In vitro evaluation of membrane stabilizing activities of leaf and root extracts of *Calliandra portoricensis (*JACQ) benth on sickle and normal human erythrocytes

Amujoyegbe[1] O. O., Agbedahunsi[1] J. M., Akinpelu[2] B. A., Oyedapo O. O[2]

[1]Drug Research and Production Unit, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Nigeria
[2]Department of Biochemistry, Faculty of Science, Obafemi Awolowo University, Ile-Ife, Nigeria

Sickle cell disease is an inherited disorder of haemoglobin, resulting in abnormal red blood cell which may block blood vessels leading to acute painful crises and other complications. Membrane stabilization activities of aqueous and ethanolic extracts of *C. portorincensis* leaf and root on sickle and normal erythrocytes exposed to both heat and hypotonic induced lyses were investigated. The percentage membrane stabilizing activities of these extracts were found to be concentration dependent. Both leaf and root extracts of *C. portoricensis* mode of protection was revealed to compete favourably with the standard drug (ibuprofen) but ethanolic root extract exhibited the best membrane stabilizing activities. This study indicates that *C. portoricensis* exhibited human erythrocyte membrane stabilizing activity and possesses potential ability for the management of sickle cell related ailments.

Keywords: Membrane-stabilizing, sickle cell diseases, erythrocytes, *Calliandra portoricensis.*

INTRODUCTION

Sickle cell anemia (SCA) or drepanocytosis is an inherited blood disorder that affects red blood cells. It is a disorder of the blood caused by inherited abnormal haemoglobin (an oxygen-carrying protein within the red blood cells). The abnormal haemoglobin causes distorted (sickled) red blood cells. Sickle cell disease is a long-term (chronic) blood disorder that causes the bone marrow to produce red blood cells with defective hemoglobin (hemoglobin S) (Platt *et al.*, 1994).

Under low oxygen tension, deoxy-HbS molecules polymerize, causing the formation of rigid and sickled erythrocytes. The deformity of the sickled erythrocyte results in their shortened survival since they become vulnerable to lysis as they penetrate the interstices of the splenic sinusoids and hence severe hemolytic anemia ensues with hemoglobin values ranging from 6 to 10 g/L.(Martins, 1981; Karayakin, 1971). The homozygous state of SCA is associated with complications and reduced life expectancy (Kutlar, 2005; Frenette and Atweh, 2007)

Membrane stabilization is a process of maintaining the integrity of biological membranes such as erythrocyte and lysosomal membranes against osmotic and heat-induced lyses (Sadique *et al.,* 1989; Oyedapo *et al.,* 2004). The erythrocyte resembles lysosomal membranes, as such it has been used as a model system by many workers in the study of interaction of drugs with membranes. The effect of drugs on the stabilization of erythrocytes could be extrapolated to the stabilization of lysosomal membranes (Kumar and Sadique, 1987; Horie *et al.,* 1979, Litman *et al.,* 1976). The membrane stabilizing activity of red blood cell membrane exhibited by some drugs, serves as a useful *in vitro* method for assessing the anti-inflammatory activity of various compounds (Naibi *et al.,* 1985).

The plant, *Calliandra portoricensis* (Figure 1) is a native of Central America, and most precisely to Mexico, Panama and the West Indies. It grows in areas where frosts are brief, moderate and warmer. It is a shrub or little tree of about 6 m tall with evergreen small bipinnate leaves.

Figure 1. Photograph of *Calliandra portoricensis*, showing twigs, leaves and flowers.

The leaves may reach 20 cm long and 15 cm wide and they fold at night (*Burkill,* 1985). Investigations on the *C. portoricensis* chemical constituents revealed the presence of saponins, steroids, tannins, glycosides, alkaloids, anthraquinones, digitalis glycosides, fatty acids, gallic acid, methyl gallate, myricitrin, quercitrin, afzelin, isoquercitrin, caffeic acid, betulinic acid and other related compounds (Akah and Nwaiwu, 1988). The phytochemical constituents of ethanolic and aqueous extracts of *Calliandra portoricensis* leaves were identified to be saponins, tannins, flavonoids and glycosides (Aguwa and Lawal, 1987).

The management or treatment goals for sickle cell disease aim to relieve pain, prevent infections, and manage complications. Some medicinal plant extracts have been demonstrated by *in vitro* investigations to reduce polymerization of HbS molecules (Chikezie, 2006, 2007) and have been established to serve as potential chemotherapeutic preparations for alleviation and management of sickle cell anaemia (Ekeke and Shode, 1985; Okpuzor *et al*., 2008).

MATERIALS AND METHODS

Materials

Collection and Identification of Plant Materials

Fresh leaves and roots of the *C. portoricensis* were collected around Mozambique Hall Obafemi Awolowo University, Ile-Ife in July 2009. The plant was identified and authenticated by Mr. A. T. Oladele of Faculty of Pharmacy herbarium, and Dr H. C. Illoh Department of Botany, Obafemi Awolowo University, Ile-Ife, Nigeria.

Reagents and chemicals

All the solutions, reagents used in this study were of analytical grades. They were procured from various sources. All the solutions, reagents and buffers were prepared with glass distilled water.

Collection of Blood Samples

Ethical clearance was obtained from the Ethical Committee of the Obafemi Awolowo University Teaching Hospital Complex (OAUTHC), Ile-Ife. Fresh normal and sickled blood samples (5ml) each were collected into heparinized bottles and mixed gently to prevent lysing. The sickled blood samples were collected from confirmed sickle cell anaemia patients who were in a steady state and attending the routine clinic. The blood samples were transported cold to the laboratory.

Standard drugs

Ibuprofen (Non steroidal anti-inflammatory drug [NSAID]) tablets (500mg) were used as the reference drug for

membrane stabilizing activity and were purchased from Campus Pharmacy of Obafemi Awolowo University, Ile-Ife, Nigeria.

Methods

Preparation of plant material

Fresh leaves of *C. portoricensis* were air dried for 24 h in the laboratory and oven dried at 40ºC for 4 h for complete dryness. The roots were washed, cut into small pieces, air dried and oven dried for 72 h. All the dried materials were separately milled into powdered form using grinding machine (Christy) and stored in well sealed amber coloured bottles.

Preparation of aqueous and ethanolic extracts

Powdered materials (200 g) each were separately extracted with (2 L) water or 70 % (v/v) ethanol (2 L) under reflux. The suspension were allowed to simmer for 3 h and finally allowed to cool. The suspensions were filtered under vacuum and concentrated to dryness at 50°C. The residual water in the dried extracts was removed using activated silica gel desiccators. Final drying was done by freeze-drying. They were finally stored separately in sample bottles until used.

Preparation of red blood cells

The red blood cells were prepared according to a procedure reported by Oyedapo and Famurewa (1995). Fresh blood sample was collected into anticoagulant bottle containing trisodium citrate (3.8% w/v) and mixed thoroughly to prevent lysing. The anticoagulated blood was poured into clean centrifuge tubes and then centrifuged on Gallenkamp Bench Centrifuge at 3000 rpm for 10 min. The supernatants were carefully removed using sterilized Pasteur pipettes. The packed erythrocytes were resuspended in fresh isosaline, mixed gently followed by centrifugation for another 5min as above. The supernatants were also removed. This process was repeated five times until clear supernatants were obtained. Then a 2% (v/v) erythrocytes suspension was prepared by diluting 2.0 ml of packed red blood cells with isosaline to 100 ml.

Preparation of extracts for membrane stabilizing assay

Different solutions of aqueous and ethanolic extracts (leaf and root) of *C. portoricensis* were prepared by weighing appropriate quantity of the extract separately. The extracts were dissolved in normal saline to serve as stock solution. The working solutions were prepared by dilutions of the stock solution to give varying concentrations (1.5 mg/ml, 2.0 mg/ml and 2.5 mg/ml).

Membrane stabilizing activity assay

The membrane stabilizing assay was carried out using a procedure previously described by Oyedapo *et al*., 2011 using 2% (v/v) on sickle erythrocytes suspension while ibuprofen was used as standard drug. The assay mixtures consisted of hyposaline (2.0 ml), 0.15 M Sodium phosphate buffer, pH 7.4, varying volumes of drugs (0.0-1.0 ml) of (extracts or standard drugs), varying volume of isosaline and 2% (v/v) erythrocyte suspension in isosaline (0.5 ml) to give a total assay volume of 4.5 ml. Erythrocyte suspension was absent in drug control while drugs were omitted in blood control. The reaction mixtures were mixed thoroughly and incubated at 56º C for 30 min on a water bath. The tubes were cooled under running water and then centrifuged at 3000 rpm on Gallenkamp Bench Centrifuge for 10 min at room temperature. The supernatants were collected into test tubes and absorbance (Abs) of the released hemoglobin was taken at 560 nm. The percentage membrane stabilizing activities were established from the expression.

% Membrane stabilizing activity =

$$\frac{100 - [(\text{Drug test value} - \text{Drug control value}) \times 100]}{\text{Control value}}$$

where the blood control represented 100% lysis

Statistical analysis

Values are expressed as mean ± SEM of 3 consistent readings. The statistical significance differences were analyzed using Student" t" test.

RESULT

Figures 2, 3 and 4 showed the membranes stabilizing profiles of leaf and root extracts of *C. portoricensis* and ibuprofen (standard drug) on human sickle and normal erythrocytes exposed to both heat and hypotonic induced lyses.

At 1.5 mg/ml (Figure 2) ethanolic and aqueous root extracts exhibited monophasic mode of protection on both sickle and normal erythrocytes i. e. the extract protected red blood cells at all concentrations used in dose dependent manner. Ethanolic leaf extract followed the same trend on both sickle and normal erythrocytes but aqueous leaf extract mode of protection on normal erythrocytes is biphasic while that of sickle erythrocyte is monophasic. At this concentration all the extracts

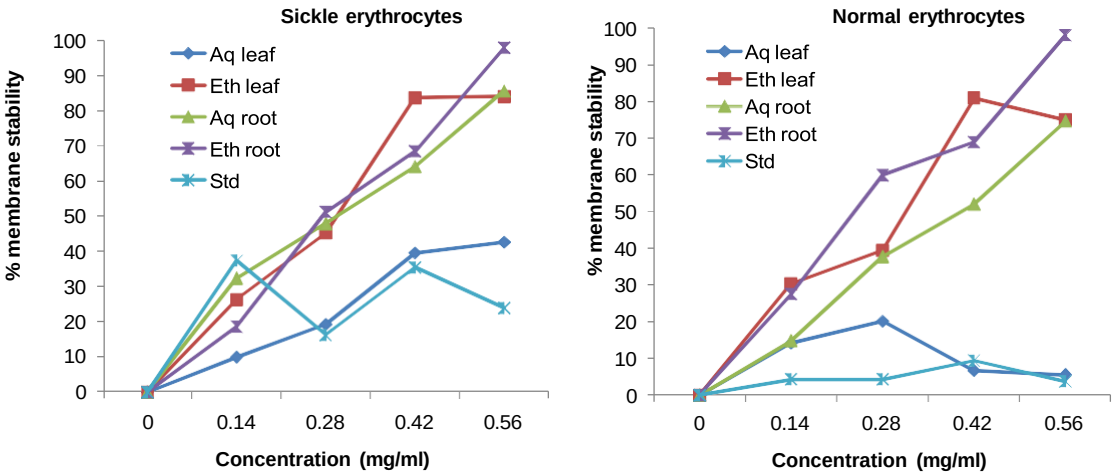

Figure 2. Membrane stabilizing profiles of leaf and root extracts of Calliandra portoricensis and ibuprofen at 1.5mg/ml. Each value represented the mean ± SEM of readings.

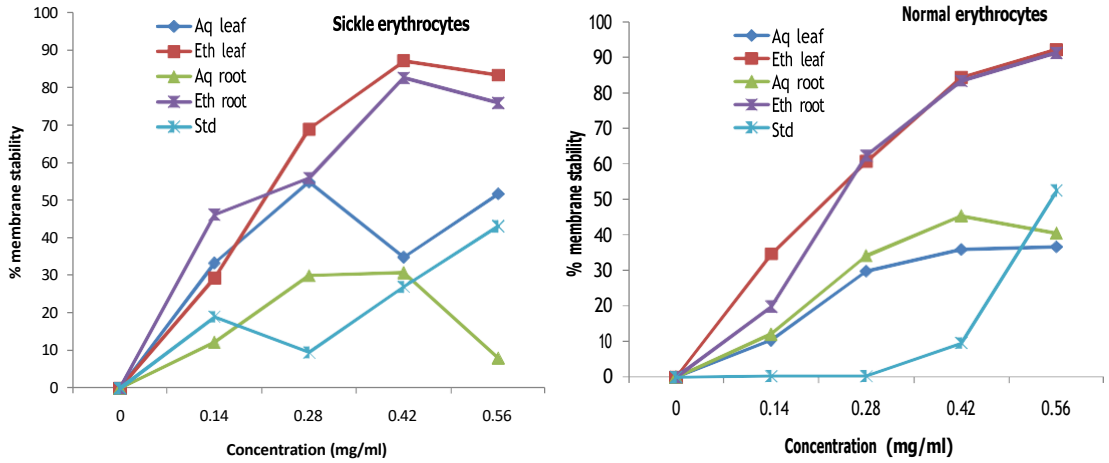

Figure 3. Membrane stabilizing profiles of leaf and root extracts of *Calliandra portoricensis* and ibuprofen at 2.0mg/ml. Each value represented the mean ± SEM of readings.

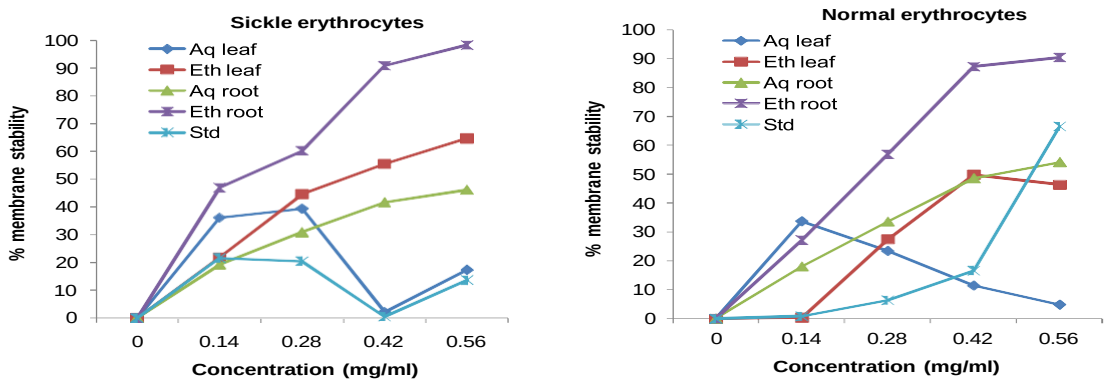

Figure 4. Membrane stabilizing profiles of leaf and root extracts of *Calliandra portoricensis* and ibuprofen at 2.5mg/ml. Each value represented the mean ± SEM of readings.

protected better than ibuprofen.

In Figure 3, both ethanolic root and leaf extracts of C. portoricensis at 2.0 mg/ml showed monophasic mode of protection on normal erythrocytes. Also, aqueous leaf

that increased.

Figures 4 showed membrane stabilizing profiles of sickle and normal erythrocytes at 2.5mg/ml. At this concentration all ethanolic extracts exhibited monophasic mode of protection on sickle erythrocytes likewise on normal erythrocytes except for ethanolic leaf whose percentage membrane stability reduced at highest concentration.

Also, at 2.5mg/ml aqueous root extract mode of protection on both sickle and normal erythrocytes is monophasic in dose dependent manner while aqueous leaf extract exhibited biphasic mode of protection.

DISCUSSION

Plant derived drug have been demonstrated to contain principles that possess ability to facilitate the stability of biological membranes when exposed to induced lysis (Sadique et al*.,* 1989,Oyedapo et al., 2004). Several reports have supported the fact that the membranes of human erythrocytes HbAA, HbAS and HbSS blood types have varying stability as determined from the mean corpuscular fragility (Onah *et al.,* 2002; Elekwa *et al.*, 2003; Okpuzor *et al.,* 2008). Therefore plant extracts that can positively affect the red cell membrane would be useful in sickle cell disease management. Furthermore, it has been suggested that the extract of the seed of the *Cajanus cajan* was effective in restoring normal morphology of erythrocytes from blood samples of patients affected by sickle cell anemia (Onah *et al.*, 2002),. The Pharmacological agents that alter membrane stability could be applied in the control of the sickling of process of the erythrocytes, a major physiological manifestation of the sickle cell disease (Dean and Schchter, 1978). In addition, many investigatorss have reported a large number of drugs that cause alterations on the shape and physiology of the erythrocytes (Ammus and Yunis, 1989; Braga *et al.,* 2000).

At 1.5 mg/ml the extracts protects red blood cells against heat and hypotonic induced lysis and compared favourably with ibuprofen in. Also,ethanolic root extract was observed to protect sickle erythrocytes better than normal erythrocytes.

At 2.0 mg/ml, ethanolic leaf extract mode of protection was better than ethanolic root extract at all concentrations used (Figure 3). It was observed that ethanolic extracts of both root and leaf protected far better than aqueous extracts at 2 mg/ml. At this concentration also, normal erythrocytes were protected better than sickle erythrocytes by ethanolic extracts**.**

The membrane stabilizing profiles of sickle and normal erythrocytes at. 2.5mg/ml (Figure 4) revealed that ethanolic extracts exhibited highest mode of protection on sickle erythrocytes compared to normal erythrocytes likewise ethanolic and aqueous leaf extracts. The mode of protection of all the extracts at this concentration compared favourably with ibuprofen in except aqueous leaf extract.

This study revealed that ethanolic root extract of C. *portoricensis* stabilized red blood cells (especially sickle erythrocytes) exposed to heat and hypotonic induced stress, likewise aqueous root extract but at lower concentrations. Also, both aqueous and ethanolic root extracts mode of protection is better than the corresponding leaf extracts.

The membrane stabilizing activities of aqueous and ethanolic leaf and root extracts of *C. portoricensis,* obtained in this study compared favourably with standard drug ibuprofen at all concentrations used.

Quite a number of extracts have been demonstrated to protect and stabilize red blood cells that were exposed to a combined hypotonic and heat induced stress. The roots of *Threobroma cacao* (Oyedapo *et al*., 2004; Falade *et al*., 2005) exerted very high membrane stability when compared with that of *Olax subscorpioides* (87.5%), *Aspilia africana* (85%), *Fagara zanthoxyloides* (83.8%) (Oyedapo and Famurewa, 1995).

The findings of this study supported the traditional used of the plant for treatment and management of inflammatory related disease as reported in literature. Therefore the use of the plant in chemotherapeutic preparations for alleviation and management of sickle cell anaemia and further investigations on the plant are hereby suggested.

REFERENCES

Aguwa CN, Lawal AM (1987). Pharmacology studies on active principles of *Callindra portoricensis* leaf extract. J. Ethnopharmacol. 22, 63-71.

Akah AP, Nwaiwu IJ (1988). Anticonvulsant activity of the root and stem of *C. portoricensis* J. Ethnopharmacol., 22: 205-210.

Ammus S, Yunis AA (1989). Drug-induced erythrocyte dyscrasias. *Blood Rev*., 3: 71-82.

Bode SO, Oyedapo OO (2011). Biological activities and phytoconstituents of the lower plant *Platycerium angolense,* Welwex Hook. J. Med. Plants Res. Vol. 5(8), pp. 1321-1329.

Braga ACS, Oliveira MBN, Feliciano GD, Reiniger IW, Oliveira JF, Silva CR, Filho MB (2000). The effect of drugs on the labeling of blood elements with technetium-99m. *Curr. Pharm. Des*., 6: 1179-1191.

Burkill HM (1985). The useful plants of west tropical Africa Families *Royal Botanic Gardens* Kew. Great Britain. Vol 3 177-266.

Chikezie PC (2006). Studies on the anti-sickling effects of *Azadirachta indica* and Vernonia *amygdalina* on HbSS erythrocytes. *Inter. J. Nat. Appl. Sci.*;2:24–8.

Chikezie PC (2007). Effect of ethanolic extracts of *Garcina kola, Cola nitida* and *Cola acuminata* on *in vitro* haemoglobin S polymerization. *Inter. J. Trop. Agric. Food Sys.*;1:220–5.

Chikezie PC (2007). Osmotic fragility index of HbAA erythrocytes in the presence of aqueous extracts of three medicinal plants (*Aframomum melegueta*, *Garina kola* and *Cymbopogon Citracus*). Glob. J. Pure Applied Sci., 13: 496-499.

Chikezie PC, Uwakwe AA, Monago CC (2009). Studies of human HbAA erythrocytes osmotic fragility index of non malarious blood in the presence of five antimalarial drugs. *J. Cell Ani. Biol.*;3:39–43.

Dean J, Schechter AN (1978). Sickle cell anemia: molecular and cellular basis of therapeutic approaches. *New England J. Med*. 229**:**753-755.

Ekeke GI, Shode FO (1985). The reversion of sickled cells by *Cajanus cajan*. *Planta Medica*.;6:504–7.

Elekwa I, Monanu MO, Anosike EO (2003). Studies on the effect of aqueous extracts of *Garcinia kola* seed on the human erythrocytes adenosine triphosphatase of HbAA, HbAS, and HbSS.

Ethnopharmacol., 102: 15-22.

Falade OS, Otumuyiwa IO, Oladipo A, Oyedapo OO, Akinpelu BA, Adewusi SRA (2005). The chemical composition and membrane stability of some herbs used in local therapy for aneamia. J.

Frenette PS, Atweh GF (2007). Sickle cell disease: Old discoveries, new concepts, and future promise. *J. Clin. Invest.*; 117:850–8.

Horie T, Sugiyama V, Awazu S, Hanano M (1979). The Correlation between drug binding to human erythrocyte and its hemolytic activity.J. Pharmacol., 4:116-122.

Imaga NOA, Gbenle GO, Okochi VI, Adenekan SO, Edeoghni SO, Kehinde MO, Bamiro SB, Ajiboye A and Obiona A (2010). Antisickling and toxicological profiles of leaf and stem of *Parquetina nigrescens* L. *J. Med. Plant Res.*;4:639–43.

Karayakin HN (1979). Cholelithiasis in children with sickle cell disease. *Am. J. Dis. Child.*;133:306–7.

Kumar PE, Sadique J (1987). The biochemical mode of action of *Gynaudronnis gynandra* in inflammation. Fitotrapia LVIII, 379-385.

Kutlar A (2005). Sickle cell disease: A multigenic perspective of a single gene disorder. *Med Princ Pract.*; 14:15–9.

Litman GW, Litman RT, Henry CJ (1976). Analysis of lipophilic carcinogen membrane interaction using human erythrocyte membrane sysmem model. Cancer Research 243:4364-4371.

Martins DW. Porphyrins and bile pigments. In: Martin DW, Mayes PA, Rodwell VW, editors. *Harper's Review of Biochemistry.* Los Altos: Lange Medical Publications; 1981. pp. 212–4.

Naibi, R.A., Sukumar, Sethuraman, V., Suluchana, N., Sadique, J.(1985): Satellite symposium on traditional medicine as Asian congress of Pharmacology.Tamil University of Thanjavur, 140.

Okpuzor J, Adebesin O (2006). Membrane stabilizing effect and antisickling activity of *Senna podocarpa* and *Senna alata*. 31st congress for European biochemical societies, Instanbul, Turkey.

Okpuzor J, Adebesin O, Ogbunugafor H, Amadi I (2008). The potential of medicinal plants in sickle cell disease control: A review. Inter. J. Biomed. Health Sci.;4:47–55.

Okpuzor J, Adebesin O, Ogbunugafor H, Amadi I. The potential of medicinal plants in sickle cell disease control: A review. *Inter. J. Biomed. Health Sci.* 2008;4:47–55.

Onah, J. O.; Akubue, P. I. and Okide, J. F. (2002): The kinetics of reversal of pre-sickled erythrocytes by the aqueous extract of *Cajanus cajan*; *Phytother. Res*. 16(8): 748-750

Oyedapo OO, Famurewa AJ (1995). Antiprotease and membrane stabilizing activities of extracts *Fagara zanthoxyloides, Olax subscorpioides* and *Tetrapleura tetraptera.* Int. J. Pharmacog., 33(1): 65-69. genotypes. *Global J. Med Sci*. 2(2). 107-114.

Oyedapo OO, Akinpelu BA, Orefuwa SO (2004). Anti-inflammatory effects of *Theobroma cacao*, L. root extract*. J.Tropical med. plants* (Malaysia) 592): 161-166.

Platt OS, Brambilla DJ, Rosse FW (1994). "Mortality in sickle cell diseases. Life expectancy and risk factors for early death". *N. Engl. J. Med.* 330 (23): 1639–44.

Sadique J, Al-Rqodah WA, Baghhath MF, El-Ginay RR (1989). The bioactivity of certain medicinal plants on the stabilization of the RBC system*. Fitoterapia* (Italy), LXVI: 525-532.