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RESEARCH ARTICLE

EVALUATION AND COMPARISON OF ANTI-SICKLING ACTIVITIES OF MACERATED SEEDS OF CAJANUS CAJAN AND PHENYLALANINE

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Abstract

Sickle cell patients' interest in traditional pharmacopoeia comes from the healing promise made by traditional practitioners and the expensive cost of medicines. The objective of this study was to evaluate the anti-sickling activity of macerated seeds of *Cajanus cajan* and compare its activity to that of phenylalanine. An induction of sickling due to 2% sodium metabisulfite was performed on 30 blood samples from SSFA₂ homozygous sickle cell patients. The macerated seeds of *Cajanus cajan* at 10 to 500 mg / ml concentrations and 10 mg / ml phenylalanine were added then. The reading was made after a contact time of 30 minutes. All extracts from the seeds of *Cajanus cajan* led to a reduction in the percentage of sickle cells which rose from 64.70% to 19.03%. The phenylalanine solution also caused a reduction in the percentage of sickling which rose from 64.70% to 22.76%. The activity of the maceration at the concentration of 400 mg / ml was higher than that of the phenylalanine. These results advocate for the use of *Cajanus cajan* seeds in the diet of sickle cell disease to reduce the occurrence of painful crisis.

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Introduction:-

Sickle cell disease, or sickle cell anemia, is a serious inherited genetic disorder with recessive autosomal transmission. It is caused by an abnormal hemoglobin (Hb S), which polymerizes under deoxygenated state resulting in a deformation of red cells which take the shape of a crescent moon. Sickle cell disease is a chronic disease; whose management is for a life time. The only current curative treatment is the hematopoietic stem cell transplant, which unfortunately cannot be performed in Ivory Coast.

Face to socio-economic problems and problems of accessibility to pharmaceutical products in Africa, 90 % of the population resort to the many remedies held by traditional practitioners. Previous studies showed that *Cajanus cajan* and *Fagara xanthoxyloids* are among the species that are used for the management of sickle cell disease (Sofowora and Isaacs - Sodeye, 1971). Previous studies (N'Draman-Donou and al., 2013) showed that the

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aqueous extract of *Cajanus cajan* seeds reduces by 50% the formation of sickles. In a study conducted by (Ekéké and al., 1990), amino acid (AA) analysis showed that the solvent extracts of *Cajanus cajan* seeds contain 26.3% phenylalanine (Phe) in the form of free amino acids, and according to them, the phenylalanine, essential amino acid would be responsible for anti-sickling activity of the plant (Ekeke and al., 1990). It has therefore been proposed as a general objective: to assess the anti-sickling activity of the maceration of the seeds of *Cajanus cajan* and the phenylalanine and to compare them.

Equipment:

It was an experimental type study initiated by the Department of Hematology, Immunology and General Biology of the Faculty of Pharmaceutical and Biological Sciences of the University of Felix Houphouët Boigny, Abidjan, Ivory Coast. It was carried out in collaboration with the department of pharmacognosy of the same Faculty and the hematology unit of the central laboratory of the University Hospital (CHU) of Yopougon over a period from January to April 2018. Extraction was carried out on the seeds contained in the mature *Cajanus cajan* pod (Fabaceae) and phenylalanine was obtained commercially. The evaluation of the anti-sickling activity is made in vitro using blood taken from purple tube containing Ethylene Diamine Tetra- Acetate elbow crease.

Methods:-

Search for anti-sickling activity:

Preparation of working solutions:

Maceration:

After harvest, the drug was dried for a week in the shadow at the laboratory for pharmacognosy at room temperature and then ground with a mortar and a pestle to obtain a coarse powder. The maceration was obtained by soaking 1 g of the drug powder with 100 ml of distilled water for 24 hours in a 500 ml erlenmeyer flask. The maceration obtained was filtered first on an ordinary filter and then on a wattman type paper filter and constituted solution 1 (S1). This solution was put in a glass flask, with a hermetic seal and stored in a refrigerator. The process to obtaining solutions S2 to S6 is summarized in Table 1.

Phenylalanine:

A solution of phenylalanine (Phe) was prepared at the concentration of 10 mg / ml of distilled water; that is 10 mg of phenylalanine powder for 1 ml of distilled water according to the method of Seck M. and al. (2015) . And it is this solution that was used for the performance of the different tests.

2% sodium metabisulfite:

The 2% solution was obtained by using 100 ml of distilled water for 2 grams of metabisulfite.

Evaluation of anti-sickling activity:

Induction of sickling and microscopic reading:

This induction of sickling is made by performing in advance a dilution of the blood Hb SSFA₂ at 1/10th with a normal saline solution in the proportions of 20.µl of blood diluted for 180.µl of saline solution. Then, a mixture at equal volumes (20.µl) of blood diluted at 1/10th and sodium metabisulfite solution at 2% in a hemolysis tube was performed. After 15 minutes of contact, observation by a microscope at 40x magnification of a drop of this mixture deposited on a blade covered with lamella was made to determine the percentage of sickle cells by counting the number of red blood cells (RBCs) and sickle cell in a field.

Saline solution test: Control test:

The purpose of this test was to search for a possible return of sickle cells to their normal shape after a lapse of time. Therefore, a mixture in a hemolysis tube with plug of 20.µl of blood diluted at 1/10th, of sodium metabisulfite 20.µl 2% and of 20.µl of saline was performed. After 30 minutes of contact, a drop of the mixture was observed between blade and lamella in the microscope with a G x 40 objective to determine the percentage of sickle cells over time.

Test with the macerated seeds of *Cajanus cajan*:

After identifying 6 cap hemolysis tubes (S1, S2, S3, S4, S5, S6), a mixture at equal volumes of blood diluted at 1/10th of 2% sodium metabisulfite solution and the different macerations was performed. The observation by an optical microscope at 40 x magnification of one drop of each mixture between blade and lamella was then made after 30 minutes and the percentage of sickle (RBCs) was determined.

Phenylalanine test:

The phenylalanine test was carried out on a mixture in a cap hemolysis tube containing 20.µl of diluted blood, 20.µl of 2% sodium metabisulfite and 20.µl of phenylalanine solution. Microscopic observation with Gx 40 objective of a drop of the mixture between blade and lamella was made after 30 minutes to determine the percentage of sickle red cells.

Data Analysis:

The test used to compare our proportions is that of t- student at the significance level $\alpha = 5\%$.

Results:-**Evaluation of anti-sickling activity of the different solutions:****Control test:**

After 30 minutes of contact between the diluted blood, 2% sodium metabisulfite and the saline solution used as a control, it was noted the presence of 64.7% sickle cell. When this incubation reached 60 minutes, the variation in the number of sickle cells over time was not significant with a $p = 0,17$.

Test with the different solutions to evaluate:

To perform the tests, each solution was mixed with the blood of a sickle cell disease patient and 2% sodium metabisulfite. A decrease in the percentage of sickle cells was observed and was accentuated in the first 30 minutes. The results are reported in Table 2. Following the same procedure as for the maceration, the addition of 10 mg / ml phenylalanine also caused a reduction in sickling (Table 2).

Comparison of anti-sickling activity of the solutions:

The activity of the maceration for concentrations of 10 mg / ml to 300 mg / ml and 500 mg / ml is substantially equal to that of the phenylalanine because there is no significant difference ($p > 0,05$). However, at a concentration of 400 mg / ml, the activity of the maceration was maximal and the effect obtained was higher than that of the phenylalanine (Table 2).

Table 1:-Method to obtain different macerations.

Powder (g)	1	10	20	30	40	50
Distilled water (ml)	100	100	100	100	100	100
Maceration	S1	S2	S3	S4	S5	S6

Table 2:- Data on the activity of the maceration and on that of the phenylalanine after 30 minutes.

Solutions	Percentage of sickle cells Mean \pm standard deviation (%)		Statistics p
	phenylalanine	Maceration at different concentrations	
			0,462 (NS)
Phe (10 mg / ml) - S1 (10 mg / ml)	22,76 \pm 7,35	24,00 \pm 7,50	
Phe (10 mg / ml) - S2 (100 mg / ml)	22,76 \pm 7,35	22,13 \pm 7,00	0,728 (NS)
Phe (10 mg / ml) - S3 (200 mg / ml)	22,76 \pm 7,35	25,63 \pm 10,50	0,153 (NS)
Phe (10 mg / ml) - S4 (300 mg / ml)	22,76 \pm 7,35	20,97 \pm 7,30	0,269 (NS)
Phe (10 mg / ml) -S5 (400 mg / ml)	22,76 \pm 7,35	19,03 \pm 8,34	0,041 (S)
Phe (10 mg / ml) -S6 (500 mg / ml)	22,76 \pm 7,35	19,97 \pm 8,58	0,060 (NS)

Discussion:-

Under anaerobic conditions, in the tube serving as control and containing the blood with 2% of sodium metabisulfite and physiological water, the presence of sickle cells was found. In fact, 64.7% of the cells had sickled. These results are similar to those found in previous studies (N'Draman-Donou and al, 2015, 2017). After 30 min of contact, the percentage of sickle cells remained practically unchanged, showing that the cells do not tend to return to their normal shape.

Test with the maceration:

After a conditioning time of 30 minutes with the maceration of the seeds of *Cajanus cajan*, there was a decrease of about 30% in sickle cells (Table 2). In the dose of 10 mg / ml, sickling which concerned 64.7% of red blood cells decreased to 24% in this period of time. Similar results were found for concentrations of 100, 200, 300 and 500 mg / ml with maceration. This inhibition was statistically significant. The maceration process of *Cajanus cajan* seeds was maximum at a dose of 400 mg / ml. Ogada and al (2002) with the methanolic extract of *Cajanus cajan* have also shown anti-sickling activity of these seeds. Similarly, N'Draman-Donou and al (2015, 2017), with their aqueous and organic extracts, all at a concentration of 333, 35 mg / ml (concentration at the limit of solubility), found similar results. The phytochemical sorting in their studies had revealed the significant presence of alkaloids both in aqueous and organic extractions (N'Draman-Donou and al., 2015, 2017).

Test with the Phenylalanine:

When replacing the maceration with the Phe at a dose of 10 mg / ml, we observed an inhibition of sickling. The percentage of sickle cells decreased from 64.70% to 22.76%. The Phe increases the capacity of erythrocytes to absorb water without lysing and there fore stabilizes their membrane (Elekwa and al., 2005). This anti-sickling activity of the Phe could be explained by studies which have shown that AA and in particular aromatic AA have the possibility of inhibiting the gelling of deoxyhemoglobin S and partially preventing the formation of sickle cells (Noguchi, 1977; Noguchi and Ackeman, 1983). The Phe esters and Phe-containing peptides have the same property (Acquaye and al., 1982; Votano and al., 1984).

Comparison between the activity of the maceration of *Cajanus cajan* and phenylalanine:

According to a study conducted in 1990, the Phe is the main AA contained in the soluble aqueous fraction of a seed extract of the plant (Ekéké and Shode, 1990). In addition, analysis of AA has shown that methanolic extracts of *Cajanus cajan* seeds contain, in the form of free AA, up to 26.3% Phe (Ekéké and Shode, 1990). It was observed during our study that the activity of the plant, regardless of the concentration to which the test was carried out (10 to 300 mg / ml and 500 mg / ml), is substantially equal to that of the Phe at 10 mg / ml after a contact time of 30 minutes. These results are similar to those found in the studies of Ekéké and al. (1990) who also demonstrated that Phe is responsible in vitro for the anti-sickling activity of the plant.

It would appear that the presence of this AA alone could account for about 70% of anti-sickling activity of the maceration. At 400 mg/ml, the activity of the plant was maximal and superior to that of the Phe. This may be due to the presence of compounds other than Phe. Indeed, earlier work by Akojie and Fung (1992) revealed the presence of phenylalanine and hydroxybenzoic acid in a methanolic extract of *Cajanus cajan*. Vanderjagt and al (1977), observed that there is a significant decrease in all AA, especially essential AA such as Phe, in sickle cell disease patients as a result of increased urinary excretion, which partly helps explain the growth retardation of patients. The seeds can there fore be recommended to sickle cell anemia patient on the one hand, to compensate for urinary losses and on the other hand, to reduce pain attacks.

Conclusion:-

Biological tests revealed that the maceration of the *Cajanus cajan*'s seeds (from 10 to 300 mg / ml) has an activity comparable to that of phenylalanine (from 10 mg / ml). After a contact time of 30 minutes, the activity was maximum at a dose of 400 mg / ml with the macerate. The alkaloids in general and phenylalanine in particular could be responsible for the ant-sickling activity of these seeds. This plant shows quite promising results in the care of sickle cell anemia. As a matter of fact, since it is an edible plant, it could be recommended as a food supplement for patients with sickle cell anemia or even be the subject of galenic preparations so as to contribute to the care of patients. Also, more in-depth studies will make it possible to assess the in-vivo effect of these seeds after administration to patients or even to know their mechanism of action in the reversibility of sickling.

Conflict of interests:

The authors declare not having any conflicts of interest in relation with this article.

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