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ARTÍCULO ORIGINAL

Antisickling effect of crude flavonoids in the methanolic leaf extract of *Persea americana* Mill

Efecto de los flavonoides aislados del extracto metanólico de las hojas de *Persea americana* Mill en la drepanocitosis

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ABSTRACT

Introduction: Sickle-cell anemia, a difficult-to-treat genetic disease, causes synthesis of abnormal hemoglobin in which erythrocytes assume a sickle shape and become susceptible to hemolysis. Research about *Persea americana* Mill has shown that this plant has an antisickling effect, but the metabolites responsible for such activity have not been identified.
Objectives: Determine the effect of a fraction of flavonoids isolated from *P. americana* in sickle-cell anemia.
Method: A methanolic leaf extract was obtained from *P. americana* using Soxhlet methodology. 55.23 % of the crude flavonoids were isolated by sequential extraction of the aqueous fraction with ether, ethyl acetate and n-butanol. Effect in sickle-cell anemia was determined by counting the sickle-shaped cells in a blood sample taken from a sickle-cell patient. Upon exposure of the cells to 2, 4, 8 and 10 mg/ml of the extract, five readings were taken every 30 min. Para-hydroxybenzoic acid (5 mg/ml) was used as positive control, whereas the negative control was 0.9 % sodium chloride. The Pearson correlation coefficient was estimated and linear regression was determined. *P*-values equal to or under 0,05 were considered to be significant. Mean effective concentration (EC_{50}) was estimated at 120 min.
Results: Crude flavonoids displayed time- and dose-dependent antisickling activity. At 120 min and 10 mg/ml of the extract, no sickle-shaped cell was detected, whereas with the para-hydroxybenzoic acid 61 abnormal cells were observed. The lowest concentrations of the extract showed a large number of abnormal cells. Significant correlations were obtained

with 10 mg/ml of the extract and with para-hydroxybenzoic acid. A notable negative linear correlation was found at this concentration between the presence of abnormal cells and time. EC_{50} was 8.050.

Conclusions: High concentrations of flavonoids in *P. americana* leaves may be responsible for the effect of the latter in sickle-cell anemia, which increases with time.

Key words: antisickling effect; *Persea americana* Mill; flavonoids, sickle-cell anemia.

RESUMEN

Introducción: La anemia drepanocítica, enfermedad genética de difícil tratamiento, provoca la síntesis de una hemoglobina anormal en la cual los eritrocitos adoptan forma de hoz y son susceptibles a la hemólisis. Las investigaciones realizadas con *Persea americana* Mill han demostrado que la planta tiene efecto contra la drepanocitosis, pero no se han identificado los metabolitos responsables de esta actividad.
Objetivos: Determinar el efecto en la drepanocitosis de una fracción de flavonoides aislados de *P. americana*.
Método: Se obtuvo el extracto metanólico de las hojas de *P. americana* mediante el método Soxhlet. Se aisló 55,23 % de flavonoides crudos mediante extracción secuencial de la fracción acuosa con éter, etilacetato y n-butanol. El efecto en la drepanocitosis se determinó mediante el conteo de las células en forma de hoz en una muestra de sangre de un paciente con anemia drepanocítica. Después de exponer las células a 2, 4, 8 y 10 mg/mL del extracto se hicieron cinco lecturas cada 30 min. Se usó el ácido para-hidroxibenzoico (5 mg/mL) como control positivo y el cloruro de sodio al 0,9 % como negativo. Se calculó el coeficiente de correlación de Pearson y se determinó la regresión lineal. Se consideran significativos los valores de *p* menores o iguales que 0,05. La concentración efectiva promedio (CE_{50}) se calculó a los 120 min.

Resultados: Los flavonoides crudos actuaron contra la drepanocitosis en dependencia del tiempo y la dosis. A los 120 min y en 10 mg/mL del extracto no se detectó ninguna célula en forma de hoz mientras que en el ácido para-hidroxibenzoico se observaron 61 células anormales. Concentraciones más bajas del extracto mostraron un número elevado de células anormales. Se obtuvieron correlaciones significativas para 10 mg/mL del extracto y para el ácido para-hidroxibenzoico, y hubo una notable correlación lineal negativa entre la presencia de células anormales y el tiempo entre los flavonoides y el control positivo a esta misma concentración. La concentración efectiva promedio fue 8,050.
Conclusiones: Las altas concentraciones de flavonoides en las hojas de *P. americana* pueden ser las responsables de su efecto en la drepanocitosis, el cual aumenta en función del tiempo.

Palabras clave: efecto en la drepanocitosis; *Persea americana* Mill; flavonoides; anemia drepanocítica.

INTRODUCTION

The sickle cell disease (SCD) is a genetic disorder fatal in nature. Thousands of children are dying off due to this health problem throughout the world.[1] It is most common among people of African and Mediterranean descent and it is also common in people from South American or Central American countries, the Caribbean and the Middle East.[2]

In Uganda, of the 1,6 million babies are born each year, 20 000 are born with the sickle cell gene and 50 to 80 % of them die before the age of 5. This is mainly due to the lack of screening and early detection of the mutation along with lack of a national treatment program. The Bamba people of western Uganda carry 45 % of the gene and have the highest frequency of the trait registered in the world.[2]

In other countries, due to the rapid development of diagnosis and clinical managements, such patients are living up to a respectable age. But as there is no permanent cure the patients are suffering from bone and joint pain, jaundice, hepato-splenomegaly to chronic

infections etc.; all beginning with a serious hemolytic anemia triggered by the exposure of sickle-shaped red cells to low oxygen tension in tissues.[1]

The main pathophysiological feature is due to the polymerization of sickle hemoglobin (sickling process) inside the red blood cell (RBC) of these patients starting in tissues areas where oxygen concentration reduces. The change of RBC from its biconcave disc shape to sickle shape is due to the polymerization of mutant hemoglobin (HbS) inside the RBC and membrane fragility during hypoxic condition. The mechanism and the process of sickling are very complex and multifactorial in nature.[1,3] To get rid from such complicacies it is necessary to suitably and accurately stop or reduce the sickling of RBC of the patients.[1]

Treatment of SCD has proved difficult and inefficient due to its genetic origin. Earlier therapies included the use of liver-based extracts and diets, oxygen, vasodilators, carbonic anhydrase inhibitors and splenectomy.[4] The conventional therapeutic management involves the use of drugs like tucaresol an immune modulator which increases oxygen affinity and reduces haemolysis;[5] hydroxyurea that reduces vaso-occlusive events, it does so via several mechanisms that ultimately increases synthesis of HbF, which promotes solubility of hemoglobin within red cells. Hydroxyurea also reduces adhesion of red cells to vascular endothelium, and suppress the production and adhesiveness of neutrophils decreasing their contribution to vascular occlusion. This drug also reduces the frequency of painful events, acute chest syndrome, and secondary strokes in sickle cell patients.[6]

The potential antisickling agents either from natural sources and/or synthetic molecules may be helpful for reducing the clinical morbidity of the patients. A lot of natural compounds from plant extracts have been tried by several workers in recent past. Most of the studies are based on in vitro red cell sickling studies and their mode of action has not been properly understood.[1]

Oxidative stress plays important role in pathophysiology of *sickle cell anaemia*. Erythrocytes from SCD patients continuously produce larger amounts of pro-oxidants than normal cells. Oxidative stress seems to primarily affect the membrane and results in hemolysis. The use of antioxidants in vitro reduces the generation of pro-oxidants.[7]

Flavonoids, secondary metabolites present in many plants like *Persea americana* Mill (*P. americana*) are potent water-soluble antioxidants and free radical scavengers preventing oxidative cell damage. Flavonoids also have beneficial antiinflammatory and inmunomodulatory effects.[(8)] It is known that the leaves and fruits of *P. americana*, commonly known as avocado, which belongs to Lauraceae family, portray antisickling properties.[9,10]

Considering that avocados are common plants in Uganda, the purpose of this study was to assess the antisickling activity of crude flavonoids isolated from *P. americana* leaves.

METHODOLOGY

The antisickling properties of the crude flavonoids were determined based on the number of sickle-shaped cells present in the blood samples after exposure to different concentrations of the extract over a period of time.

Plan material

The *P. americana* leaves (one kg) were collected in TASO village, Mbarara district and taken to the Faculty of Science, Department of Biology for identification by the botanist Dr. Eunice Olet and it was given a voucher number: EDWARD LUKYAMUZI 001.

The leaves were washed with distilled water and shade dried. The washed and air dried leaves were powdered by a blender and weighed; 170 g of the powdered leaf was obtained.

The powdered sample was soxhlet extracted[(11)] in 80 % methanol for 24 h and then filtered; 18.54 g of crude methanolic extract was obtained at a percentage yield of 10.90 %.

Identification and isolation of crude flavonoids

Flavonoids identification was carried out in order to ascertain their presence; ammonia test was done.[12] The methanolic dry extract was dissolved in 100 ml of distilled water producing the aqueous fraction of the crude extract.

Isolation of the flavonoids was carried out by first the aqueous fraction being fractioned by sequential extraction with 100 mL of ether at 40-60 °C (fraction I), 100 ml of ethyl acetate (fraction II) and 100 ml of n-butanol (fraction III) in succession. Each step was repeated twice to ensure complete extraction. Fraction I was rejected due to large presence of fatty substances, whereas fraction II was analyzed for free flavonoids and fraction III for bound flavonoids.[13] Since ethyl acetate extracts more of the free flavonoids while n-butanol extracts more of the bound flavonoids, a total of both would allow for exhaustive extraction of crude flavonoids in general.

Upon isolation, the percentage yield of flavonoids from the methanolic extract was 12.60 %. This was inclusive of both the bound and the free flavonoids (fraction II and fraction III).

The percentage yield was derived from the fraction of total volume of bound and free flavonoids and the total volume of the crude aqueous extract and was calculated according to the following formula:

$$\% \text{ yield of flavonoids} = \frac{\text{(fraction II + fraction III)}}{\text{volume of aqueous crude extract}}$$

The flavonoids extracted were dried in vacuo. The powder was dissolved in normal saline (negative control) to generate the concentrations (2, 4, 6, 8 and 10mg/ml).

Blood collection and preparation

Five milliliters of blood were obtained by venipuncture from a patient affected by SCD (HbSS) from Mbarara Regional Referral Hospital (MRRH). Blood was collected in disodium ethylenediaminetetraacetic acid (EDTA) bottles and the content thoroughly mixed by gently rolling the bottle. All experiments were performed with fresh blood.

Determination of antisickling activity[9]

The fresh blood (0.5 ml) was mixed each with the crude flavonoids extracts of 2, 4, 6, 8 and 10 mg/ml in uncovered test tubes. Samples were taken from the different mixtures. Each sample was smeared on a microscope slide. 0.2 ml of 2 % sodium metabisulphite was added to deoxygenate the system, mixed thoroughly and sealed with wax. Each sample was examined under the light microscope. The samples were focused under x10 magnification and the cells were examined for sickling under x40 magnification. At least 500 red blood cells in each sample from five different fields of view across the slide were counted. The numbers of both sickle and not sickle-shape red blood cells were counted. The slides were then incubated under moisture at 37 °C and the readings taken again at 30 min interval until five readings were obtained.

Two types of control were employed in this biological testing. A positive control used was 5 mg/ml para-hydroxybenzoic acid (PHBA), a compound known to reverse the sickling in HbSS blood cells. As negative control sodium chloride 0.9 % (normal saline) was used.

Results were obtained and expressed as percentage of sickle cells remaining after incubation of HbSS blood samples with the crude flavonoids in the leaf extract, normal saline and PHBA taking into account the baseline in each sample in order to compare the difference. The normal cells were also counted in all samples at all times to ensure that the apparent reduction was not partially caused by hemolysis of sickle cells previously observed.

The half maximal effective concentration (EC50) was calculated using the AAT Bioquest Calculator which determines the EC50 value based on the responses to three different

concentrations of the extract at specified time. The calculator generates a graph (response-concentration curve). The data sets included the responses at 120 min for the three most effective concentrations (6, 8 and 10 mg/ml) among the extract concentrations used.

Statistical analysis

Statistical analysis of the data was carried out using SPSS (version 18.0) software. Pearson correlation coefficient was calculated to know the closeness of the linear relationship and linear regression was used to describe changes of antisickling effect with time. The *p* level at 0.05 or below was considered statistical significant.

Ethical considerations

A patient was identified and suggested by a physician of Medicine Department at MRRH. Consent from the participant was sought and a consent form signed (Appendix I). The participant was informed in detail about the study. Extreme care and caution was taken to ensure confidentially at all stages of data collection, analysis, report writing and dissemination of results.

RESULTS

Flavonoids identification

The presence of flavonoids was confirmed in the methanolic leaf extract of *P. americana*.

Antisickling activity

A dose dependent reduction of sickle cells was observed on exposure of the blood to the crude flavonoids at varying concentrations. The higher concentration of the extract exhibited a greater and significant antisickling effect. The results also showed a time dependent antisickling effect for both, the plant extract and the standard PHBA (Fig. 1).

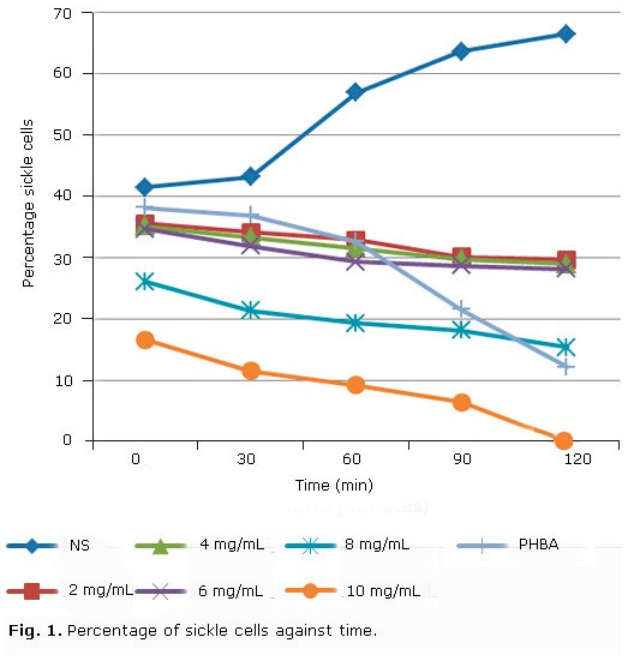

Fig. 1. Percentage of sickle cells against time.

The greatest reversal in sickle cells for the plant extract was at 10 mg/mL that completely reversed the sickling at 120 min. This result is comparable to the standard drug where at this time of exposure only 12.2 % of sickle cells was observed since the initial percentage of sickle cells on the sample exposed to PHBA (5mg/mL) was higher than that of the extract. However, at 30 min the results observed for 10 mg/ml of the extract were better than those obtained for PHBA.

The lowest results were seen at 2 mg/ml which gave a 29.67 % of sickled cells at 120 min however possessed a similar trend to 4mg/ml and 6 mg/ml achieving 28.96 % and 28.18 % respectively at 120 min. The lower concentrations produced less significant sets of results. (p< 0.05).

Pearson correlation coefficient

The Pearson correlation was significant(**) at 0,01 (bilateral) for 10 mg/ml of the extract. There was inverse correlation, so as the time increased one unit (min) the number of sickle-shaped cells reduced. Significant correlation(*) was also obtained for PHBA at 0,05 (bilateral) (Table 1).

Table 1. Pearson correlation coeficiente

Correlations			
		T	10mg
T	Pearson	1	-.986**
	Sig. (bilateral)		.002
	N	5	5
10 mg	Pearson	-.986**	1
	Sig. (bilateral)	.002	
	N	5	5
		T	PHBA
T	Pearson	1	-.922*
	Sig. (bilateral)		.026
	N	5	5
PHBA	Pearson	-.922*	1
	Sig. (bilateral)	.026	
	N	5	5

Linear regression analysis

Table 2 shows the results of the linear regression. The results obtained in terms of presence of sickle cells against time at concentrations of 2 mg/ml, 4 mg/ml, 6 mg/ml, and 8 mg/ml of crude flavonoids were not significant from the statistical analysis with a significance level at 0.05.

At high concentration of 10 mg/ml there was a strong inverse correlation between the presence of sickle-shaped cells and time; every minute the number of sickle cells reduced in 0.79 at this concentration.

Similar findings were found for the PHBA (positive control). As the time increased one minute the number of sickle cells reduced in 1.3 (in basic pH). The EC50 at 120 min was calculated using the responses obtained for the concentrations of 6, 8 and 10 mg/ml giving a value of 8.050 expressed in molar concentration. The response-concentration curve is shown in Figure 2 where the EC50 value is also indicated.

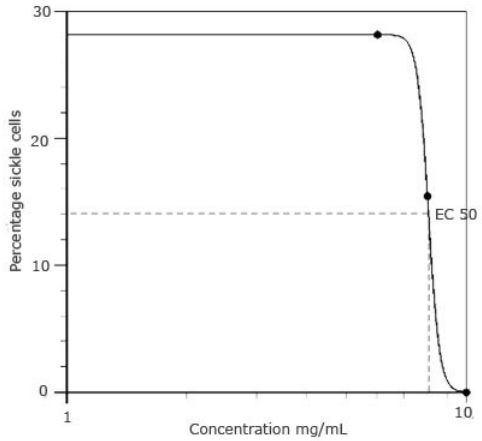

Fig. 2. Percentage of sickle cells against concentration at 120 min.

DISCUSSION

The screened *P. americana* leaf extract confirmed the presence of flavonoids which was expected. *Adeyemi O.O* and collaborators conducted a phytochemical screening of aqueous leaf extract of the plant and found a positive reaction to this phytochemical.[14] *Arukwe* and collaborators quantified the concentration of flavonoids in a *P. americana* leaf extract giving a high concentration of 8.11±0.14 mg/100 g which was higher than that one found in fruits and seed extracts being 4.25±0.16 mg/100 g and 1.90±0,07 mg/100 g respectively; they also found that in the plant leaf extract, flavonoids predominate among the others chemicals quantified.[10]

Several investigations on plant material have professed antisickling activity,[9,15-17] which include a research conducted by *Iwela* and collaborators confirming the antisickling property of the fruit extract of *P. americana*; the percentage of sickle cells being 5 in the alkaline extract, 84 in the alcoholic extract and 90 in the water extract and this was accounted to some of the active constituents such as essential oils, flavonoids and carotenoids.[9]

Dash BP and collaborators in their search for antisickling agents from plants, as well as *Ameh* and collaborators showed that among the most promising antisickling agents were the flavonoids.[1,18] The crude flavonoids isolated in this study produced a dose and time dependent antisickling effect on the RBC, no sickle-shaped cells were found with the highest concentration used at the longest time. Similar results were obtained in a research conducted by *Ramdé-Tiendrébéogo* and collaborators who demonstrated that the highest percentage of antisickling of *Ficus sycomorus* crude leaf extract was found at the highest concentration thus affirming the dose dependent effect.[16] *Ogunyemi* and collaborators confirmed that the antisickling effect of fermented *Carica papaya* fruit pulp were released greatest on the 5th day of exposure which was the longest time on their research which match with the results of our study.[19]

The similar trend of the positive control (PHBA) and the crude flavonoids at 10 mg/ml might suggest a similar mechanism of action. PHBA modifies the cell membrane stability while phenolic compounds like flavonoids form complexes with the cell walls and bind to adhesins.[20]

The association between red cell membrane deformation and changes in membrane permeability affecting Ca^{2+} and other ions has been documented for a number of physiological and pathological processes in the past, based mostly on experimentation with

RBC suspensions. Physiological shear stress in the circulation has been claimed to cause a reversible increase in Ca^{2+} permeability. Recent evidence supported the view that the increasing density of aging human RBCs, attributed to a progressive loss of KCl and osmotic water, results from the cumulative effects of declining Ca^{2+} extrusion capacity of the plasma membrane Ca^{2+} pump, aided by minor episodes of increased Ca $^{2+}$ permeability in the circulation. In sickle cell anemia, deoxygenation of red blood cells in the circulation reversibly increases their membrane permeability to Na^{+}, K^{+}, Ca $^{2+}$ and Mg^{2+}, and this increase has been attributed to the activation of P sickle, a poorly selective cation permeability pathway thought to be generated by the protruding deformation of the RBC membrane on contact with polymers of deoxy-hemoglobin S. The increase in [Ca^{2+}] resulting from P sickle activation in turn activates the Ca $^{2+}$-sensitive K^{+} channel of the red cell membrane, a critical stage in the mechanism of sickle cell dehydration.[21]

Complexation of flavonoids with the cell walls[(20)] can result into inhibition of the Ca^{2+} activated K^{+} channels which normalizes the deranged water transport across the mucosal cells resulting in rehydration of RBCs, cell swelling, decrease in HbSS concentration and decreased sickling.[21]

Furthermore, it has been studied the *in vitro* mechanism of dense cell formation caused by deoxy-oxy cycling and found that nutritional antioxidants could inhibit the formation of dense cells when they are employed in combination with vitamin C, vitamin E and aged garlic extracts.[19] It has been reported the antioxidant activities of flavonoids[8] which could also contribute to its observed antisickling properties.

Taking into account our findings it can be concluded that high concentrations of flavonoids may be responsible for the antisickling activity of the *P. americana* methanolic leaf extract and this activity increases with time.

Conflicto de intereses

Los autores del artículo científico "Antisickling effect of crude flavonoids in the methanolic leaf extract of *Persea Americana* Mill" que se publicará en la Revista Cubana de Plantas Medicinales hacemos constar que no existe conflicto de intereses en cuanto al contenido ni a la autoría del trabajo.

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Appendix 1: Consent form

Mbarara University of Science and Technology

Study title: Antisickling effect of crude flavonoids in the methanolic leaf extract of *Persea americana* Mill

This is a medical research study, and you do not have to take part. Mr. Lukyamuzi Edward John from the Department of Pharmacy will explain this study to you. If you have any questions, you may ask him.

You are being asked to take part in this study because you have and you possess the sickle cell gene.

In this study, the researchers are collecting a blood sample to learn more about the possibility of reversing the sickle cell nature of the cells with the crude flavonoids in the methanolic extract of *Persea americana* (Avocado) leaves.

1. What will happen if I take part in this study?

If you agree to be in this study, you will go to Mbarara Regional Referral Hospital (MRRH) and give a blood sample. The blood will be drawn by putting a needle into a vein in your arm. One small tube of blood will be taken. This will take about five minutes.

Your tests result for the sickle cell gene will need to be accessed from the medical records. Any use of herbal preparations and any medications will be required for review.

2. Are there risks?

The needle stick may hurt and there is a small risk of bruising.

3. Are there benefits?

There is no benefit to you. The blood will be used only for research but if you are interested on the preliminary results you will be informed.

4. **Can I say "No"?**

Yes, you do not have to donate a blood sample for this study. If you decide not to be in this study you will not lose any of your regular benefits, and you can still receive medical care from MRRH.

5. Will my medical information be kept confidential?

We will do our best to protect the information we collect from you. Information which identifies you will be kept secure and restricted. However, your personal information may be given out if required by law. If information from this research is published or presented at scientific meetings, your name and other identifiers will not be used. Information which identifies you will be destroyed when this research is complete.

6. Are there any costs or payments?

You will not be charged or paid for the blood sample.

7. What if I get injured?

Tell the laboratory scientist or the Principle Investigator if you feel that you have been injured because of being in this research. You can tell the doctor in person.

8. Who can answer my questions about the study?

You can talk to the study student about any questions, concerns, or complaints you have about this study. Contact the Principal Investigator, Edward Lukyamuzi John at 0705555726 / 0774555726.

If you wish to ask questions about the study or your rights as a research participant to someone other than the researchers or if you wish to voice any problems or concerns you may have about the study, please visit the office of the Head of Pharmacy Department, Faculty of Medicine at MUST.

CONSENT

You have been given copies of this consent form.

If you wish to be in this study, please sign below.

Date Participant's Signature for Consent

Date Person Obtaining Consent

Date Witness - Only required if the participant is a non-English speaker

Edward Lukyamuzi John. Mbarara University of Science and Technology, Uganda. Correo electrónico: eddy.lukyamuzi@gmail.com