM), a potent endogenous catecholamine, in order to test their contractile capacity. The presence of functional endothelium was assessed in all preparations by determining the ability of acetylcholine (10 µM) to induce more than 50% relaxation of rings precontracted with phenylephrine (1 µM).

**Record of isometric vascular tone:** After washing and returning to baseline tension, aortic rings with functional endothelium were precontracted with phenylephrine (1 µM). When the contraction reached a steady state, increasing doses of plant extracts were added cumulatively. To characterize the involvement of endothelial-NO, some arteries with functional endothelium were exposed to the NO-synthase inhibitor, L-NAME (0.3 mM), 30 min prior to contraction with phenylephrine. In this case, the concentration of the agonist was adjusted in order to obtain the same level of precontraction as in the absence of L-NAME.

**Acute toxicity:** The general acute toxicity was evaluated according to Litchfield and Wilcoxon (1949) method. Healthy male Naval Medical Research Institute (NMRI) mice bred in the animal house of the Institute and fasted for at least 18 h before the experience were used. Six homogeneous groups of 6 mice each, of which one untreated control group were selected. The test animals were given increasing doses (800-3000 mg kg<sup>-1</sup> b.wt.) of the extract through an intraperitoneal route. The control group received only the solvent (distilled water) used for diluting the extracts. Two hours after the doses administration, the animals were fed and observed for 72 h. The LD<sub>50</sub> was calculated at the end of 72 h.

### STATISTICS

The inhibition induced by the plant extracts on red blood cells was expressed as a percentage of sickled cells

(n = 5). The Relaxations of aortic rings with functional endothelium in the absence of L-NAME (n = 4) and in the presence of L-NAME (n = 8) were expressed as percentage of the level of precontraction. The sensitivity of vessels to each fraction was expressed as the EC<sub>50</sub> value (i.e., the concentration of fraction required to produce 50% of the maximal relaxation). All results were expressed as Mean±SEM of n experiments. ANOVA was used to compare the EC<sub>50</sub> values. A p value level of 0.05 or less was considered significant. For the acute toxicity, the values of LD<sub>50</sub>, LD<sub>1</sub>, LD<sub>99</sub> were determined graphically from a log-probit graphic. From these data the security index was calculated and the class of toxicity determined in reference with the WHO level of toxicity (WHO, 2010).

### RESULTS

Figure 1 illustrates the antisickling activities of the different extracts of leaves of *Ficus sycomorus*. As shown, the three types of extracts (decoction, macerated extract and ethanolic extract) inhibit the sickling of erythrocytes induced by sodium metabisulfite on the two types of sickle cell (SS and SC); the ethanolic extract being the most active. Its effect at 20 mg mL<sup>-1</sup> is comparable to that of the two controls, Hydreia (10 mg mL<sup>-1</sup>) and Torental (12 mg mL<sup>-1</sup>) after 90 min for SS cells (Fig. 1c) and 60 min for SC cells (Fig. 1d). Figure 2 shows the morphology of red blood cells of both type of sickle cells before and after treatment with the ethanolic extract. The relaxation effect of the different extracts (on rat aortae precontracted by phenylephrine is shown in Fig. 3. The alcoholic extract showed the most vasorelaxant activity with IC<sub>50</sub> values of 6.86±0.13 and of 9.04±0.14 mg mL<sup>-1</sup> on the aortic rings (with functional endothelial), respectively in the absence and presence of L-NAME (Table 1). The acute toxicity determination using a decoction of leaves (which is the traditional form of use) has led to a LD<sub>50</sub> value of 1553.61 mg kg<sup>-1</sup> b.wt. The LD<sub>1</sub> and LD<sub>99</sub> values obtained were of 427.56 and 5645.22 mg kg<sup>-1</sup> b.wt., respectively.

Table 1: IC<sub>50</sub> value (mg mL<sup>-1</sup>) obtained with *Ficus sycomorus* leaf extracts in pherylephrine-precontracted rat aortic rings with functional endothelium with and without L-NAME

Extract	IC <sub>50</sub> (mg mL <sup>-1</sup> )	
	Without L-NAME	With L-NAME
EtOH 95%	6.86±0.13	9.04±0.14 <sup>a</sup>
ML	18.78±0.38 <sup>a,b</sup>	nd
DL	28.56±1.27 <sup>a,b,c</sup>	nd

Values are expressed as Mean±SEM (n = 4-8). (a) Significant difference compared to the EtOH95% extract without L-NAME (b) Significant difference compared with EtOH95% extract with L-NAME (c) Significant difference in comparison with ML extract in the absence of L-NAME, DL: Decoction of the leaves, ML: Macerated extract of the leaves, EtOH95%: 95% ethanol extract

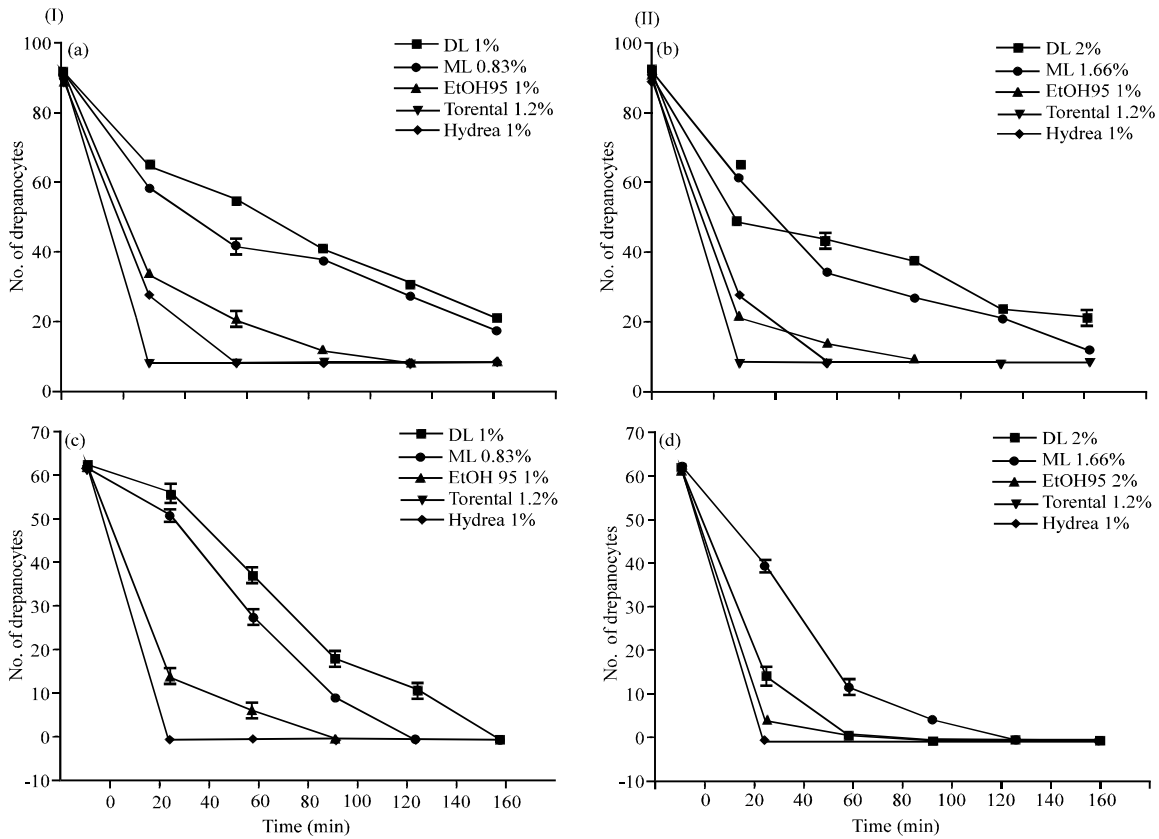


Fig. 1(a-d): Effect vs. time of *Ficus sycomorus* leaf extracts at various doses on sickling induced by the sodium metabisulfite 2% on erythrocytes of subjects with sickle cell disease, (a): Effect of extract on red blood cells of a homozygous SS subject; (b): Effect of extract on red blood cell of a heterozygous SC subject; DL: Decoction of leaves; ML, macerated extract of leaves; EtOH95: 95% ethanol extract. The ethanol extract (2%) completely inhibited sickling of SS cells after 90 min (Fig. 1c) and that of SC cells after 60 min (Fig. 1d), thus showing an effect comparable to that of positive controls (Torental and Hydrea). The decoction as traditional form of use of *Ficus sycomorus* leaves showed a moderate activity. It could not inhibit sickling SS cells (Fig.1c) but was able to inhibit that of SC cells after 60 min (Fig. 1d)

**DISCUSSION**

The Pathophysiology of sickle cell disease, a complex process, is based on the polymerization of the abnormal hemoglobin (HbS) leading to sickling and rigidity of erythrocyte that accumulate in the capillaries. This blocks the vessels and creates micro-vascular occlusions. This phenomenon gives rise to painful crises as a result of poor blood circulation and poor oxygenation of organs (Giroto and Begue, 2003; Rees *et al.*, 2010). The genetic character of sickle cell disease is the major obstacle to its therapeutic management. Treatments are generally supportive care, which mainly focus on the

malformation of red blood cells and the occurrence of vaso-occlusive crises (Guissou *et al.*, 1995). The three types of leaf extracts of *Ficus sycomorus* inhibit sickling of SS and SC erythrocytes in the presence of sodium metabisulfite (cell sickling factor). These results prove the antisickling activity of extracts of leaves of *Ficus sycomorus* and thus they support the traditional use of the plant in the management of sickle cell disease. Traditional healers usually use the decoction as form of extraction. According to the results hereby obtained, the decoction would be less active than the ethanolic extract against the sickling. Indeed, at a concentration of 2%, ethanol extract completely inhibits sickling of SS cells (treated with metabisulphite) after 90 min (Fig. 1c) and

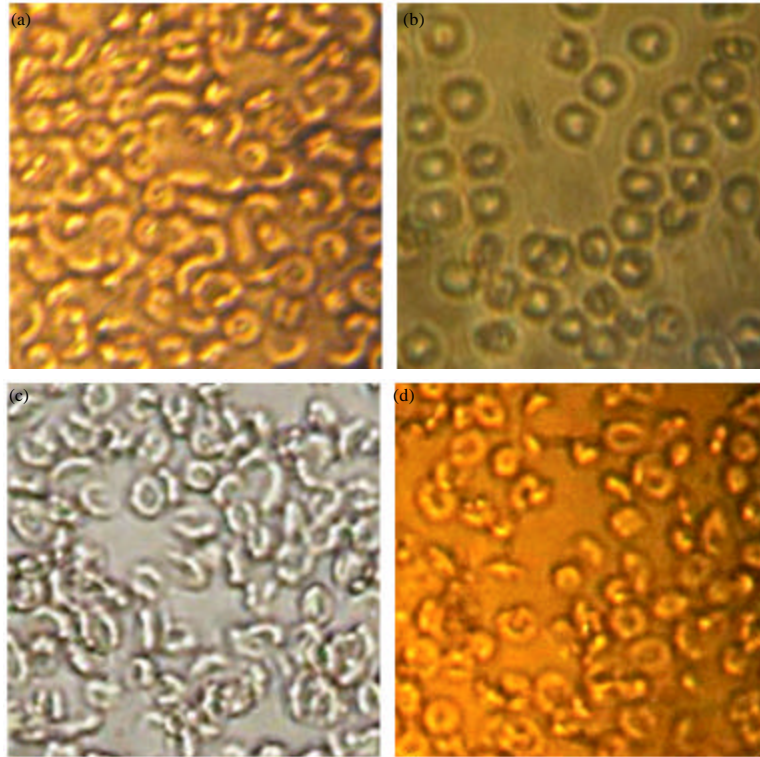


Fig. 2(a-d): Inhibition of red blood-cell sickling (induced by sodium metabisulfite 2%) with 2% solution of ethanolic extract, (a) SC erythrocytes in contact with 2% sodium metabisulfite (SC Control), (b) SC erythrocytes in presence of sodium metabisulfite (2%) and after 60 min of contact with the extract, (c) SS erythrocytes in contact with sodium metabisulfite 2% (SS Control), (d) SS erythrocytes in presence of sodium metabisulfite (2%) and after 60 min of contact with the extract

that of SC cells after 60 min (Fig. 1d), thus showing an effect comparable to that of positive controls (Torental and Hydrea). At the same dose (2%) the decoction could not inhibit sickling SS cells (Fig. 1c) and was able to inhibit that of SC cells only after 60 min (Fig. 1d). The high content of anthocyanins in *Ficus sycomorus* is well documented (Abdel-Hameed, 2009; Sandabe *et al.*, 2006). The anti-sickling properties of these pigments have been reported (Mpiana *et al.*, 2008) and they could support this antisickling activity. The present study suggests that an ethanolic extract contains more active compounds against sickling than the decoction or the macerated extract. This finding could therefore be of interest for a bioguided-fractionation in the search for new anti-sickling molecules. The antisickling activity of the acetone extract of the bark of *Ficus sycomorus* was reported by Nongonierma *et al.* (2005), however the traditional preparation, of recipes, which uses leaves should be encouraged as it could contribute to the protection and conservation of biodiversity.

The three types of extracts have also showed vasorelaxant activity on rat aortic rings (with functional endothelium) precontracted with phenylephrine. However, the aqueous extracts activities were meaningless ( $p < 0.05$ ) in comparison to the ethanolic extract which has induced a dose-dependent relaxation up to 100% (Fig. 3). The relaxation of aortic ring (with functional endothelium) is generally mediated by vasorelaxant endothelial cell mediators such as nitric oxide (NO), prostacyclin (PGI<sub>2</sub>) and/or derived hyperpolarizing factor to endothelium (EDHF) (Furchgott and Zawadzki, 1980). During sickle cell crisis, the concentration of NO, main factor of relaxation, is often strongly decreased either by the presence of endothelial superoxide that disables it, or by its capture by free hemoglobin released during hemolysis (Charbonney *et al.*, 2006; Reiter *et al.*, 2002), increasing considerably the risk of vaso-occlusive crisis. To check whether the extracts are able to preserve their vasorelaxant potential, even in the absence of NO, L-NAME was used to inhibit the NO synthase. The results showed that in the absence of NO, the

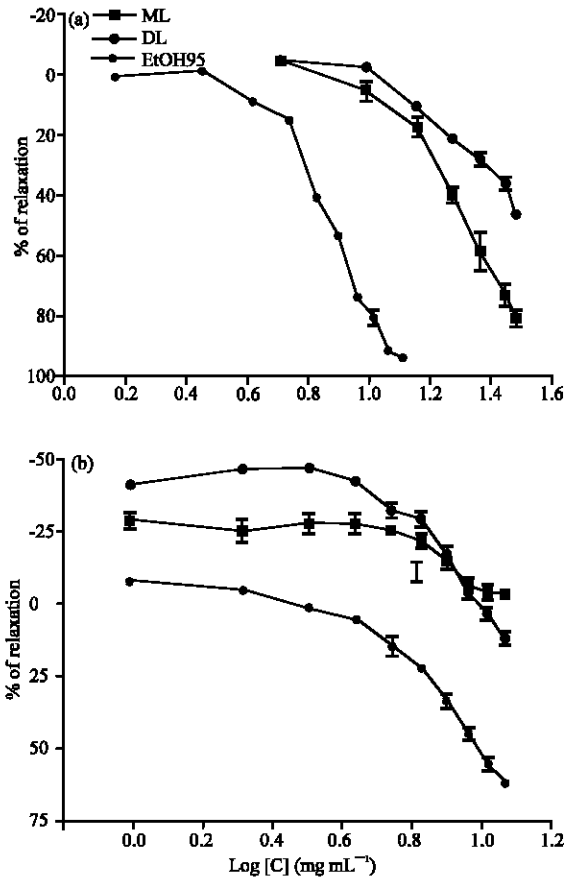


Fig. 3(a-b): Concentration-response curves for the crude extracts of leaves of *Ficus sycomorus* in phenylephrine-precontracted rat aortic rings, (a) With functional endothelium without L-NAME, (b) With functional endothelium +L-NAME. The ethanol extract was the most active exhibiting 100% of relaxation in absence of L-NAME, this activity remain high, up to 73% of relaxation in presence of L-NAME. Values are Means $\pm$ SEM, (a. n = 4, b. n = 8), DL: Decoction of leaves, ML: Macerated extract of the leaves; EtOH95: 95% ethanol extract

vasorelaxant property of ethanolic extract, although attenuated, remains high (up to 73% of relaxation, Fig. 3b). At the same doses, the vasorelaxant activity of the two aqueous extracts was insignificant, even causing vasoconstriction at low concentrations. This vasorelaxant property in the absence of NO of the ethanol extract is of great interest in the search for molecules that prevent vaso-occlusive accidents during sickle cell crisis. Thus, securing the use of traditional phytotherapy recipes is a

real concern since medicinal plants are not always exempt of risk (Hirt and M'Pia, 1995). This is particularly important in the case of sickle cell disease because the therapy is for life. The general acute toxicity of aqueous decoction, evaluated intraperitoneally lead to an LD<sub>50</sub> value of 1553.61 mg kg<sup>-1</sup> b.wt. According to the WHO (2010) scale of toxicity such a drug could be classified as of low toxicity, reflecting the weakness of risk associated with the use of this traditional therapeutic form. Furthermore, the security index (LD<sub>99</sub>/LD<sub>1</sub> ratio) is 13.20, indicating a possibility of good handling of the drug.

### CONCLUSION

This study showed pharmacological properties of *Ficus sycomorus* against red blood-cell sickling and vasoconstriction, two key components in the pathophysiology of sickle cell disease. These results reinforce those previously obtained on the importance of extracts from this plant in the management of sickle cell disease. Further studies by bioguided-fractionation on the most active extract are required in order to isolate and identify new potential antisickling molecules.

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