Studies on the in-Vitro Haematoxicity of *Vernonia amygdalyna* Leaf Extract on Human Erythrocyte

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ABSTRACT

Ethnomedicinal information from some traditional medicine practitioners revealed that V. amygdalina constitutes the bulk of the polyherbal combinations used in herbal concoction. Most of these studies are carried out in-vivo using animal models; none has been done in-vitro using erythrocyte as a model. To investigate the in-vitro haematoxicity effects of Vernonia amygdalina leaf extracts on human red blood cells using the standard percentage haemolysis of phytotoxicity determinant. The percentage haemolysis test was carried out by challenging various concentrations of Vernonia amygdalina bitter leaf extract at 125µg, 250µg, 500µg and 1000µg suspended in human red blood cells at room temperature, then viewed microscopically using the X10, X20 and x40 objective lenses respectively to study the morphology of the red blood cells .The presence of haemolysis was noted, then further measurement was done using the spectrophotometer to determine the absorbance values at 540nm. The haematotoxicity effect of bitter leaves showed observable effect from the 125µg to 1000µg with maximum effect as represented by the graded haemolysis which increased with the increase in bitter leaves extract concentrations (P<0.05). These findings suggest the ability of the tested plant leaves to cause harm to human erythrocytes though they belong to the group generally regarded as safe materials (GRAS). The GRAS tag may be subject to the route of exposure and concentration gradient. This may explain why oral exposure of human beings to bitter leaves may not pose health risk. Also, this may be due to the differences between in-vivo/in-vitro reactions.

Keywords: Haematotoxicity, Vernonia amygdalina, Ethnomedicinal, In-vitro

INTRODUCTION

Ethnomedicine is an integral part of the culture, a good number of people rely on traditional medicine for health care delivery. Majority of the people still patronize herbal remedies despite the availability of orthodox medicine in their management of some diseases and ailments.[1-6] Besides, polyherbal therapy (practice) is a common practice in traditional medicine as combination of roots, leaves and stem barks of various plants are often used in the treatment of a single disease.[1-6]

Vernonia amygdalina has a variety of names in various languages it is popularly known as bitter leaf because of its characteristic bitter taste. In English, it is referred to as bitter leaf.[7-9] "Ewuro" in Yoruba, "Etidot" in Efik, Ijaw and Ibibio. The Igbos and the Etche people of Rivers State call it "Onugbo" or "Olubu". The bitter taste is due to anti-nutritional factors such as alkaloids, saponins, tannins and glycosides. Its nutritional, medicinal uses and scientific studies have respectively been articulated in two extensive reviews.[10]

Moreover, polyherbal therapy is said to be a current pharmacological principle having the advantage of producing maximum ef?cacy with minimum side effects.[11-12] Ethnomedicinal information from some traditional medicine practitioners revealed that *V. amygdalina* constitutes the bulk of the polyherbal combinations used in herbal concoction.[13] For toxicity studies, the liver is concerned with the biodegradation and regulation of a wide variety of biochemicals including the breakdown of complex molecules, many of which are central for vital functions.[13-14] Most of these studies are carried out in-vivo using animal models none has been done in-vitro using erythrocyte as a model, leaving the paucity of information on in-vitro response to these phyto substances.[13-14]

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Date manuscript was received: 12/3/2021
Date manuscript was accepted: 19/5/2021

In this study, we investigated the in-vitro hematotoxicity effects of *Vernonia amygdalina* leaf extracts on human red blood cells using the standard percentage haemolysis of phytotoxicity determinant.[11]

MATERIALS AND METHODS

Study Area

This study was carried out in the University of Benin, Health Services Department, Laboratory Unit, University of Benin and the Laboratory in the Department of Medical Laboratory Science, University of Benin, which is located within longitude 50 35'E and 50 41'E and latitude 60 26'E and 60 31'E in Ovia North East Local Government of Edo State.

Collection and Extraction of Plant

The Leaves of *Vernonia amygdalina* plant were collected and identified in the Department of Plant Biology and Biotechnology, Faculty of Sciences University of Benin and was dried in the shade and powdered using mortar and pestle. The powdered processed leaves were stored in airtight containers and labelled properly. Each of the dried grounded material weighed 500g. Extraction of the Phyto product was carried out using 2L of normal saline by cold maceration for 7 days in large amber bottles with intermittent shaking. Filtration using the Whatman filter paper (No 42) was used to separate the artifacts and macro substance.[11]

Specimen collection

Red blood cell with the ABO blood group types was from an apparently healthy individual to determine the in vitro haematotoxicity.

Sample Size Determination

The minimum sample size in this study was determined by.[11-12]

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

n=minimum sample size

z = is the value of the normal curve corresponding to 95% confidence interval = 1.96 p = prevalence of LD50 of Pumpkin extract is at=5% =.[13]
0.05and q =1-p i.e. 10.05=0.95
d =level of significance or error margin = 5%

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

Minimum sample number = 73.

Haemolysis Study

The properties of bitter leaves extract that provide compatibility of the formulation to the cells are the lipids having biocompatibility to the blood cells. The red cell suspension was prepared by washing the cells in phosphate buffered saline, centrifuging at 3000rpm, for 5 times, equal concentration 20µ of red cell was added to each concentration of bitter leave extract.[13]

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 of the compatibility with blood cells.[11-12]

Microscopy

The homogenized erythrocytes and bitter leaves extract was incubated at room temperature then viewed under the microscope using the x20, and x40 objective lens to observe for haemolysis and morphology of the red blood cells, micrographs were taken at the various concentrations.[11-12]

Percentage Haemolysis:

Haemolytic toxicity of bitter leaf was checked by incubating the formulations with Red Blood Cells separated from Human blood by centrifugation at low speed and analyzing the samples for haemoglobin release at 541nm. Haemolysis with different formulations was compared with that obtained with Triton -X100 as a positive control.[14]

Cell Viability Test

Haemolysis potentials of the bitter leaf 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 was centrifuged at 3000rpm for 5 min to separate the pellet. The supernatant was analyzed for absorbance at 540nm in UV spectrophotometer against normal saline as blank. Percentage of haemolysis was determined for different samples considering the absorbance value of sample treated with 0.5% Triton-X100 to represent 100 % haemolysis and normal saline treated samples to serve as negative control. % relative haemolysis was determined by the following expression.

$$\frac{\%100-(\text{Abs Sample - Abs Negative})}{(\text{Abs Neg-Abs Posi})-100.}$$ [11-12]

Statistical Analysis

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

RESULTS

The haematotoxicity effect of bitter leaves extract was determined by carrying out a haemolytic assay on Human red blood cells of the various blood groups. This showed observable effect from the 125µg to 1000µg with maximum effect as represented by the graded haemolysis which increased with the increase in bitter leaves extract as shown in Table 1. The absorbance of each concentration of bitter leaves extract is shown in Table 2. These findings suggest that bitter leaves produced harmful effects on human red blood cells In-vitro.

The result in Figure 1 shows the effect of different concentrations of *Vernonia amygdalina* (bitter leaf) on human red blood cells in-vitro at room temperature (27±1°C). There where observable haemolysis from the least concentration of the leaf extract. These findings suggest that *V.amygdalina* produced harmful effects on human red blood cells in-vitro.

The human red blood cells morphological changes found in *Vernonia amygdalina* leaves extract treated samples are shown in plates 1-2. There were Macrocytic (swollen) and lysed (ruptured) red blood cells which increased with an increase in the concentration of the leaf extract.

Table 1: Determination of lethal toxicity dosage of bitter leave extract on human red blood cells in vitro using concentration versus the absorbance value

Dose	10^{10}	10^{20}	10^{30}	P-value
125mg	0.4078±0.002	0.4955±0.002	0.5567±0.009	0.0001
250mg	0.4037±0.002	0.4184±0.002	0.4470±0.008	0.0001
500mg	0.8710±0.008	1.033±0.020	1.078±0.0233	0.0001
1000mg	1.133±0.003	1.773±0.064	2.019±0.038	0.0001

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. A similar number of asterisk shows no difference in the exposed groups.

Table 2: Susceptibility of the ABO blood groups to the various concentration of *Vernonia amygdalina*

BLOOD GROUPS	125µg/ml	250µg/ml	500µg/ml	1000µg/ml	P-value
BITTER LEAVES					
O	0.4818±0.003	0.8072±0.01	1.203±0.03	1.943±0.02	0.0002
A	0.4808±0.017	0.800±0.012	1.222±0.03	2.519±0.22	
B	0.4983±0.011	0.7576±0.01	1.315±0.04	2.043±0.04	
AB	0.4818±0.050	0.8170±0.01	1.220±0.03	2.763±0.19	

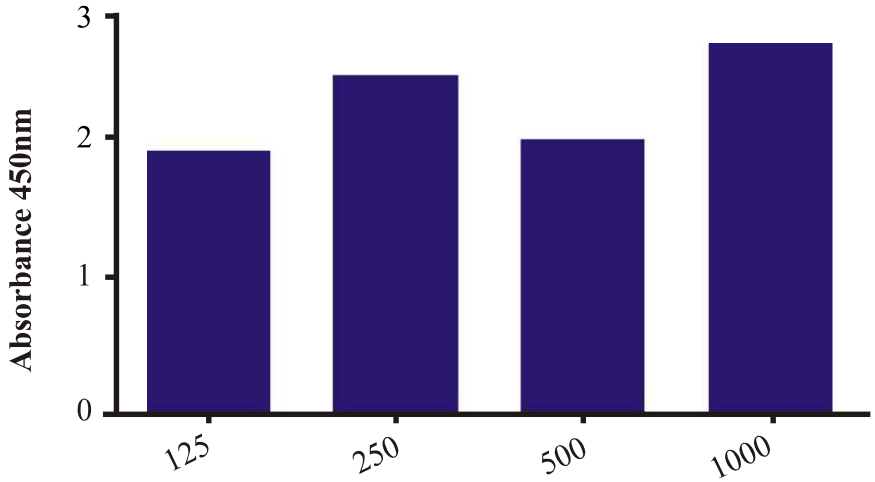

Figure 1: Comparative effect of 1000mg dose of *Vernonia amygdalina* (Pumpkin, Scent and Bitter leaves) extract on the human ABO blood groups to determine its toxicity at absorbance values.

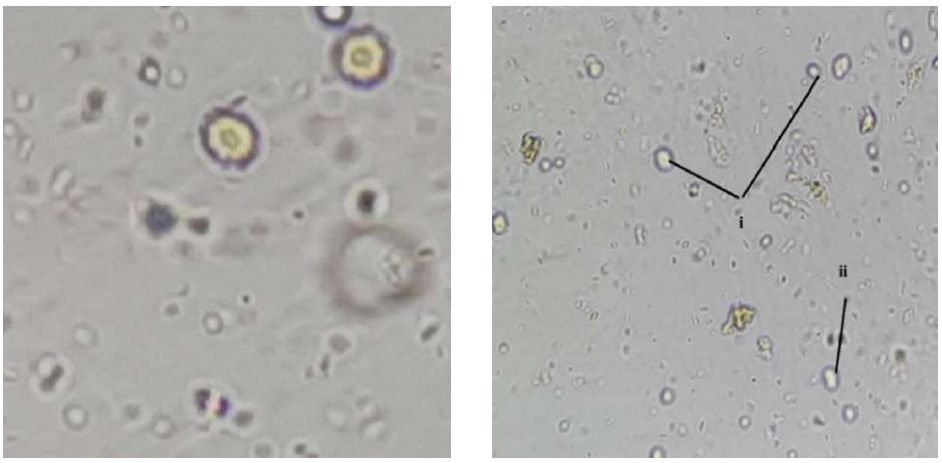

Plate 1: Phytotoxic effects of *Vernonia amygdalina* leaf extract on human red blood cells in-vitro, the red blood cells shrink and are lysed on the other slide, while the first slide looks swollen and crenated.

DISCUSSION

The effects of different concentrations of bitter leaves extract on human red blood cells were evaluated. The first marker was haemolysis of erythrocytes. It is well documented in literature that the erythrocytes test are highly sensitive to environmental factors like heat, pH, acidity, alkalinity and change in environmental tonicity.[6-7] In the present study, the observed haemolysis of

human erythrocytes exposed to extracts of bitter leaves may be due to the impact of the extract on the cell membrane which affects its integrity thereby causing an unregulated influx of fluid into the cells which led to cell swelling and dysregulation. [2] This view is in agreement with the physiological changes applicable to erythrocytes under severe conditions like change in the tonicity of the suspending environment.[2-4] this view is supported by the absence of cell lysis in the negative control cells suspended in phosphate-buffered saline (PBS). Erythrocyte lysis could also occur if the cells lose internal fluid to the exterior, thereby causing shrinkage of the cells.[3] However, cytological studies on the morphological changes of the cells under investigation may provide further information that may help in determining whether the human erythrocytes experienced plasmolysis or not.[15-16.]

The haemolysis experienced by human erythrocytes exposed to bitter leaves was concentration-dependent, increasing with an increase in leaf extract concentration. This outcome suggests that the observed effect on the erythrocytes was due to the bitter leaves and less so to other extraneous factors. This view is in agreement with substance toxicity expression.[4-10] The current study further buttress the haematotoxic properties of bitter leaf at all concentration, and as such caution must be taken when it is used as a herbal concoction. Some may argue about the health improvement due to their daily consumption in soup and delicacies, cooking may inactivate some of these properties for those who consume it in meals. Although most of the haematotoxic effects were carried out with crude extracts at various concentrations.

The absorbance of the human erythrocytes exposed to bitter leaf extracts changed with an increase in the leaf extract concentration. This may be due to the presence of haemoglobin which is known to absorb ultra-violet wavelength and can be colorimetrically measured.[9] Variations in the concentration of haemoglobin in its test units will produce variation in their absorbance. The release of such haemoglobin in this study is another marker for bitter leaves toxicity on human erythrocytes. Thus the open verbose of its use over the belief that herbal extract do not have any toxicological importance on humans and animals is false, as this study clearly observes the haematotoxic potentials of bitter leave. And also to buttress the alternative use of cells, like the erythrocyte for toxicity studies other than laboratory animals.[13-14]

CONCLUSION

These findings strongly suggest the ability of the tested plant leaves to cause harm to human erythrocytes though they belong to the group of generally regarded as safe materials (GRAS). The GRAS tag may be subject to the route of exposure and concentration gradient. This may explain why oral exposure of human beings to bitter leaf may not pose a health risk. Also, this may be due to the differences between in-vivo/in-vitro reactions. In the latter, there is less compartmentalization and more direct contact by reactant.

Acknowledgement

All thanks go to the National Academy for the Advancement of Science and staff and members of the Health Services department in the University of Benin for the support rendered throughout this study.

Conflict of Interest: There was no conflict of interest all through this study.

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