International Journal of Scientific Advances
ISSN: 2708-7972
Volume: 6 | Issue: 1 | Jan – Feb 2025 Available Online: www.ijscia.com
DOI: 10.51542/ijscia.v6i1.29

Synergistic Effect of One Annonaceae and Two Rutaceae on The Stability of HbSS Red Blood Cells

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ABSTRACT

One of the consequences of the sickle cell is chronic hemolysis. The aim of this study was to determine the membrane-stabilizing potential of extracts of three plants: *Zanthoxylum leprieurii*, *Xylopia aethiopica,* and *Harungara madagascariensis*. The 70% hydroethanolic extract (EZHm) and the decocted (DZHm) were used to evaluate the protective effect of both extracts against the membranes HbSS hemolysis in the presence of increasing concentrations of NaCl respectively. The spectrophotometric method was employed in determining the osmotic fragility index of HbSS erythrocytes. From these results, it appears that the test extracts have the capacity to stabilize the membrane of HbSS erythrocytes. In the presence of the tested substances at the concentration of 0.625 mg/mL, the IC50 was 4, 3.23, and 3.9 for Phenylalanine, DZHm, and EZHm respectively, while at the concentration of 1.25 mg/mL, the IC50 was 3.30 2.43 and 2.39 for the same substances. There was a significant difference ($p<0.01$) between the NaCl-induced hemolysis inhibitory activity of DZHm and EZHm at 0.625 mg/mL compared to Phenylalanine at 0.625 mg/mL. The present study showed that the extracts from the combination of the three plants exhibited membrane stability potential on sickle cell erythrocytes.

Keywords: Antihemolytic activity*; Zanthoxylum leprieurii; Xylopia aethiopica; Harungara madagascariensis.*

1 INTRODUCTION

The disease was discovered in 1910 by Dr. Herrick. He spotted the presence of sickle cells in the blood of a black student from the Caribbean with chronic anemia. This sickle characteristic of red blood cells gave the name of sickle cell disease to the ailment [1]. Sickle cell disease is the first most common genetic disorder in the world [2]. The molecular cause of this disease is a point mutation of the 17th of the β globin gene, which leads to the replacement of glutamic acid by valine at position 6 in the β globin chain. This is followed by a series of structural changes leading to reduced solubility, polymerization of hemoglobin, dehydration, and the shape change of the erythrocyte HBSS [3].

Sickle cell disease is a major public health problem in Africa with a prevalence of carriers of the sickle cell trait varying between 15 to 25% depending on the region [3]. In Côte d'Ivoire, the prevalence is more than 14% of the total population, of which more than 4% are homozygous [4].

The sickle cells are osmotically and mechanically more fragile [5]. This situation leads to chronic anemia and vaso-occlusive crises, resulting from the adhesion of erythrocytes to the epithelium of blood vessels. The created Oxidative environment may play a role in the pathogenesis of sickle cell disease. The common clinical manifestations of sickle cell anemia are hemolysis, hypercoagulable state, recurrent bacterial infections, and vaso-occlusive.

The combination of *Harungara madagascariensis* (LAM), *Zanthoxylum* leprieurii (GUILL) and *Xylopia aethiopica* was a recipe proposed by traditional healers in eastern Côte d'Ivoire [6]. According to the work of Uwakwe [7] extracts of *Xylopia aethiopica*

would have protected the membrane of SS erythrocytes against oxidative damage caused by reactive oxygen species (ROS). These extracts also prevented the HbSS red blood cell membrane deformation and their hemolysis. This activity was believed to be due to the presence of some amino acids, alkaloids, and phenolic compounds in the extracts of *Xylopia aethiopica*. Biapa [8] also showed that the hydroethanolic extract of *Harungara madagascariensis* protected the membrane of SS erythrocytes. It was noted that the aqueous extract of Zanthoxylum macrophyla which is another species of Zanthoxylum stabilized the membrane of SS erythrocytes against hemolysis [9]. The aim of this study was to examine the synergistic effect of the three plants on human sickle cells.

2 MATERIAL AND METHODS

2. 1 Plant Material

The plant material was composed of leaves of *Harungara madagascariensis* (LAM), the bark of the trunk of Zanthoxylum leprieurii (GUILL), and fruits of *Xylopia aethiopica*. These plants were listed and harvested from December 2017 to February 2018 in the region of Abengourou, (eastern Côte d'Ivoire). They were identified at the National Floristic Center of Félix Houphouët-Boigny University in February 2018 by the late Professeur Aké Assi Jean.

2.2 Preparation of 70% Hydroethanolic extract

The hydroethanolic extract was prepared according to the method of Zirihi [10]. One hundred grams (100 grams) of vegetable powder were soaked in one liter of hydroalcoholic 70% ethanol. The mixture was homogenized 10 times for 2 minutes per revolution using a Severin ® brand blender. The obtained homogenate was filtered using a square of cotton cloth then successively three times on cotton wool and then once with Whatman paper (3 mm). The filtrate was evaporated at 45 °C using a Venticell® type oven for 24 hours. The dry powder obtained was codified EZHm.

2.3 Preparation of the aqueous extract by decoction

According to the method of Konkon's [11] one hundred grams (100g) of vegetable powder were brought to a boil for 20 min in 2 L of distilled water. The obtained mixture was cooled at room temperature (25 ° C) and was filtered three times on cotton wool and once on Whatman 3 mm. The obtained filtrate was dried at 50 °C in the oven of the Venticell® type. The powder obtained was the total aqueous extract codified DZHm.

- ***Human Material***

To be included in the study, the blood should come from homozygous sickle cell voluntary patients regardless of age and gender. The voluntary patients shouldn't have undergone a blood transfusion for at least two months prior to the blood test and they must not be in crisis. In addition, the volunteers have given their consent on an ethical issue. Venous blood sampling of each patient was collected in the tube (EDTA). The blood samples were placed in a cooler containing cold accumulators at 4°C and then conveyed to the Pasteur Institute in Côte d'Ivoire.

2.4 Osmotic Fragility Tests

This method comes from the procedure developed by Parpart [12] and modified by Elekwa [13]. It allowed us to determine the activity of the plant extracts on the membrane of erythrocytes.

A stock solution of 100 g/L of NaCl (0.9%) Buffer solution at pH 7.4 with 150 mM phosphate was prepared. A series of nine (9) solutions were prepared by dilution to obtain concentrations equivalent to 9.0, 7.0, 6.0, 5.5, 5.0, 4.0, 3.0, 2.0, and 1.0 g/L of NaCl. The controls were prepared by adding 0.05 mL of washed erythrocytes to 5.0 mL NaCl (9.0, 7.0, 6.0, 5.0, 4.0, 3.0, 2.0, 1.0, and 0 g/L). In another series of nine tubes, a volume of 0.5 mL of the plant extracts at concentrations: 0.625 and 1.25 mg/mL was added to 4.5 mL of each of the nine (9) NaCl solutions previously prepared. Then 0.05 mL of the blood sample was added to the mixture. The total volume was 5.05 mL.

Each tube was shaken by inverting the tubes several times. The tubes were left to stand for 30 minutes at room temperature before centrifuged for 5 minutes at 1500 rpm. The absorbance of the supernatant from each tube was read at 540 nm and the 9.0 g/L NaCl tube was used as blank. The average of three measurements of the blood sample was taken. A positive control was performed with phenylalanine. From measurements of optical density, the rate of hemolysis inhibition of the different extracts was determined with the following formula:

Hemolysis inhibition% = (DO0 - DOi)/ DO0 X 100

doi: Absorbance of sample extract; DO0: Optical density of the control solution.

The characteristic point IC50 was determined schematically. It is the concentration of NaCl corresponding to the inhibition of lysis of 50% erythrocytes. The IC50 values were interpolated from the erythrocyte osmotic fragility curves obtained by plotting the percentage of lysis inhibition against saline concentrations. The more IC50 was small the more the plant extract had the anti-hemolysis activity and also the capacity to protect the erythrocytes against hemolysis.

3 RESULTS AND DISCUSSION

3.1 Results

Effect of extracts on means corpuscular fragility of SS red blood cells. The percentage of Hemolysis inhibition of the HbSS erythrocytes treated with DZHm and EZHm at the concentrations of 0.625 and 1.25mg/mL were determined using the earlier described formula with the different optical density measurements. The curves obtained by plotting the percentage of lysis inhibition against saline concentrations in the presence of the extracts were summarized in Figures 2a and 2b. The results of this study showed that the more, the concentration of NaCl decreased, the more the hemolysis was promoted (Figure 2). The interpretation of the capacity of the two extracts to stabilize the HbSS red Blood cell membrane against hemolysis was made

based on the IC50 values presented in Table 1. The more the IC50 value was relatively low, the more the plant extracts had a protective effect against the hemolysis of HbSS erythrocytes. The results also displayed that the different concentrations of DZHm and EZHm (0.625, 1.25 mg/mL) produced a positive inhibition (greater than 10%) of the hemolysis of the HbSS red blood cells even at the lowest concentration of NaCl (Figure 2 a,b). The IC50 values of DZHm and EZHm at 0.625 mg/mL were 3.23 and 3.9. Which were relatively lower than 2.43 and 2.39 the CIH 50 values of DZH and EZH at 1.25mg/mL respectively. The protective effect against HbSS erythrocytes hemolysis of both extracts was better when the concentration was increased from 0.625 to 1.25 mg/mL (table 1). There was a significant difference ($p<0.01$) between the NaCl-induced hemolysis inhibitory activity of DZHm and EZHm at 0.625 mg/mL compared to Phenylalanine at 0.625 mg/mL.

TABLE 1: Effect of extracts on mean corpuscular fragility of SS red blood cells.

	Phénylalanine		DZHm		EZHm	
Concentrations	0,62	1,25	0,625	1,25	0,62	1,25
IC 50	4	3,30	3,23	2,43	3,9	2,39

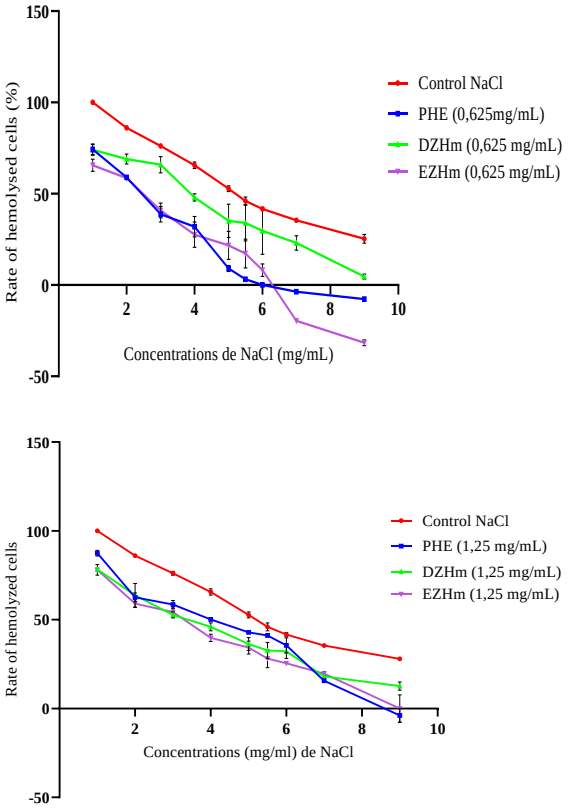

FIGURE 1: Effect of extracts on mean corpuscular fragility of SS red blood cells.

3.2 Discussion

Indeed, sickle cell disease is known as hemolytic anemia because of the early hemolysis of erythrocytes in sickle cell blood which, by thus evacuating hemoglobin into an unsuitable environment, destines it to early destruction that leads to a decrease in the level of this vital blood protein, thus to anemia. Any substance that would prevent or reduce this hemolysis would be beneficial for sickle cell patients [14]. The results obtained with NaCl alone, show the behavior of blood cells when the osmotic pressure is changed from a very hypotonic to an isotonic medium. During this observation, the number of lysed cells gradually decreases. In comparison to this phenomenon, the results show that ZHm extracts and phenylalanine also decrease the number of lysed cells under the same conditions as described above.

In general, with all three substances, the number of lysed cells is strongly reduced. Among these substances tested, the best performance was generated by the hydroethanol extract (EZHm). Since it is known, that phenylalanine protects the erythrocyte cells from bursting, then it appears from these results that the hydroethanolic extract generates a protection of the erythrocyte membrane which is clearly better than those of phenylalanine and the aqueous extract (DZHm). This protection of the membrane would be due to the presence of flavonoids in the extracts. This assertion is in agreement with the results of [15]. These authors showed in their studies that the anti-hemolytic and anti-lipid peroxidation activity of Ficus sycomorus was due to the action of phenolic compounds such as flavonoids. These compounds are known for their potential to protect red blood cells against membrane lysis and lipid peroxidation. In addition to these authors, the work of [7], on extracts from *X. aethiopica* on the membrane of red blood cells, showed that these extracts protect the SS erythrocyte membrane against oxidative damage caused by reactive oxygen species (ROS), thus preventing membrane deformation and hemolysis. This activity is thought to be due to the presence of amino acids, alkaloids and phenolic compounds in *X. aethiopica* extracts. [8] Also showed that the hydroethanol extract of *H. madagascariensis* protected the membrane of SS erythrocytes. [9] It also showed that the aqueous extract of *Z. macrophylla* which is of the same family as *Z. leprieurii* would stabilize the membrane of SS erythrocytes against hemolysis. In sum, all the plants in the ZHm recipe contain metabolites such as amino acids, flavonoids, and polyphenols that protect the erythrocyte membrane against lysis. Indeed, several studies have proven that phenolic compounds especially flavonoids possess anti-free radical properties, which allow them to neutralize or scavenge free radicals [7, 16, and 17]. In addition, polyphenols are known to be chelators of transition metals, thereby reducing the rate of the Fenton reaction. They can also prevent free radical-induced oxidations across the erythrocyte membrane [18]. Regarding the proteins in the extracts, their interaction with erythrocyte membrane lipids protects these cells from destruction and oxidation [19, 20].

Given the performance of ZHm extracts better than that of phenylalanine, these extracts, although crude, are a hope for the discovery of many antihemolytic active ingredients. Thus, the extracts of the ZHm recipe would prevent complications related to sickle cell disease such as anemia, pain, and inflammation.

4 CONCLUSION

The present study showed that DZHm and EZHm protected the red blood cell membrane in very hypotonic solutions and generated IC_{50} significantly lower than those of phenylalanine and NaCl control, implying that these extracts possess a stabilizing effect on the HbSS erythrocyte membrane. The observed anti-hemolytic effect of ZHm extracts suggests a synergistic effect of the plants. ZHm could constitute a phyto-drug for the management of sickle cell anemia and its associated pathologies.

Acknowledgments

We would like to thank the officials and staff of the Hematology Unit of the Yopougon University Hospital, the Pasteur Institute of Cote d'Ivoire, not to mention those of the Pharmacognosy Laboratory of the UFR of Pharmaceutical and Biological Sciences of Felix Houphouet Boigny University in Cocody for their availability and assistance in carrying out the work. Also, we would also like to thank all the patients who have agreed to participate in this study.

Competing interests

The authors have declared that no competing interests exist.

Consent

An agreement was obtained from the ethics committee and an informed consent was approved by each patient wishing to participate in the study.

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DEFINITIONS, ACRONYMS, ABBREVIATIONS

DZHm: decocted extract of combination *Zanthoxylum leprieurii, Xylopia aethiopica, and Harungara madagascariensis.*

EZHm: ethanolic extract of combination *Zanthoxylum leprieurii, Xylopia aethiopica, and Harungara madagascariensis.*