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DETERMINATION OF PUMPKIN LEAVES EQUIVALENT; AS A MEASURE OF PYHTO TOXICITY ON HUMAN ERYTHROCYTES

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ABSTRACT

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. 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. Human erythrocytes of the A,B,O. blood groups and Rhesus positive and negative were obtained from blood donors in the University of Benin Teaching Hospital, Benin City. Pumpkin leaves weighing 250g was extract in 250ml 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$\pm$ 1^{o}c)for 30 minutes. The heamolysis occurring in the tubes was read spectrophometrically (Cornings) at 540nm wavelength along with the neat and saline control. A drop of erythrocyte suspension (0.02ml) from each tube was placed on a clean grease- free slide, covered with a coverslip and examined under the microscope (Olympus) using X10 and X40 objective lenses. Then photomicrographs to show cell structures were obtained using a motic camera with extension lense. The lowest concentration of pumpkin leaves extract to yield heamolysis was 125 µg/m/ irrespective of blood group and Rhesus status. 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). The data obtained from this study may have value in determining phytotoxicity with pumpkin leaves.

INTRODUCTION

Telfairia occidentalis is a vital staple vegetable big in Nigeria. Recent studies have shown that *Telfairia occidentalis* leaf is wealthy in mineral components (such as iron, potassium, sodium, phosphorus, metal and magnesium), antioxidants, vitamins (such as vitamin B, riboflavin, ascorbate), nicotinamide and phytochemicals (Longe *et. al*., 1983).

The amino acid profile of *T. occidentalis* seeds had conjointly been shown to be nutritious with the amino acid, aspartate, glycine, glutamine, histidine, lysine, methionine, tryptophan, cystine, leucine, arginine, serine, threonine, essential amino acid, valine, amino acid and essential amino acid (Tindall, 1968; Fasuyi, 2007).

Following a line of investigation, facts have been established on the biological significance of *Telfairia Occidentalis* seeds. The seeds are extremely healthy, moreover it helps loosen the alimentary. They are eaten up, either roasted or stewed. They are conjointly used as soup thickeners (Okoli and Mgbeog, 1983). The seed is extremely rich in oil, particularly unsaturated fatty acids. 61% of the oil (Okoli and Nyanayo, 1988;Odoemena and Onyeneke, 1998). Akubue *et al*., (1980) and Taylor *et al*., (1983) have documented that Fluted pumpkin seeds are a honest supply of 4 mineral components (Ca, K, Na, Zn) needed in human nutrition. The report showed that the seed contained 29 oil and half-hour supermolecule (Asiegbu, 1987). Fluted pumpkin seed contain 47 oil and 31 supermolecule. The supermolecule is aforesaid to be markedly deficient with the sulphur-containing aminoalkanoic acid.

Longe *et al*., (1983) has it that Fluted pumpkin seeds had 53% fat, 22% supermolecule, 3% fibre, 15% carbohydrates and 2% ash. Oyolu (1980) determined that vegetables can still stay the first supply of proteins, minerals and vitamins in African countries, he noted that leaves and edible shoots of Fluted pumpkin along contain 85% liquid content, whereas the dry portion of what's

sometimes consumed contains 11% Martinmas crude protein, 25% carbohydrate, 3% oil, 11% ash and the maximum amount of iron as 700 ppm.

Medicinal Plant Toxicity:

In geographic region, ancient medication is widely utilized in rural and concrete areas conjointly. this can be basically due to the preventive value of pharmaceutical-based medication and also the low incomes of a serious a part of the population additionally, the efficacies of the many of those ancient and plant-based medicines, however the actual fact remains that some plants utilized in ancient medication have cytotoxic effects (Craig, *et al*., 2001). It's during this perspective that we tend to investigate the literature on the toxicity of plants utilized in ancient medication. It's crucial to achieve data on these plant-based medicines prescribed by practitioners, significantly in terms of toxicity, composition, specific effectuality of malady and to advise practitioners of this practice of medicine on the protection and security of patients (Craig, *et al*., 2001).

The World Health Organization calculate that maybe 80% of the inhabitants of the planet believe mainly on ancient medicines. It, therefore, approved the employment of flavored merchandise for national policies and drug regulative measures so as to strengthen analysis and analysis of the protection and effectuality of flavored merchandise. The report has steered up to 119 plant derived drug listed by World Health Organization study, seventy four (74) were discovered as a results of chemical studies to isolate the active compounds accountable for the employment of original plant in ancient medication (Farnsworth *et al*., 1984).The employment of plants for

Acute toxicity:

Acute cytotoxicity is outlined because the toxic effects created by single exposure of medication by any route for a brief amount of your time (UWCSDG, 1999). Acute toxicity studies in animals

are required for any pharmaceutical meant for human use. The most objective of acute toxicity studies is to spot one dose inflicting major adverse effects or life threatening toxicity, which frequently involves associate degree estimation of the minimum dose inflicting morbidity. The studies are typically meted out in rodents and carries with it one dose. In pharmaceutical drug development, this can be the sole study kind wherever pathology or critical toxicity is associate degree end as documented in current regulative pointers (Poole et al., 1989). To measure the toxicity of a compound in animals, varied routes could also be used, however 2 most typically used modes of administration for animals studies are via intraperitoneal injection or the oral route (Poole *et al*., 1989).

Usually acute (single dose) toxicity study is meted out on laboratory animals by victimization high dose (sufficient to supply death or morbidity) of the substance in question and/or supported previous report on its toxicity or toxicity of structurally connected compounds (Demma *et al*., 2007). Acute toxicity studies are unremarkably wont to verify LD50 of drug or chemicals (Ozbek *et al*., 2004). The acute study provides a suggestion for choosing doses for the sub-acute and chronic low dose study, which can be clinically additional relevant (Janbaz *et al*., 2002).

Sub-acute toxicity:

In sub-acute toxicity studies, repeated doses of drug are given in sub-lethal quantity for a period of 14 to 21 days. Sub-acute toxicity studies are used to determine effect of drug on biochemical and hematological parameters of blood as well as to determine histopathological changes (Ozbek *et al*., 2004).

Chronic toxicity:

In chronic toxicity studies, drug is given in different doses for a period of 90 days to over a year to determine carcinogenic and mutagenic potential of drug (Ozbek *et al*., 2004). The parameters of chronic toxicity studies are same as that of sub-acute study. Multiple dose studies are necessary to assure the safety of natural products(Ozbek *et al*., 2004).

On the other hand clinical observations of acute assays are valuable tools to define the doses to be tested in multiple dose experiments, along with pharmacological studies in animals and in humans (Alvarez *et al*., 2004 and Hasumura *et al*., 2004).

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 thedried grounded material weighed 500g. Extraction of the phyto product was carried out using 2Lof methanol and normal salineby cold maceration for7days in large amber bottles with intermittent shaking. Filtration using the Whatman filter paper (No 42) was used to separate the artifacts and macro substance.

Sample size

The minimum sample size in this study is determined by (Daniel *et al*., 1999).

$$n = Z^2 pq / d^2$$

n=minimum sample size

z=is thevalue ofthe normal curve corresponding to 95% confidence interval = 1.96 p

=prevalenceof LD50 of Pumpkin extract is at=5%= 0.05and q =1-p i.e. 1–0.05=0.95

d =level of significance or error margin = 5%

$n = 1.96^2 \times 0.05 \times 0.95 / 0.05^2 = 72..9$

minimum sample number=73.

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) (Egunyomi *et al*., 2009).The washed packed cells were used for the toxicity test by in vitro red cell hemolysis

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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.

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 control (Oboh *et al*.,

2010)

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.

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.

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

Determination of Pumpkin Leaf Equivalent

Pumpkin Leave Equivalent (PLE) = U/P x100

U= (Hemolytic concentration of the unknown)

P=(Minimal hemolytic concentration of Pumpkin leaves)

PLE is in µg/ml and from the toxicological point of view useful values range from

> 1 = More toxic than *Telfaira occidentalis*

≤ 1= Less Toxic or as phyotoxic as *Telfaira occidentalis* (Ibeh *et al.,* 2019).

Statistical Analysis

Statistical analysis including descriptive statistics will be 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) will be used to determine significant difference in test and control groups ($p<0.05$) at confidence limit will be set at 95%.

RESULTS

The Pumpkin equivalent was determined comparing the least and the highest dose concentration gradient (125,250,500 and 1000 µg). The concentration gradient is compared with an equivalent hemolytic assay of Human red blood cells in Phosphate buffered saline was determined and termed the pumpkin equivalent (Table 4.1 and 4.2).

The absorbance values of the aqueous extract of pumpkin leaves, bitter leave and scent leave at 125µg concentration and the various ABO (O,A,B and AB) blood grouping system were (0.3953±0.005, 0.3963±0.007, 0.3722±0.010 and 0.3953±0.005).The absorbance values of Aqueous extract of bitter leave at 125 µg were (0.4818±0.003, 0.4808±0.017, 0.4983±0.011 and 0.4818±0.050). Comparatively the highest absorbance values at 125 µg concentrations were observed in the Scent leave extract with (0.4860±0.019, 0.5069±0.025, 0.5012±0.016 and 0.4860±0.019). There were observable significant difference ($p<0.005$) when comparing the effect of the various leave extract on the ABO blood groups (O,A,B and AB) with the pumpkin extract showing the least toxicity in vitro on the Human red blood cells (Table 4.3).

At 250µg concentration of the aqueous leaves extract on the ABO (O, A, B and AB) blood grouping system the absorbance values were (0.3424±0.008, 0.3504±0.010, 0.3478±0.007 and 0.3575±0.010).Aqueous extract of bitter leave at 250 µg were (0.8072±0.01, 0.800±0.012, 0.7576±0.01 and 0.8170±0.01). The highest absorbance values at 250 µg concentration were

observed in the Scent leave extract with (0.7202±0.020, 0.7281±0.020, 0.6972±0.016 and 0.7039±0.020). There were observable significant difference ($p<0.005$) when comparing the effect of the various leave extract on the ABO blood groups (O,A,B and AB) with the pumpkin extract showing the least toxicity in vitro on the Human red blood cells (Table 4.3).

At 500µg concentration of the aqueous leaves extract on the ABO (O,A,B and AB) blood grouping system the absorbance values were (1.078±0.02, 1.102±0.03, 1.081±0.02 and 1.087±0.02).Aqueous extract of bitter leave at 500 µg were (1.203±0.03, 1.222±0.03, 1.315±0.04 and 1.220±0.03). The highest absorbance values at 500 µg concentration were observed in the Scent leave extract with (1.160±0.02, 1.162±0.02, 1.116±0.02 and 1.162±0.02). There were observable significant difference ($p<0.005$) when comparing the effect of the various leave extract on the ABO blood groups (O,A,B and AB) with the pumpkin extract showing the least toxicity in vitro on the Human red blood cells (Table 4.4).

At 1000µg concentration of the aqueous leaves extract on the ABO (O,A,B and AB) blood grouping system the absorbance values were (1.662±0.01, 1.782±0.06, 1.677±0.01 and 1.783±0.03).Aqueous extract of bitter leave at 500 µg were (1.943±0.02, 2.519±0.22, 2.043±0.04 and 2.763±0.19). The highest absorbance values at 500 µg concentration were observed in the Scent leave extract with (1.881±0.01, 1.959±0.02, 1.925±0.01 and 1.933±0.02). There were observable significant difference ($p<0.005$) when comparing the effect of the various leave extract on the ABO blood groups (O,A,B and AB) with the pumpkin extract showing the least toxicity in vitro on the Human red blood cells (Table 4.5).

The percentage hemolysis of aqueous extract of scent leave equivalent at 125,250,500 and 1000 µg on the ABO blood groups (O,A,B and AB) were, 125 µg (6.7,7.4,7.3 and 6.7), 500 µg (15.3,

13.6, 14.5 and 14.7), 500 µg(38.4,38.4,38.6 and 38.4%) and 1000 µg (58,60,59.7 and 60.0) (Table 4.10, 4.11, 4.12 and 4.13).

The percentage hemolysis of aqueous extract of bitter leave equivalent at 125,250,500 and 1000 µg on the ABO blood groups (O,A,B and AB) were, 125 µg (6,6.5,7.2 and 6.2%), 250 µg (18.5,16.3.16.7 and 18.9), 500 µg(33.8,37.2,33.7, and 33.1) and 1000 µg (60,81.5,64.0 and 90.5%) (Table 4.14, 4.15, 4.16 and 4.17).

The mean±SEM comparative percentage hemolysis of the various extract of (Pumpkin, Scent and Bitter leave) at 125,250,500 and 1000 µg on Human red blood cells were 125 µg (12.85±0.184, 16.30±0.178 and 16.05±0.1500), 250 µg (11.55±0.104, 23.5±0.240 and 26.28±0.44), 500µg (35.8±0.09, 38.45±0.05 and 41.0±0.84) and 1000µg (57.0±1.15, 63.63±0.59 and 76.68±6.43). As the concentration gradient increased there is a significant difference in their various levels of hemolysis. At 250 µg of pumpkin extract the hemolysis is at 11.55% which is significantly lower than the other concentration gradient ($P<0.005$) and determined as the equivalent dose. (Table 4.18 and 4.19).

Table 4.18: Effect of Pumpkin leaves, Scent Leaves and Bitter Leaves extract on the percentage Heamolysis Human erythrocytes, comparing the control groups and the treated groups across the various concentration gradient

	TRITON X (n=5)	**PBS (n=5)**	**125mg (n=5)**	**250mg (n=5)**	**500mg (n=5)**	**1000mg (n=5)**	**Pvalue**
PUMPKIN LEAVES	100±0.00	0.0±0.00**	3.2±0.184***	1.7±0.104***	28.7±0.09****1	52.1.0±1.15****	0.0001
SCENT LEAVES	100±0.00	0.0±0.00*	7.02±0.178**	15.0±0.240***	36.7±0.05***	63.6±0.59****	0.0001
BITTER LEAVES	100±0.00	0.0±0.00*	16.05±0.150**	26.28±0.44***	41.0±0.84***	76.68±6.43****	0.0001

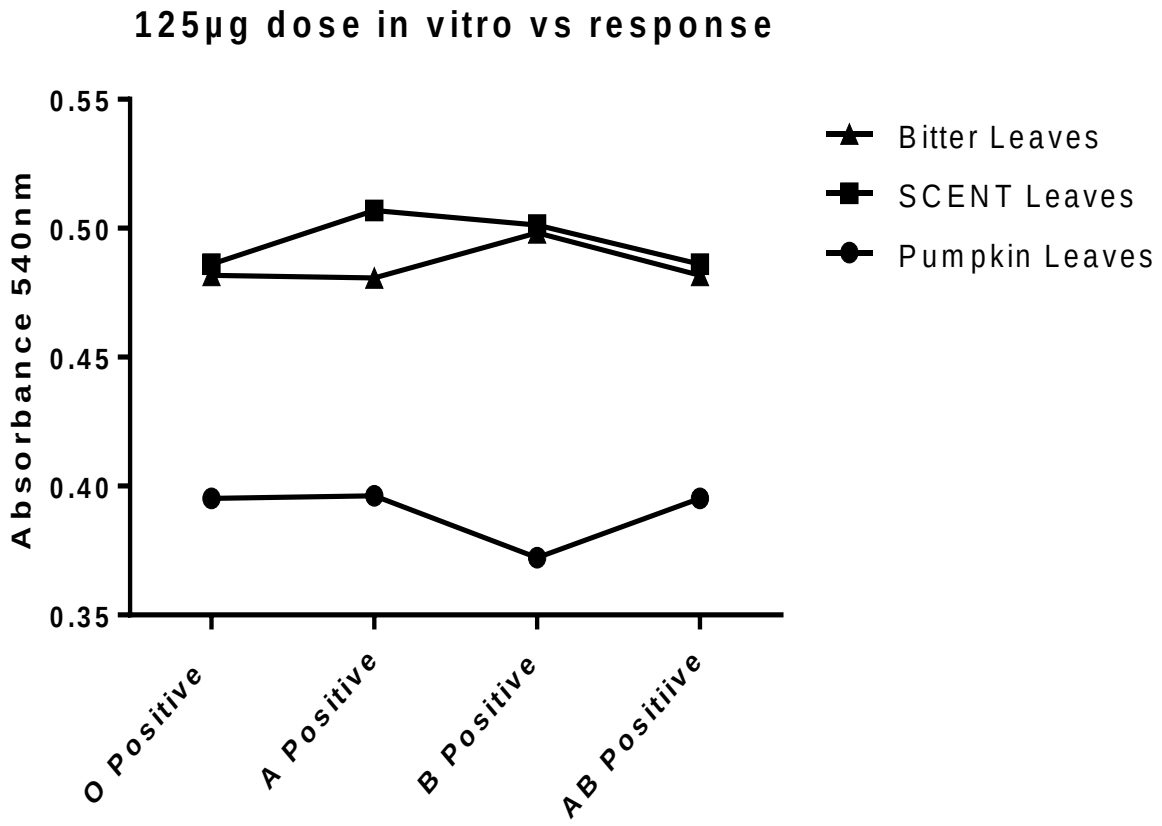

Figure 1.0. Comparative effect of 125mg dose of *Telfaira ocidentalis, Ocimum gratissimum,* and *Vernonia amygdalina* (Pumpkin, Scent and Bitter leaves) extract on the human ABO blood groups to determine its toxicity at absorbance values.

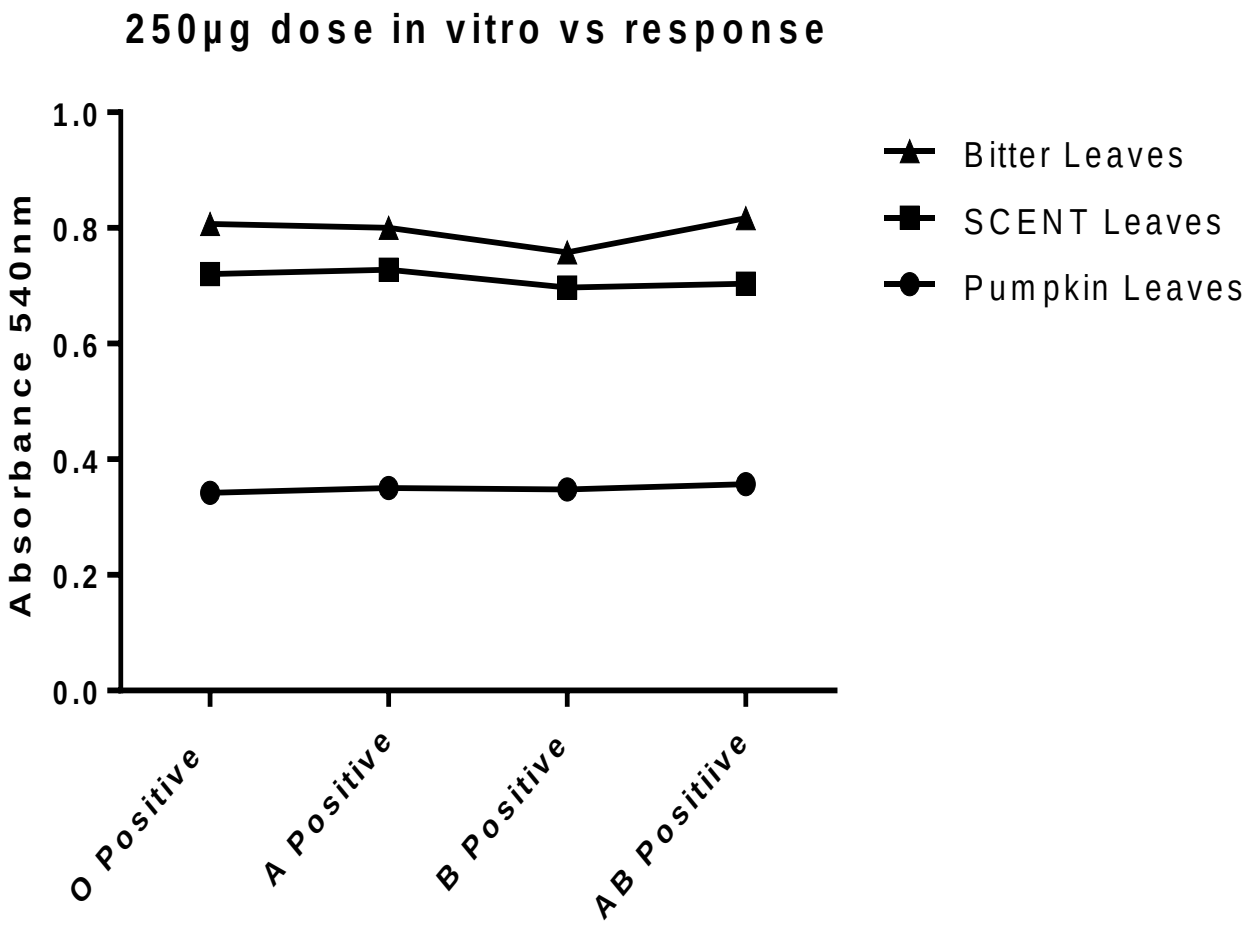

Figure 2.0. Comparative effect of 250µg dose of *Telfaira ocidentalis, Ocimum gratissimum*, and *Vernonia amygdalina* (Pumpkin, Scent and Bitter leaves) extract on the human ABO blood groups to determine its toxicity at absorbance values.

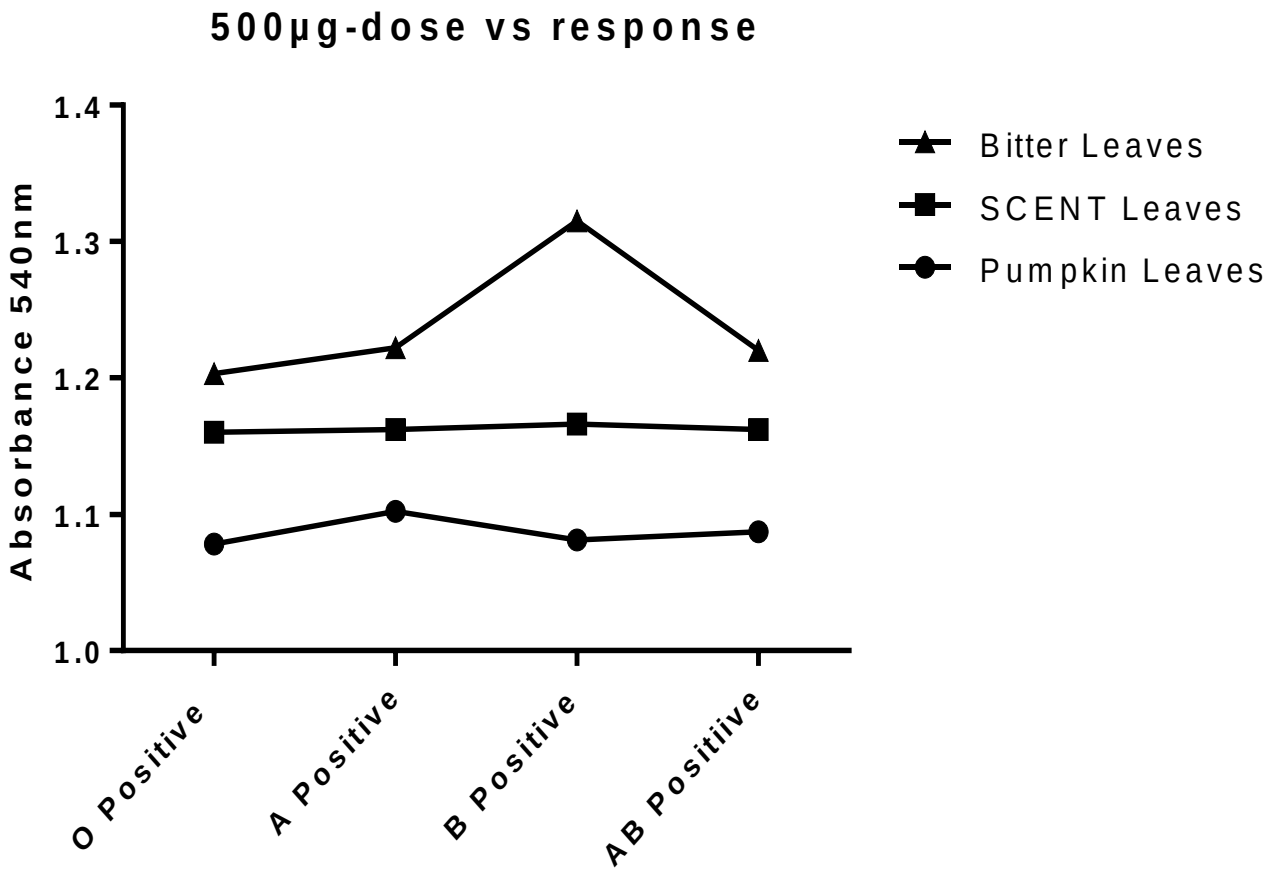

Figure 3.0. Comparative effect of 500mg dose of *Telfaira ocidentalis, Ocimum gratissimum,* and *Vernonia amygdalina* (Pumpkin, Scent and Bitter leaves) extract on the human ABO blood groups to determine its toxicity at absorbance values.

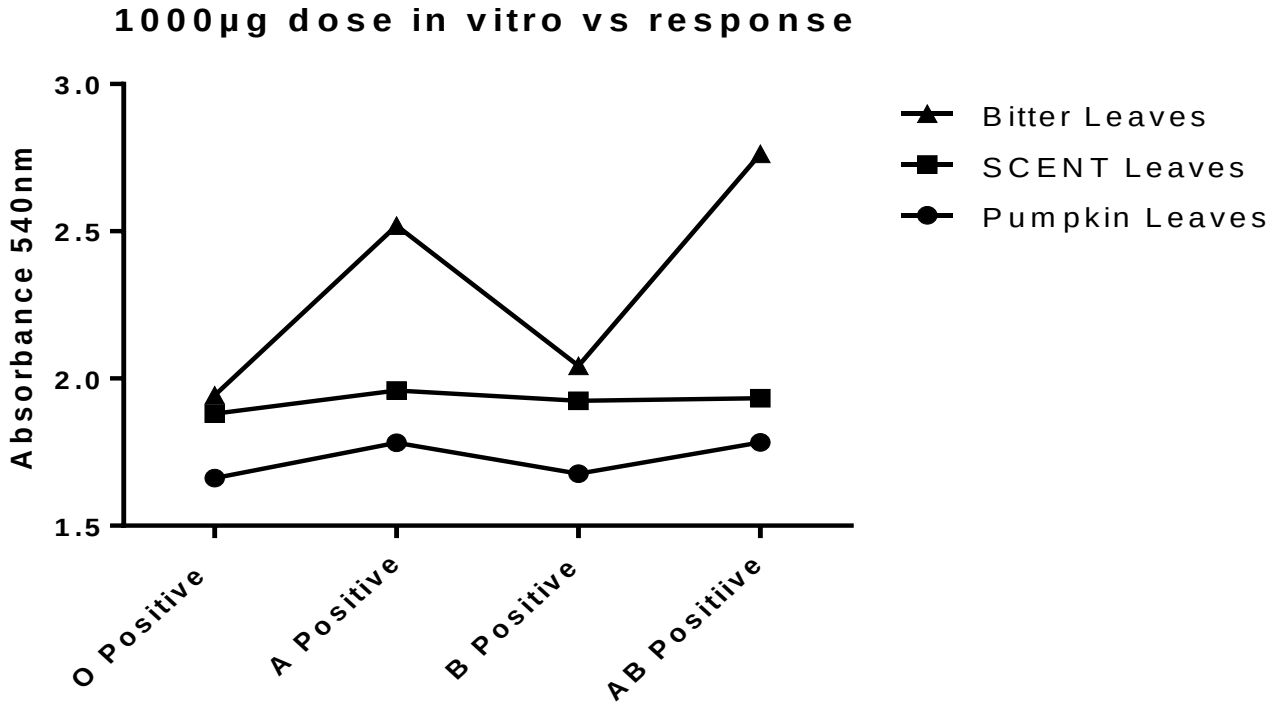

Figure 4.0. Comparative effect of 1000mg dose of *Telfaira ocidentalis, Ocimum gratissimum,* and *Vernonia amygdalina* (Pumpkin, Scent and Bitter leaves) extract on the human ABO blood groups to determine its toxicity at absorbance values.

DISCUSSION

This study showed an elevated the hemolytic effect on the human erythrocyte as the concentration increased which showed that at a higher concentration *Telfaira occidentalis* shows some level of toxicity on human erythrocyte conferring with the dose dependent toxicity this is synonymous with studies carried out by (Rathinam *et al.*, 2012) which discovered a lethal concentration of *Telfaira occidentalis* at high concentration.

The in vitro effect of *Telfaira occidentalis, Vernonia amygdalina* and *Occimium gratissimium* there was a significant difference on all the concentration gradient on human erythrocytes

comparatively ($p<0.05$). *Telfaira occidentalis* had a less toxic effect on human erythrocyte with the least percentage hemolysis of (3.4, 1.7, 28.7 and 52.1%) as compared with *Vernonia amygdalina* which showed an increased toxicity with percentage hemolysis of (16.05, 26.2, 41.0 and 76.6%) this is synonymous with studies carried out by (Kailesilvi *et al* ., 2013). *Occimium gratissimium* leave extract showed a reduced hemolysis as compared with *Vernonia amygdalina* but significantly higher than that of *Telfaira occidentalis* in vitro (7.02, 15.0, 36.7 and 63.6%) at 125, 250,500 and 1000µg concentration respectively.

The stabilizing effect of *Telfaira occidentalis* on human erythrocyte as observed in this study may be due to the action of the extracts (leaf, stem and seed) which is proposed to likely be due to prevention of two main erythrocyte sickling pathways; the Ca high K+ and Mg2+2+ activated K+ efflux and K-Cl co-transport Channels which promotes the rehydration of the cells and also exhibit synergy with the protective phyto-chemicals like the haematopoietic glycosides, saponins and the alkaloids present in the aqueous pumpkin leave extract (Mpiana *et al*., 2010).

The essence of using pumpkin as an equivalent for determining the phyotoxicity of substance on human erythrocyte stems from the stabilisng effect *Telfaira occidentalis* has on the human erythrocyte which prompt its usage thus the least concentration at 250µg is determined as the pumpkin equivalent. To determine the equivalent of other phyto-product using the equivalent is calculated using the least concentration which had the least hemolysis on *Telfaira occidentalis* divided by the least concentration of other phyto-extract. The equivalent greater than one shows that the toxicity is higher than *Telfaira occidentalis*, the equivalent less than one shows a toxicity lower than pumpkin equivalent but the when is it equals to one , it is equals to that of pumpkin (Ibeh *et al.,* 2019).

Pumpkin Leave Equivalent (PLE) = U/P x100

U= (Hemolytic concentration of the unknown)

P=(Minimal hemolytic concentration of Pumpkin leaves)

PLE is in µg/ml and from the toxicological point of view useful values range from

> 1 = More toxic than *Telfaira occidentalis*

≤ 1= Less Toxic or as phyotoxic as *Telfaira occidentalis* (Ibeh *et al.,* 2019).

CONCLUSION

From the results obtained in this study *Telfiara occidentalis* has a potential as a tool for determining phyto-toxicity. However there is still need for further studies to evaluate its sensitivity, specificity and accuracy.

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CONFLICT OF INTEREST

There were no conflict of interest.

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