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Research Article

Phyto-Toxicity and Biokinetics of Pumpkin Leaves Extract on Human Erythrocytes, *In Vitro* Study

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Abstract

Background of Study: Pumpkin leaves (*Telfairia occidentalis*) are extensively used in homes in Nigeria and some West African Countries for soup making and sometimes extracted in water for drinking as medicament for stimulating erythropoiesis.

Aim and Objectives: The present study aims at evaluating the *in-vitro* effect of pumpkin leaves extract on human erythrocytes with an interest in obtaining base data on the phyto-toxicity.

Methods: Pumpkin leaves weighing 250g was extract in 250 ml cold distilled water to obtain a stock solution of 1gm/ml pumpkin leaves extract. The stock solution was further diluted in sterile test tubes to obtain a concentration range of 10µg/m/, 20µg/m/, 40µg/m/ to 640µg/m/ using cold sterile distilled water. The test proper required the addition of 0.5ml of each pumpkin leaves extracted concentration to a sterile test tube containing 2.5ml of 5% human erythrocyte in sterile physiological saline and allowed to remain at room temperature (27 + 1oc)for 30 minutes. The heamolysis occurring in the tubes was read spectrophometrically (Cornings) at 540nm wavelength along with the neat and saline control.

Result: The absorbance reading reflected corresponding increases in the concentration of pumpkin leaves extract using phosphate buffer as negative control and Triton X as positive control (100% heamolysis) this showed a significant increase in toxicity in vitro as the concentration gradient is increased, the least concentration with stability of the erythrocyte *in vitro* as compared with the phosphate buffer is termed an equivalent for the determination of toxicity in erythrocyte *in vitro - in vivo* by other phyto products.

Conclusion: The data obtained from this study has value in determining phytotoxicity with pumpkin leaves and any other phyto products on human erythrocytes with all its determining variables in consideration.

Keywords: *In vitro*/*In vivo*; Hemolysis; Phyto Toxicity; Erythrocytes; *Telfairia occidentalis*; Spectrophotometrically; Biokinetics; Triton x

Introduction

Biomaterials are either derived from nature or synthesized using polymers, ceramics, metals, and composite materials. Specifically, biomaterials have been extensively applied to controlled release systems since 1976 that continually released macromolecules to inhibit angiogenesis [1]. There have been many studies on drug delivery, but *in vitro* toxicity assessments a carrier must be developed. Most reports have assessed cytotoxicity using only target cells or non-specifc cells from animals. This approach cannot represent the overall toxicity for humans because of the differences between many of the cells used in these studies and human cells [2].

Charles Telfair (1778-1835), an Irish botanist who lived in Mauritius, sent an African genus of the cucumber family (Cucurbitaceae) from Mauritius to Sir Williams Jackson Hooker (1785- 1865) for identification. In honor of Dr Telfair, the plant *Telfairia occidentalis* was named after him by Sir Hooker [2]. However, the earliest reference to Telfairia was made by Oliver in 1871 and it recorded its presence in Upper Guinea areas of Sierra Leone, Fernando Po, and Abeokuta (Nigeria) [3-5].

Telfairia occidentalis which belongs to the family Cucurbitaceae is one of the leafy vegetables widely consumed in Nigeria for its nutritional and therapeutic benefits. It is widely cultivated in the south eastern, south western and south southern parts of Nigeria and utilized in the preparation of soups and medicines. Dietary intake of *Telfairia occidentalis* could reduce garlic induced haemolysis in rats [3,4]. The aqueous extracts of *Telfairia occidentalis* has been found to reduce blood glucose levels and also have anti diabetic effects in glucose induced hyperglycemic and streptozotocin induced diabetic mice [5,6]. Also aqueous extracts of *Telfairia occidentalis* has been reported to assist in the purging of the gastro intestinal tract as revealed by the purgative effects of the aqueous extracts of *Telfairia occidentalis* leaf on isolated Guinea pig ileum [7-10].

The leaves are rich in vitamins and minerals such as Ca, P, Fe etc. The seed is also eaten as food. The oil obtained from the seed is used in cooking [4,5,10]. *Telfairia occidentalis* has been reported to possess antioxidant property. The aqueous extract had a high total phenol, reducing power and free radical scavenging ability (12.2%, 1.9 OD700 and 92%, respectively) than the ethanolic extract which had total phenol, reducing power and free radical scavenging ability of 5.5%, 1.5 OD700 and 25%, respectively The free soluble polyphenols content in the leaf of the plant which was higher than the bound polyphenols had higher antioxidant activity as typified by their higher reducing power and free radical scavenging ability than the bound polyphenols [6,7]. *Telfairia occidentalis* leaf contained a significantly high amount of vitamin C, total flavonoids and phenolics than Psidiumguajava stem bark. The leaf inhibited more free radicals than *Psidium guajava* stem bark [10,11]. The n-hexane fraction had the highest flavonoid content and free radical scavenging activity comparable to that of the commercial antioxidant BHT [12,13]. The ability of the leaf of *Telfairia occidentalis* to reduce iron (III) to iron (II) was also reported. The antioxidant property of *Telfairia occidentalis* is attributable to the high content of polyphenols, especially flavonoids. In the search for methods to evaluate the toxicological risk of chemicals without employing animal experimentation, much emphasis has been put on the replacement of acute toxicity (LD50) determinations. One important assumption was that acute toxicity is related to a compound's basal cytotoxicity [13]. The erythrocytes of humans and mammals represent a good model to evaluate the cytotoxicity of molecules, organic and inorganic, natural or synthetic, by cellular damage measure [14-16]. Indeed, before any investigation on the mechanism of action of different molecules, it is important to perform a cytotoxicity assay [7]. Among the different cytotoxicity assays that assess a possible toxicity in the red blood cells is the rate of haemolysis [7]. This essay is based on the evaluation of the alterations of red cell membranes in the presence of an eventual xenobiotic [17,18]. Red blood cells are the main cells in circulation, and they are responsible for transporting oxygen; in fact, any alterations of this process could be lethal. The present study aims at evaluating the *in-vitro* effect of pumpkin leaves extract on human erythrocytes with an interest in obtaining base data on the phytotoxicity [19-23].

Materials and Methods

Study area

This study was carried out between the month of March and September 2018 in the University of Benin, Health Services Department, Laboratory Unit, University of Benin and the Laboratory in the Department of Medical Laboratory Sciences, University of Benin.

Inclusion criteria

Red blood cell with the ABO blood group types from healthy patients to determine the *in vitro* phyto toxicity of pumpkin leaves.

Exclusion criteria

Blood samples from patients who are on any form of medication which could interfere with the toxicity studies.

Ethical approval

Permission shall be sought and obtained from the Ministry of Health, Benin City, Edo State.

Sample collection

Blood sample

Arterial/Venous blood is collected aseptically from the cubital fossa and dispensed into a EDTA coagulated specimen bottles. (5mls) was deposited.

Methodology

Phyto material extraction

The Leaves of the plant were collected, dried in the shade and powdered using mortar and pestle. The powdered processed leaves were stored in airtight containers and labeled properly. Each of the dried grounded material weighed 500g. Extraction of the phyto product was carried out using 2 Lof methanol and normal saline by cold maceration for 7days in large amber bottles with intermittent shaking. Filtration using the Whatman filter paper (No 42) was used to separate the artifacts and macro substance [13,14].

Collection of Blood and Preparation for analysis

Using a sterile five milliliter syringe, five milliliters of blood was collected by veni-puncture from the cubital fossa of healthy patients without gender discrimination. The blood was dispensed into sodium EDTA specimen bottles (green cap), it was mixed gently and thoroughly rolling the bottle. Centrifugation was carried out to separate plasma from the packed erythrocytes. The separated packed erythrocytes were washed 3 times with phosphate buffered normal saline and the supernatant decanted. The time of centrifugation was 5 mins at a speed of 626 (xg) [8,9]. The washed packed cells were used for the toxicity test by *in vitro* red cell hemolysis.

Haemolysis study

The properties of Pumpkin extract that provide compatibility of the formulation to the cells are the lipids having biocompatibility to the blood cells. Toxicity on the blood cells gives a primary idea of the effect of the pumpkin extract on the red blood cells of the body apart from giving an apparent idea on the compatibility with blood cells. RBCs may cause electrolyte loss as well as inducing immunological reactions inside the cell leading to RBC death which usually follows loss of hemoglobin from RBCs [15,16].

Thus hemolysis potential of the liposomes is necessitated to be evaluated. Hemolytic toxicity of pumpkin equivalent was checked by incubating the formulations with Red Blood Cells separated from Human blood by centrifugation at low speed and analyzing the samples for hemoglobin release at 541 nm. Hemolysis with different formulations were compared with that obtained with Triton -X100 as a positive.

Cell viability test

Hemolysis potentials of the pumpkin leave extract equivalent were added to the RBC concentrate and gently mixed. The concentrate was then incubated at 37°C for 30 min in incubator [17].

After incubation it is again at 3000 rpm for 5 min to separate the pellet. The supernatant was analyzed for absorbance at 540 nm in UV spectrophotometer against normal saline as blank.

Percentage of hemolysis was determined for different samples considering the absorbance value of sample treated with 0.5% Triton-X100 to represent 100% hemolysis and normal saline treated samples to serve as negative control. % relative hemolysis was determined by following expression [18-20].

$$\% \text{ Relative Hemolysis} = \frac{\% \ 100\text{-(Abs Sample- Abs Negative)}}{\text{(Abs Neg-Abs Posi)-100}}$$

Statistical analysis

Statistical analysis including descriptive statistics carried out using the Statistical Package (Graph Pad Prism). All values will be expressed as Mean ± S.E (Mean standard error of mean). The analysis of variance (ANOVA) used to determine significant difference in test and control groups ($p<0.05$) at confidence limit will be set at 95%.

Results

The Phytotoxic effect of pumpkin leaves equivalent was determined by carrying out a hemolytic assay on Human red blood cells. 5mls of the various ABO blood group was collected from 292 healthy human subjects and dispensed into the EDTA bottle this selection was void of gender bias. *Telfairia occidentalis* were extracted at varied concentration, 125 µg, 250 µg, 500 µg and 1000 µg using water as the solvent of extraction. Human red blood cells were exposed to the various concentration of the extract firstly to record the observable effects in other to determine the toxicity equivalent of pumpkin leave extract as a determinant for the equivalent of the other two leave extract (Bitter leave and Scent leave).

The Pumpkin equivalent was determined comparing the least and the highest dose concentration gradient (125,250,500 and 1000 µg), there is a significant difference due to dose and concentration gradient, with 250 µg showing the closest when compared with phosphate buffered saline (2.3 percent hemolysis) (Table 1 and 2).

The pumpkin equivalent was determined comparing the equivalent with Phosphate buffered saline (0%) hemolysis and Trition X (100%) hemolysis. The pumpkin equivalent closet to the phosphate buffered saline was at 250 µg with no significant difference across the ABO blood groups ($P>0.005$).

Dose	10^1	10^2	10^3	10^4	10^5	10^6	10^7	10^8	10^9	P. value
125 mg	0.3306± 0.001	0.3304± 0.002	0.3375± 0.002	0.3394± 0.002	0.3716± 0.004	0.4034± 0.002	0.4078± 0.002	0.4955± 0.002	0.5567± 0.009	0.0001
250 mg	0.3067± 0.003	0.3124± 0.002	0.3206± 0.004	0.3394± 0.003	0.3598± 0.015	0.3953± 0.005	0.4037± 0.002	0.4184± 0.002	0.4470± 0.008	0.0001
500 mg	0.4955± 0.002	0.4975± 0.001	0.5001± 0.000	0.5019± 0.001	0.6800± 0.006	0.7600± 0.005	0.8710± 0.008	1.033± 0.020	1.078± 0.0233	0.0001
1000 mg	0.8599± 0.005	0.8757± 0.006	0.9731± 0.001	1.033± 0.020	1.048± 0.021	1.078± 0.023	1.133± 0.003	1.773± 0.064	2.019± 0.038	0.0001

Table 1: Showing the Determination of lethal toxicity dosage of pumpkin extract on human red blood cells *in vitro* using a doubling dilution concentration versus the absorbance value.

Key: Mean ± SEM and Analysis of variance (ANOVA) where P≤0.005. P≤ 0.0001**** highly significant, *** moderately Significant, ** Significant, P≥0.005 no significance. Similar number of asterisk shows no difference in the exposed groups.

At varied Concentration the Lethal dosage of pumpkin leaves extract was determined with doubling dilutions of 10^1-10^9, using the heamolytic assay to determine cytotoxicity on human red blood cells. The absorbance values at 540 nm as measure of its toxicity.

At 125 mg, 250 mg, 500 mg and 1000 mg doses respectively, the concentration gradient from 10^1 to 10^9, the effect of pumpkin leave extract determine its equivalent (X). A steady increase in absorbance values as the concentration of the pumpkin leave extract continued to increase exponential.

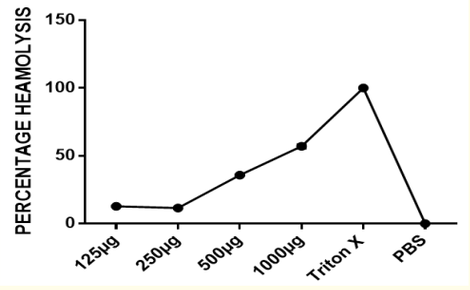

Figure 1: Comparative effect of dose response of Telfairia occidentalis (Pumpkin leaves) extract on the human red blood cells to determine its percentage heamolysis.

Discussion

The *in-vitro* effect of pumpkin leaves extract on human erythrocyte was determined comparing its haemolytic effects on human erythrocyte as a measure of its toxicity; this led to the discovery of an equivalent lethal toxic concentration [12,13]. *Telfairia occidentalis* leaves extract showed significant less toxic effect *in-vitro* at room temperature when compared with *Ocimium gratissimum* and *Vernonia amygdalina* ($P<0.005$) there was similarity when compared with works carried out on the stabilizing effect of pumkin leave extract on sickle cell erythrocyte [25,29].

From the observations of this study, *Telfairia occidentalis* extract showed a dose dependent increase in heamolytic activity, there was low to mild heamolytic effect towards human erythrocyte, the dose of 125 µg showed an absorbance value of 0.3969 (3.4%) on the erythrocyte which was close to the absorbance value of phosphate buffered saline erythrocyte, 0.3023 (0%) [12,13].

All of the concentration of *Telfairia occidentalis* leave extract showed minimal heamolytic effect on human erythrocyte, 3.4, 2.3, 15 and 32.9% respectively as compared with Triton X and PBS solution, this coincides with previous studies which shows the stabilizing effect of *Telfairia occidentalis* leave extract on human erythrocyte in vitro [14,15].

This study showed an elevation in the hemolytic effect on the human erythrocyte as the concentration increased which showed that at a higher concentration *Telfairia occidentalis* shows some level of toxicity on human erythrocyte conferring with the dose dependent toxicity this is synonymous with studies carried out by [18-22] which discovered a lethal concentration of *Telfairia occidentalis* at high concentration [23-29].

Conclusion

From the results obtained in this study *Telfairia occidentalis* has a potential as a tool for determining phyto-toxicity, this is due to its stabilizing effect on the erythrocyte at a lower concentration. The pumpkin leave equivalent will provide an alternative from using laboratory animals for toxicity studies but to replace them with erythrocyte *in vitro* as a measure *in vivo* for determine toxic concentration across a concentration gradient in any phyto product. However there is still need for further studies to evaluate its sensitivity, specificity and accuracy. This study has discovered a significant similarity with phtyotoxicity studies done *in vitro* comparing with *in vivo* using human erythrocyte as alternative instead of laboratory animals and bring to the forefront the use of cells as an alternative for laboratory animals in both one phase and multi phase toxicity studies considering sequestration points and active sites.

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Conflict of Interest

There is no conflict of interest.

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