

Safety and Efficacy of *Prunus africana* and *Warburgia ugandensis* Against Induced Asthma in BALB/c Mice

L. W. Karani^{1*}, F. M. Tolo², S. M. Karanja¹ and C. W. Khayeka¹

¹College of Health Sciences, Jomo Kenyatta University of Agriculture and Technology (JKUAT), Nairobi, Kenya.

²Centre for Traditional Medicine and Drug Research, Kenya Medical Research Institute, Nairobi Kenya.

Authors' contributions

This work was carried out in collaboration between all authors. Authors LWK, FMT and SMK were the project leaders and responsible for experimental and project design. Authors CWK, LWK and FMT performed the experiments and data analysis. All the authors participated in the writing of the manuscript. All authors read and approved the final manuscript.

Research Article

Received 11th March 2013
Accepted 17th April 2013
Published 27th April 2013

ABSTRACT

Aim: *In-Vitro* and *In-Vivo* safety and anti-asthmatic activity of stem bark extracts of *Prunus africana* and *Warburgia ugandensis* against induced asthma in BALB/c mice.

Methodology: Cytotoxicity on Vero E6 cells were investigated using MTT assay. Acute toxicity was determined by administering single oral gavages of extracts to five groups of BALB/c at 500, 889.56, 1581.64, 2812.15 and 5000mg/kg body weight doses. Efficacy against induced asthma was determined by assaying heart blood serum for ovalbumin specific immunoglobulin E (IgE) antibodies and quantification of eosinophil proportion in Bronchoalveolar lavage fluid (BALF). Eight sensitized groups were used, 2 were controls, 3 were treated with *P. africana* extract and 3 with *W. ugandensis*; each treatment group received one dose concentration of 125, 250 or 500mg/kg body weight of either plant extracts.

Results: *P. africana* CC₅₀ was 104.08µg/ml while *W. ugandensis* had CC₅₀ > 250 µg/ml. In acute toxicity, mortality and signs of toxicity were recorded within 24 hours and the mice monitored for 14 days. There was 20%, 60% and 100% mortality within 24 hours for mice that received *P. africana* extracts at 1581.64, 2812.15 and 5000mg/kg body weight

*Corresponding author: Email: lucynyauma@gmail.com;

respectively. Lethal dose (LD₅₀) for *P. africana* was 2201.207mg/kg body weight. *W. ugandensis* extracts had no mortality recorded and the LD₅₀ was >5000mg/kg body weight. Treatment with *P. africana* extracts at 500mg/kg body weight reduced the IgE and BALF Eosinophil to 0.100±0.0001 and 2.80±0.20 respectively which were significantly different from positive controls $P<0.05$. *W. ugandensis* extracts at the same concentration reduced the IgE and BALF eosinophils to 0.134±0.00016 and 3.80±0.20 respectively and were significantly different from positive controls $P<0.05$.

Conclusion: The results attested that *P. africana* and *W. ugandensis* stem bark extracts have anti-asthmatic property though there is need for further validation of anti-asthmatic chemical compounds to augment the findings.

Keywords: *Prunus africana*; *Warburgia ugandensis*; cytotoxicity; vero cells; acute toxicity; lethal dose and efficacy.

1. INTRODUCTION

More than 60% of the world's population and over 80% of the people in developing countries depend upon traditional medicine for their primary health care [1,2]. Millions of people in the rural communities rely on Medicinal plants. Knowledge of such plants and their roles in treatment of diseases is as old as man. There is individually held knowledge by the traditional health practitioners (THP) who are revered and trusted people in the community. The knowledge on the use of medicinal plants was bequeathed to them by their fathers orally from generation to generation. Self-medication is also a common practice in most rural communities. Herbal medicine is thought to be more effective and having fewer side effects compared to synthetic medicines. Some herbal remedies have had adverse effects and sometimes life-threatening conditions which have been recorded among some ethnic communities [3,4]. Few scientist studies have been undertaken to ascertain the safety of traditional remedies prior to putting them to clinical use despite several scientific reports showing that many plants used as food and traditional medicine are potentially toxic, mutagenic and carcinogenic [5], it is therefore necessary to ascertain both efficacy and toxicity profile of medicinal plants.

World health organization (WHO) is fully aware of the significance of herbal medicines to many of its member states and supports the use of medicinal plants and their products [6,7,8]. Nonetheless, the organization has raised great concern on; identification, preparation, methods for proving the safety and efficacy, utilization, regulation and conservation of medicinal plants to prevent extinction. In its policy, WHO emphasizes on necessity to make a systematic inventory and assessment (pre-clinical and clinical) of medicinal plants; to introduce measures on the regulation of herbal medicines to ensure quality control of herbal products by using modern techniques, applying suitable standards and good manufacturing practices; and to include herbal medicines in the national standard [6,9]. WHO also endorses the call for international cooperation and coordination to establish programmes for the conservation of medicinal plants to ensure that adequate quantities are available for future generations [6].

Asthma illness involves the respiratory system in which the airway occasionally constricts, becomes inflamed, and is lined with excessive amounts of mucus. This results in chronic recurrent airflow obstruction causing intermittent wheezing, breathlessness, chest tightness and cough, sometimes with sputum production [10,11]. There are two types of bronchial asthma; extrinsic asthma (atopic) and intrinsic (non-atopic) asthma. Extrinsic asthma triggers

are mainly external such as allergens which include pollen, cigarette smoke, cockroaches, dust mites, mold, pets and animals while intrinsic asthma is from internal factors emanating from exercise, stress, respiratory infection, air pollution, chemical exposures and many more [10,11]. Individuals suffering from asthma tend to react strongly to certain substances (allergens) that are inhaled [12]. In this disease, the airway wall is infiltrated with mononuclear cells, which are mostly CD4 T cells and with eosinophils [13,14]. Structural changes in the airway wall collectively referred to as airway remodeling may be as a result of either the interaction of inflammatory mediators with stromal cells or tissue injury [10,13,15]. Airway remodeling and inflammation result in airway hyperresponsiveness (AHR) and airway obstruction which cause breathlessness and wheezing in asthmatic individuals [15]. AHR is defined as an increased broncho-constrictor response to a nonspecific stimulus and is sometimes referred to as “twitchy” airways [10,15]. Elevated levels of IgE in bronchoalveolar lavage fluid and in serum have also been noted in asthmatic patients [10,16]. Corticosteroids are the main anti-inflammatory medications [17,18]. These drugs are slow acting and have detrimental effects on the gastric mucosa [19], therefore more alternative therapies for asthma management need to be developed.

Prunus africana (Hook.F) is commonly known as Pygeum or African cherry and it is in the Family Rosaceae [20,21]. It is widely distributed in several provinces of Kenya and especially in Mount Kenya forest. An extract prepared from the bark of *Prunus africana*, is used as an alternative medicine in patients with asthma without screening for its safety and efficacy. Available literature with regard to *P. africana* indicates that phytochemical studies have led to isolation of triterpenic acids including derivatives of ursolic and oleanolic acids [21]. Since then, a growing interest in the use of the bark extracts to treat benign prostatic hyperplasia (BPH) has prompted numerous studies of the stem bark secondary chemistry [20,22]. *Warburgia ugandensis* (Sprague) is commonly known as East African green wood/greenheart/pepper bark tree and it is in the family Canellaceae [23]. The tree is common in East African forests. *W. ugandensis* is a species of evergreen tree native to Africa. The stem and roots of *W. ugandensis* are used as expectorant, for common cold and against fever, malaria, stomachache, constipation and diarrhoea though there is limited information on phytochemistry [23]. People customarily use the plants-derived preparations and consider them to be effective in management of asthma without any scientific base to explain the action of such plants. *P. africana* and *W. ugandensis* are such common plants whose barks have a reputation of being effective in management of asthma in Kenya. In view of the many health benefits of the stem barks of the two plants, there was apparent need to carry out an assessment of their anti-asthmatic activity and also conduct comprehensive study on their toxicity to highlight any hidden toxic activity. This study was therefore designed to investigate the *In-vitro* effect of the stem bark extracts on the Vero E6 cells, acute toxicity to BALB/c mice and their efficacy against asthma induced in BALB/c mice.

2. MATERIALS AND METHODS

2.1 Experimental Animals

Eight week old healthy female BALB/c mice with a mean body weight of 20 ± 2 g bred at KEMRI, Nairobi, Kenya were used for the study. They were fed with pellets (Mice pellets UNGA® feeds) and water *ad libitum*. Guidelines on care and handling of the animals were adhered to as stipulated by the Animal Care and Use Committee-KEMRI (ACUC-KEMRI, Kenya).

2.2 Experimental Procedures

2.2.1 Collection of the medicinal plants

Five kilograms of the medicinal plant parts were collected from Meru forest Kenya with the assistance of a plant taxonomist and their voucher specimens were deposited at the East African Herbarium, National Museums of Kenya, Nairobi, Kenya (*Prunus africana* Tfm10 and *Warburgia ugandensis* Tfm11).

2.2.2 Initial processing of the medicinal plant parts

Clean barks were air-dried at room temperature under shade for 14 days and pulverized using a laboratory mill (Christy & Norris Ltd., Chelmsford, England) and packed in air tight polythene Bags. One hundred and fifty grams of the powdered plant material was extracted with 1500 ml of distilled water in a water bath at 60°C for 1 hour. The extracts were then filtered and freeze dried using a Freeze Dryer (Edwards freeze dryer Modulyo) then weighed and stored.

2.2.3 Preparation of plant extracts for *in vitro* bioassays

The water extracts were dissolved in distilled water so that the final highest concentration in the microtitre plates was 250µg/ml. For these experiments 250 mg of the water extract was dissolved in 1 ml DMSO to form a stock solution of 10000µg/ml in 100% DMSO. This was diluted in maintenance media in a ratio of 1:100 which is 10 µl of extract in 990 of media to give a start concentration of 250µg/ml in 1% DMSO.

2.2.4 Cytotoxicity assay of the extracts in cell culture

In vitro cytotoxicity assay was carried out following a modified rapid calorimetric assay as previously described by Mosmann [24], using actively dividing sub-confluent Vero E6 cells. The Vero cells were maintained in Eagle's Minimum Essential Medium (MEM) containing 10% fetal bovine serum (FBS), in T-75 cell culture flasks incubated at 37°C in 5% CO₂. Upon attainment of confluence, cells were detached by trypsinization and pooled in a 50ml tube. The cells were then re-suspended in 40ml fresh MEM. One hundred microliters of Cell suspension of 2×10^5 were seeded in 96- well microtiter plates in columns 1, 2, 4, 5, 7, 8, 10 and 11 in wells of row A-H and incubated at 37°C / 5% CO₂ for 12 hours. Cells without drugs (row A) served as negative controls while wells with no cells (columns 3, 6, 9 and 12) served as blanks. The cells were incubated overnight (24 hours period) to allow cells to attach. After 24 hrs, MEM was removed from row H and care was taken not to leave cells without media for too long. Addition of 150µL of 250 µg/1ml DMSO of the highest drug concentration in triplicate (4 drugs per plate) was done in row H. Serial dilutions using a multi-channel pipette by removing 50 µl from wells of row H and adding to wells of row G was carried out. After mixing, another 50µl was transferred from row G to wells of row F and mixed. This was continued up to row B discarding the last 50µl of this row; leaving out row A (a threefold dilution is achieved). Row wells were exempted since they served as controls (wells without drugs). Thus row H wells had a concentration of 250µg/ml, G wells 83.3 µg/ml, F wells 27.77µg/ml, E wells 9.26 µg/ml, D wells 3.08 µg/ml, C wells 1.03 µg/ml and B wells 0.342 µg/ml wells. Consequently row H had 100% drug concentration while those of B had only 0.137% of the test sample. The plates were incubated for 48 hours at 37°C, 5% CO₂ to allow drug uptake by the growing cells. Addition of 10µL of MTT dye to each well was done after ensuring normal growth of the cells in the plate under an inverted microscope. Plates were

incubated for another 4 hours at 37°C. MEM was removed from the wells and 100 µl of DMSO added to dissolve the formazin. The plates were read on a scanning multiwell spectrophotometer (Multiskan Ex labssystems) at 562 nm and 620 nm as reference. Chloroquine was used as a positive control.

2.2.5 In vivo assay for the determination of safety (acute toxicity)

Mice were deprived of feed 12 hours before and 3 hours after administration of the plant extract. A total of 55 mice were used in the safety tests and these were divided into 11 groups of 5 mice each. One group received water by oral administration and therefore served as a control. Five groups for each extract received only one dose concentrations of 500, 889.56, 1581.6, 2812.15 or 5000 mg/kg once. Dose levels administered were calculated as previously described [25]. Animals were observed individually after dosing at least once within the first 30 minutes, then periodically during the first 24 hours and twice daily thereafter for a total of 14 days. The parameters of interest were; changes in skin and fur, eyes and mucous membranes, rate of breathing, tremors, convulsions, salivation, diarrhoea, lethargy and coma [26]. The log dose that responded to probit 5 (50% deaths) was calculated from the graph equation and its anti-log gave the LD₅₀ [27]. The mice were weighed on a daily basis and their weights recorded.

2.2.6 BALB/c asthmatic mouse model and treatment protocol

One week before sensitization the eight weeks old female BALB/c mice were allocated 9 treatment groups. Eight groups were sensitized with Ovalbumin and one non-sensitized group served as negative control. Three of the sensitized groups were treated with extracts of *P. africana*, 3 groups with *W. ugandensis*. Each group received one dose concentration of 125, 250 or 500 mg/kg body weight; 1 group was treated with reference drug and 1 group received no drug treatment serving as a positive control. The treatment took four days. The treatment groups are shown in Table 2.1.

Table 2.1. Treatment groups

Sensitized groups							Negative control	
Treated with <i>P. Africana</i> mg/kg			Treated with <i>W. ugandensis</i> mg/kg			Reference drug	+ve control	-
125	250	500	125	250	500		-	-
GP1	GP2	GP3	GP4	GP5	GP6	GP7	GP8	GP9

On day 0, 7 and day 14 mice were sensitized with 20 µg/mouse OVA (grade VI; Sigma, Steinheim, Germany) emulsified in 2mg Aluminum hydroxide (Alum) (Pierce, Rockford, IL, USA) in 200µl phosphate-buffered saline (PBS) [28,29] by intraperitoneal (IP) injection. On days 21 and 22 exposures to nebulizer 1% OVA in PBS (in a 5.9 L Pyrex glass box) for 30 minutes each day was given [28, 29]. Treatment with extracts, standard drug and placebo was done on day 22, 23, 24 and 25 days. The challenge and treatment protocols were as shown in Figure 2.1.

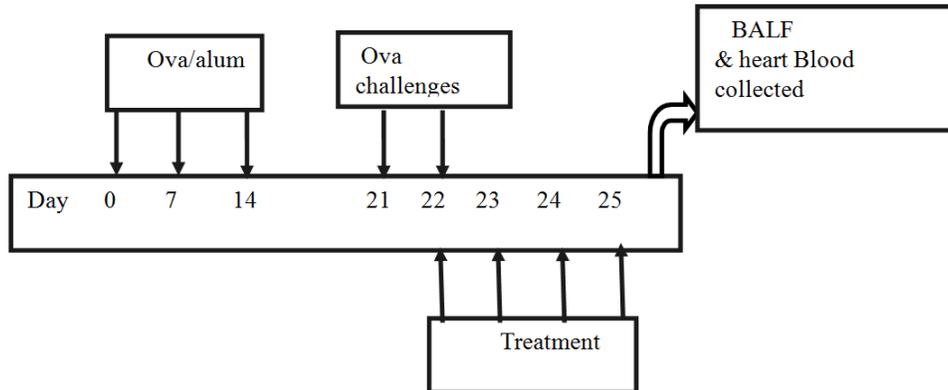


Figure 2.1. Sensitization, challenge and treatment protocol

2.2.7 Collection of blood and preparation of serum

Collection of heart blood for serum was done 24 hours after the last day of treatment. The blood obtained from cardiac puncture from every mouse was allowed to stand for 3 hours at room temperature to form a clot in a microfuge. The blood was then centrifuged in a Microfuge (Sorvall RT 600D, made in Japan) at 7000rpm for 20min. The clear supernatant (serum) was transferred to a sterile Nunc tube. The serum was stored at -20°C until use in IgE ELISA.

2.2.8 Measurement of ovalbumin-specific Immunoglobulin (IgE) by sandwich ELISA

Anti-OVA specific antibodies were measured by enzyme-linked immunosorbent assay (ELISA) as previously described [30]. Briefly, Maxisorp 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated overnight with 5 µg/mL of OVA in a 0.1M bicarbonate buffer (pH 9.6) at 4°C. After washing three times with PBST (0.05%Tween 20 in PBS) the nonspecific binding was blocked with 10% Foetal bovine serum for 2 hour at 37°C. After the washes a100 µl plasma (diluted 1:3 in blocking buffer) was added to the wells and kept at 37 °C for 2 hours. The wells after this were washed three times with PBST and 50 µl of horseradish peroxidase labeled goat anti-mouse IgE antibody (1:1000 dilution, Pharmingen, USA) was added and kept at 37°C for 30 minute after which washing was done four times with PBS. The color reaction was developed by adding 100µl/well of OPD (Sigma) in pH 4.5 citrate phosphate buffer plus 30% H₂O₂ for 10 min and stopped with 50 µl of 2 M sulfuric acid per well. The plates were read using an ELISA reader (Spectramax 190, Molecular Devices, USA) at 450 nm and results given in Optical Density (OD₄₅₀ nm).

2.2.9 Determination of the eosinophil levels in BALF

Twenty four hours after the last drug treatment, mice trachea was cannulated and the lungs lavaged three times with 0.3 ml of PBS into the trachea and aspirated with a syringe. BALF samples were then centrifuged for two minutes at 1000rpm. After removing the supernatant, the cells were re-suspended in PBS and then BAL fluid smears were stained with May-Grünwald Giemsa stain. Two investigators counted 100 inflammatory cells under a Nikon optiphot light microscope at a 100x magnification in the microscopic field.

2.3 Data Management and Statistical Analysis

Probit-log analysis was used for LD₅₀ determination. Means on the same scale were compared using student T-test. All values were expressed as mean ± standard error of mean and differences between the parameters of estimate were deemed statistically significant at $P < 0.05$.

3. RESULTS

3.1 Yields of Water Extracts

The percentage yield of the water extract in *Prunus africana* and *Warburgia ugandensis* are tabulated in Table 3.1.

Table 3.1. Plant species and percentage yields of water extracts

Botanical name	Dried material used (g)	Freeze dried product (g)	(%) extract yield
<i>Prunus africana</i>	150	13.452	8.968
<i>Warburgia ugandensis</i>	150	19.481	12.987

3.2 Cytotoxicity Assay

P. africana stem bark extracts had CC₅₀ of 104.08 µg/ml while stem bark extracts of *W. ugandensis* at the highest concentration tested did not kill 50% of the cells and therefore the CC₅₀ > 250 µg/ml.

3.3 Acute Toxicity

3.3.1 Acute toxicity in *Prunus africana*

The results of mortality at each dose level are shown in Table 3.2. There was no mortality observed within 24 hours for mice that received *P. africana* extracts at 500 and 889.56 mg/kg, while there was 20% mortality at 1581.64 mg/kg, 60% at 2812.15 mg/kg and 100% mortality at 5000 mg/kg body weight. Other signs of toxicity observed in mice treated with *P. africana* include hypo-activity, pilo-erection, low appetite and hyperventilation. LD₅₀ of *P. africana* was 2201.207 mg/kg body weight from probit analysis. The mice surviving after 24 hrs survived for the entire 14 days of observation and their weights were recorded and plotted (Figure 3.1). The weights of mice that received 500, 889.56 and 1581.64 mg/kg continued to increase and had no significant difference with that of controls ($P < 0.05$). Mice that received 2812.15 mg/kg body weight had an initial decrease in weight however continued to increase in weight in the consecutive days of the study and had significant difference in weight from that of control mice ($P > 0.05$).

Table 3.2. Acute toxicity of *P. africana* extracts administered to BALB/c mice orally at doses 500-5000mg/kg body weight

Concentration of drug mg/kg	Mortality	Total	% mortality	Log concentration	Probit
500	0	5	0	0	
889.56	0	5	0	2.949175	
1581.64	1	5	20	3.199108	4.16
2812.15	3	5	60	3.449038	5.25
5000	5	5	100	3.698970	5.25

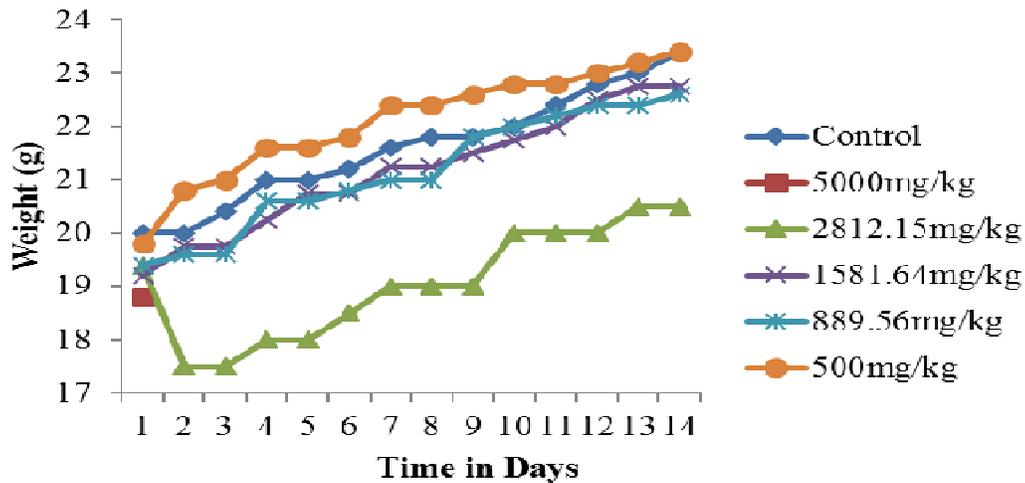


Figure 3.1. Weight of mice taken for fourteen days after oral administration with different doses of *P. Africana*

3.3.2 Acute toxicity in *Warburgia ugandensis*

There was no mortality observed within 48 hours in mice that received *W. ugandensis* extracts in all dose levels. However, the mice had physical signs such as crowding together in the cage. *W. ugandensis* had an LD₅₀ >5000 mg/kg body weight. There was increase in weight in all groups of mice treated with *W. ugandensis* in all dose levels (Figure 3.2) and their weights were not significantly different from that of control mice ($P < 0.05$).

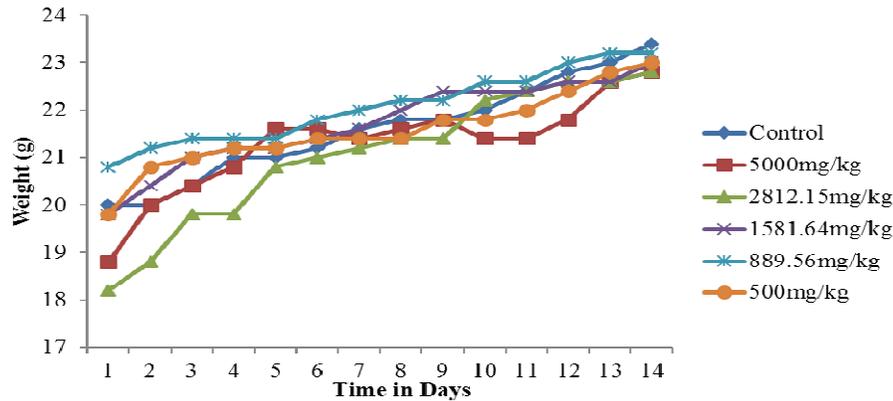


Figure 3.2. Weight of mice taken for fourteen days after oral administration with different doses of *W. ugandensis*

3.4 Determination of Anti-asthmatic Activity

3.4.1 Induction of asthma in BALB /c mice

Mice were sensitized with Ovalbumin and OVA challenged. Ova-specific IgE antibodies in sensitized mice were (O.D 0.414±0.00034) and were significantly higher than those of negative controls ($P < 0.05$). BALF Eosinophil levels were (22.60±0.245%) in sensitized mice (positive control) and were significantly different from those of negative controls ($P < 0.05$) as shown in Table 3.3.

Table 3.3. Comparison of Ovalbumin antibodies (IgE) and BALF Eosinophil levels in positive and negative controls

Treatment	IgE Optical Density (O.D)	Eosinophils (%)
Positive control	0.414±0.00034*	22.60±0.245*
Negative control	0.098±0.00013	0.40±0.245

Mean ± SEM, (n=5) *p<0.05

3.4.2 Anti-asthmatic activity of *P. africana* and *W. ugandensis* extracts

The levels of Ovalbumin specific antibodies in different treatment groups were determined by enzyme-linked immunosorbent assay ELISA. The IgE levels were presented as absorbance (OD) at 450 nm (Table 3.4) and their trends were plotted (Figure 3.3). All treatment groups had significant difference in the levels of IgE antibodies compared to the positive controls ($P < 0.05$). The group treated with 125 mg/kg of extracts of *W. ugandensis* had the highest Ovalbumin-specific IgE (0.314±0.00046) followed by group treated with 125mg/kg of *P. africana* extracts (0.274±0.00029). The lowest levels of IgE were recorded in the group treated with 500 mg/kg of *P. africana* extracts (0.100±0.0001). BALF Eosinophil counts in different treatment groups compared to the positive controls are recorded in Table 3.4 and their trends are shown in Figure 3.4. All treatment groups of mice had significant difference in the levels of BALF Eosinophil compared to the positive controls ($P < 0.05$). Mice treated with 125 mg/kg of extracts of *W. ugandensis* had the highest BALF Eosinophil (8.80±0.20) followed by mice treated with 250 mg/kg of *W. ugandensis* extracts (7.40±0.245). The lowest levels of BALF Eosinophil were recorded in the group treated with 500mg/kg of *P. africana*

extracts (2.80±0.20). All the groups treated with extracts had IgE level and BALF Eosinophils being significantly different ($P < 0.05$) from those treated with reference drug except *P. africana* extracts at 500 mg/kg (0.100±0.0001) which was significantly different ($P > 0.05$) as shown in Table 3.5.

Table 3.4. Levels of OVA-specific IgE antibodies in groups of Ova-sensitized BALB/c mice treated with *P. africana* and *W. ugandensis* extracts at each dose concentration compared to positive control

Plant species	Dose (mg/kg)	IgE (O.D)	Eosinophils (%)
<i>P. africana</i>	125	0.274±0.00029*	7.20±0.20*
<i>P. africana</i>	250	0.184±0.0005*	5.40±0.245*
<i>P. africana</i>	500	0.100±0.0001*	2.80±0.20*
<i>W. ugandensis</i>	125	0.314±0.00046*	8.80±0.20*
<i>W. ugandensis</i>	250	0.233±0.00024*	7.40±0.245*
<i>W. ugandensis</i>	500	0.134±0.00016*	3.80±0.20*
Positive control	Distilled water	0.414±0.00034	22.60±0.245

Mean ± SEM, (n=5) * $P < 0.05$

Table 3.5. Levels of IgE and BALF Eosinophils in groups of mice treated with different doses of *P. africana* and *W. ugandensis* extracts compared to Reference drug

Plant species	Dose (mg/kg)	IgE (O.D)	Eosinophils (%)
<i>P. africana</i>	125	0.274±0.00029*	7.20±0.20*
<i>P. africana</i>	250	0.184±0.0005*	5.40±0.245*
<i>P. africana</i>	500	0.100±0.0001	2.80±0.20*
<i>W. ugandensis</i>	125	0.314±0.00046*	8.80±0.20*
<i>W. ugandensis</i>	250	0.233±0.00024*	7.40±0.245*
<i>W. ugandensis</i>	500	0.134±0.00016*	3.80±0.20*
Reference drug	2	0.100±0.00012	3.60±0.245

Mean ± SEM, (n=5) * $P < 0.05$

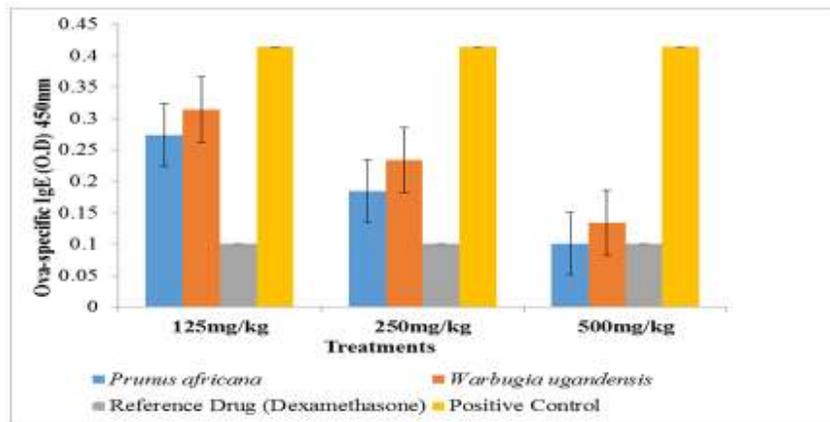


Figure 3.3. Anti-OVA specific IgE antibodies levels in different groups of BALB/c mice sensitized with Ovalbumin and treated with different concentrations of extracts from *P. africana* and *W. ugandensis* in comparison to those treated with reference drug and positive control

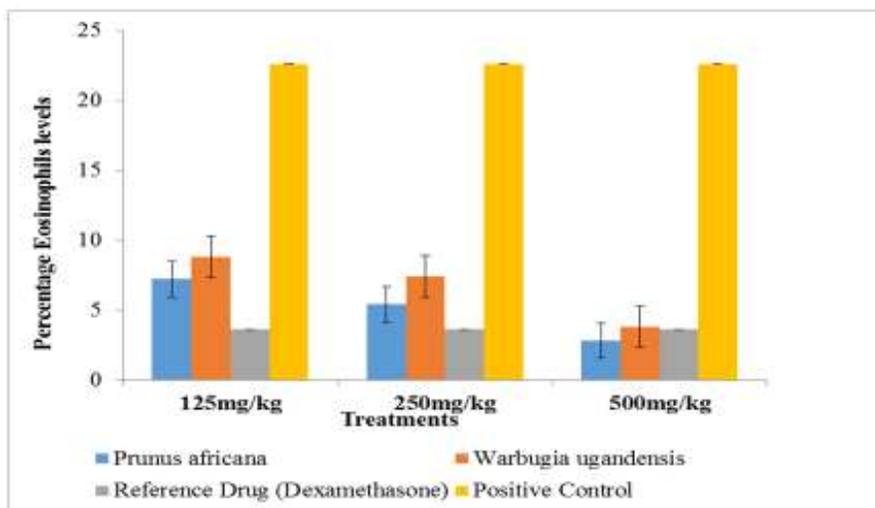


Figure 3.4. Percentage levels of Eosinophils from Bronchoalveolar lavage fluid (BALF) in groups of BALB/c mice sensitized with Ovalbumin and treated with different concentrations of water extracts from *P. africana* and *W. ugandensis* in comparison to those treated with reference drug and positive control

4. DISCUSSION

4.1 Yields of Water Extracts

From 150g of ground dried barks of *P. africana*, a yield of 13.452 g freeze dried water extract was obtained. This extraction was done using 1500 ml of water equivalent to six glasses of water (250ml glass equivalent). Each glass of water extract therefore contains 2.242 g of freeze dried *P. africana* water extract. Ground dry barks yield in *W. ugandensis* was 19.481 g and using the same argument a glass of water extract contains 3.25 g of freeze dried extract (Appendix IV). *W. ugandensis* extracts did yield 4.0193% more than those of *P. africana* in every 150 g of ground dried barks. Therefore if the same glass measure is used in treatment of ailments, the patients receive more concentration of *W. ugandensis* than *P. africana* per glass in boiled plant extract. However this does not imply that *W. ugandensis* has more activity compared to *P. africana*.

4.2 Cytotoxicity Assay

From MTT assay results, CC_{50} of *P. africana* was 104.08 μ g/ml which is the concentration of the dose of the compound/extract that kills 50% of the cells. According to Rukunga and Simons [31], *P. africana* extract was classified as not cytotoxic; whereby Cytotoxicity was classified as Cytotoxic at $CC_{50} < 2 \mu$ g/ml, moderately cytotoxic at $CC_{50} 2 - 89 \mu$ g/ml and not cytotoxic at $CC_{50} > 90 \mu$ g/ml. A recent study focused on toxicity, established *P. africana* extracts as non-toxic where cytotoxicity was $> 100 \mu$ g/ml [32]. Using the classification of Rukunga and Simons, *W. ugandensis* was also not cytotoxic even at 250 μ g/ml and thus classified as not cytotoxic at $\geq 100 \mu$ g/ml. Therefore CC_{50} for *W. ugandensis* was $> 250 \mu$ g/ml. This was in agreement with investigations in macrophages where the *W. ugandensis* stem bark extracts had no significant cytotoxicity effect ($P < 0.05$) on infected BALB/c

macrophages *in vitro* at 1000 ug/ml and were recommended as safe for use in further *in vivo* studies [33]. In complementation, previous study by Ngure *et al.* indicated *W. ugandensis* extracts being not cytotoxic to Vero cells at 100ug/ml [34]. However, a cytotoxic sesquiterpene, characterized as muzigadial was isolated from *W. ugandensis* and it was highly toxic in the brine shrimp assay [35]. In all these studies, it is critical to note that the two stem bark extracts of *P. africana* and *W. ugandensis* were not cytotoxic to Vero cells despite the fact that the stem barks in the present study were sourced from a different locality in Kenya. The CC₅₀ values of the two plants bark extracts showed that the extracts were not toxic to the Vero cells and thus considered safe to use as herbal remedies.

4.3 Acute Toxicity

4.3.1 Acute toxicity of *W. ugandensis* extracts

Several scientific reports show that many plants used as food and traditional medicine are potentially toxic, mutagenic and carcinogenic and there is very little evidence of scientific screening done on such plants [5]. Therefore it was necessary to screen the plants in this study to ascertain their safety before determination of their efficacy in asthma since there is very little information on their toxic or lethal levels. On administration of single doses of varied concentrations of *W. ugandensis* to mice, no mortality was observed even at the highest concentration tested. Based on the scale of Loomis and Hayes [36] classification of toxicity, *W. ugandensis* was classified as relatively harmless with LD₅₀ >5000 mg/kg body weight. Another pointer to the safety of the extracts tested was that all the animals that received the extracts stayed alive for the entire period of study and the progressive weight was not significantly different from that of controls. This indicates that the stem bark water extracts of *W. ugandensis* were safe to use in mice as herbal medicine at the doses tested. The findings concur with previous pointers that indicate all parts of *W. ugandensis* are edible and that the leaves, barks, young shoots and fruits are used as food and as medicine suggesting that the plant is safe for use in humans [37, 38]. Besides, *W. ugandensis* has been considered as a medicinal plant whose bark extracts have been used for ages in treatment of stomach worms and malaria in Baringo Kenya with no adverse effects and this point out its safety for use as a herbal extract [39]. Nonetheless, *W. ugandensis* stem bark extracts have been established to be non-toxic to BALB/c macrophages in previous studies [33].

4.3.2 Acute toxicity of *P. africana* extracts

Results of this study indicated that the extracts of *P. africana* at lower doses of 500 and 889.56 mg/kg body weight had no mortality noted. Lethal dose (LD₅₀) for *P. africana* was calculated by probit analysis to be 2201.207 mg/kg body weight. Based on the scale of Loomis and Hayes [36] classification of toxicity, *P. africana* was classified as slightly toxic with LD₅₀ of 2201.207mg/kg body weight and this was in agreement with other studies on rats whereby repeated daily administration of 1000 mg/kg caused mild toxicity [40]. Less progressive weight gain in the animals treated with 2812.15 mg/kg compared to controls could be as a result of the drug exhibiting inhibitory effects in the organ systems in the mice or interference in cell functioning when high doses are administered. This is in agreement with biochemical and haematological studies carried out in mice whereby it was suggested that the liver and the heart could have had subcellular changes when repeated dose of 1000 mg/kg body weight was administered [40]. Further, the results in the present study were in agreement with other findings which indicated that the stem bark extracts of *P. africana* were not toxic at therapeutic doses of 500 mg/kg body weight [32]. All the other mice that survived

the entire 14 days of study in acute toxicity assay had their weight not different from those of control indicative that the lower doses were safe to use.

Mortality was noted with administration of higher doses of *P. africana* (≥ 1581.64 mg/kg) and signs of toxicity were evident in the animals. This implies that doses above the LD₅₀ were having adverse effects in the mice, a factor that would be good to look into in future studies. Among the toxic signs noted was mean weight loss for the first few days in the group of mice that received oral dosages of this extract at 2812.15 mg/kg body weight. However, weight gain or loss can be caused by a number of factors including motivation, eating behaviors, amount of activity, overall health metabolism and stress [41]. In concurrence, previous studies have investigated how loss in body weight can be caused by decreased food intake [42,43]. Even so, one of the mortality signs noted in this study was low appetite in mice administered with higher dose level of extract. On consideration, other studies indicate that weight changes that occur especially in relation to stress are usually in response to internal changes in animal physiology as induced by stress exposure [44]. In one study using restraint stress, rats lost weight and remained hypophagic until a few days after the stress had ended [45]. If stress would have caused weight loss in mice in this study, then all the groups could have lost weight and would have had a significant difference with that of the control but this was not the case in this study. Therefore, loss of weight in the first few days and less progressive weight gain in the animals treated with 2812.15 mg/kg compared to controls and other treated groups would be as a result of the drug exhibiting inhibitory effects in the organ systems in the mice or interference in cell functioning when high doses are administered [46]. The effect would further be attested by the fact that, biochemical and haematological studies carried out in mice suggest that the liver and the heart could have had subcellular changes when a repeated dose of 1000 mg/kg body weight of *P. africana* back extracts was administered [46].

Survival of mice in this present study after treatment with *P. africana* extracts at doses less than the LD₅₀ is supported by other *In vivo* tests in rats indicating that *P. africana* stem bark water extracts do not cause clinical or pathological abnormalities in rats at daily doses of up to 1000 mg/kg for 8 weeks [47]. All the other mice that survived the entire 14 days of study in acute toxicity assay had their weight not different from those of control indicative that the lower doses are safe to use in mice and this concur with other studies where the *P. africana* extracts are currently being used for treatment of prostrate hyperplasia [20].

4.4 Determination of Anti-asthmatic Activity

4.4.1 Induction of asthma

One of the hallmarks in allergic disease is infiltration of the tissues with increased numbers of eosinophil. This is as a result of the coordinated action of cytokines, particularly IL-5, CCR3 binding chemokines and adhesion molecules, acting in concert to cause selective trafficking of eosinophils into allergic tissue [48,49]. Th-2 allergen specific lymphocyte orchestrates the process. Although there is little data to support the view that eosinophils ameliorate the allergic process, it is important to note that they are important diagnostic markers and guide to the management of allergic diseases [48,49]. Another hallmark of allergic disease is elevation of allergen specific IgE levels in the serum; IgE is one of the immunological components for mast cell degranulation and allergy, it is directly correlated with IL-4, which is involved in IgE isotype switch [49]. These two hallmarks were quantified in this study, where elevated levels indicated induction of asthma in BALB/c mice and reduction in their levels was a measure of efficacy of the plant extracts used. The plant extracts used

in this study have a reputation in the Kenyan communities for treatment of asthma [37]. Treatment is done without scientific base to explain the action of such plants. This study was therefore carried out to assess their acclaimed efficacy employing the murine asthmatic model.

Sensitization of BALB/c mice with Ovalbumin was noted as being successful since clinical symptoms of asthma were proven as well as immunological symptoms such as elevation of the two of the hallmarks of asthma in the Ovalbumin-sensitized mice. The clinical symptoms of asthma included reduced physical activity, general discomfort/restlessness, difficulty in breathing and wheezing; these observations were in agreement with previous studies in asthmatic murine model [41,50]. Serum IgE levels (O.D) in sensitized mice in this study was elevated thus serving as one of the markers of asthma and this was in concurrence with studies that previously used the murine model of asthma and considered IgE as a key pointer of asthma [16,51,52]. Further, BALF Eosinophil levels (%) in Ovalbumin-sensitized mice were also elevated; another hallmark of asthma whose level of elevation was well in agreement with studies that have used the murine model of asthma in BALB/c mice [53,54,55,56]. Eight weeks old female BALB/c mice were susceptible to Ovalbumin induced asthma and this was in accord with studies that have recently demonstrated BALB/c mice model manages to show good pattern of the human airway disease better than other animal models [50, 57]. There were virtually no Eosinophils (0-1%) in the control group (non-sensitized) compared to the sensitized group indicating efficacy of the sensitization and challenge protocol.

4.4.2 Efficacy of the plant extracts in management of asthma induced BALB/c mice

Extracts or the decoctions of the flowering and fruiting plants have long been used and are still being used in East Africa and West Africa for treatment of chest inflammations [37]. In this study two of the hallmarks of asthma were quantified; elevated levels of IgE (O.D) and increased BALF Eosinophil (%) [14,55,58]. Therefore efficacy of the plant extracts was evaluated in terms of reduction of Ovalbumin IgE levels (O.D) and BALF Eosinophil (%) as well as reduction or elimination of clinical symptoms of asthma. The clinical symptoms of asthma which included reduced physical activity, general discomfort/restlessness, wheezing and difficulty in breathing which were present after nebulization, eased off gradually in the sensitized mice following treatment with dexamethasone or plant extracts. The observation was in consistency with studies that have used Hydrocortisone as a standard drug and other herbal extracts in treatment of asthma [41]. By use of the hallmarks of interest in this study, it was clear that the non-asthmatic mice were having 0-3% BALF eosinophil and the asthmatics were having 23% of the same. Intervention that reduced the levels of these hallmarks towards the normal ranges was likely to be effective in management of asthma [59].

The two extracts used were tested at three dose levels for their *In-Vivo* anti-asthmatic properties. The treatment doses were arrived at by choosing a dose below the toxic levels determined in acute toxicity and in comparison of what other researchers have used in previous studies in murine model [32,46,60]. Oral administration of *P. africana* and *W. ugandensis* water extracts to asthmatic mice significantly reduced the levels of serum IgE (O.D) and BALF Eosinophil (%) in a dose wise manner. The greatest reduction in these hallmarks was recorded in mice treated with extracts at 500 mg/kg body weight while the lowest reduction was in mice treated with extracts at 125 mg/kg body weight. This indicates that the best efficacy was in the highest dose tested. Reduction of BALF Eosinophil (%) in all the extracts did not reach the level of reduction by Dexamethasone. This would be due to

the fact that the extracts were crude/total extracts containing active constituents in minute concentration which work in synergism and thus need to be purified and concentrated for better efficacy.

Reduction of Ova-specific IgE (O.D) levels in all plant extract treatment groups did not reach the reduction levels by Dexamethasone for the same reasons stated already except for treatment with *P. africana* extracts at 500 mg/kg body weight. This difference in treatment with *P. africana* at 500 mg/kg body weight can be attributed to higher levels of steroids and lipids that have been previously isolated from barks of *P. africana* and are believed to block leukotrienes, reduce oedema and inflammation as well as diminished histamine and therefore ease asthmatic symptoms [20]. In addition, ferulic acid esters for hypocholesterolaemic and phytosterols for anti-inflammation have been demonstrated in *P. africana* [20]. The present study findings further confirmed recent findings which indicated some medicinal plants at certain dose concentrations have no significant difference in their treatment with that of reference drug as presented by *Euphorbia hirta* extracts compared to Hydrocortisone [61]. The two plants used in the present study reduced asthmatic symptoms similarly to that of Dexamethasone as seen in reduction of IgE and BALF eosinophil levels. These findings were in agreement with studies where treatment of asthmatic mice with *Euphorbia hirta* extracts reduced the white blood cell count in a similar way to reduction by Hydrocortisone [61]. Corticosteroids have a puissant anti-inflammatory action and have a central role in treatment of asthma [62]. However, they have been reported as having detrimental effects on the gastric mucosa and have systemic and local side effects when used for a long time and at higher doses [19, 62, 63, 64] while studies using herbal medicine have demonstrated that herbal extracts have no detrimental side effects compared to corticosteroids [61]. This would imply that the herbal medicine used in this study for asthma management in mice when formulated and developed for human use would offer safe alternative medicine for asthma management although more studies need to be done to establish the long term effects of using the herbal extracts.

In this study there was no significant difference in weight of mice treated with the extracts and that of controls pointing out that the extracts were safe to use in management of asthma in mice. Therefore these extracts would be used as alternatives to some of the corticosteroids which were found to cause a reduction in body weight of mice after treatment [65]. In other findings it has also been noted that herbal medicines do not impact on weight gain as severely as hydrocortisone [41]. Phytochemical and pharmacological studies have identified many potential anti-inflammatory substances especially those derived from plants used in folk medicines, hence natural products are becoming increasingly important as a source of therapeutics, either for treatment of infectious or non-infectious lung diseases [66,67,68]. Formulation and development of cost effective herbal based medicines from plant extracts will offer more solutions in asthma management since studies have indicated that other asthma medications are known to be unaffordable to people in developing countries [69]. Notwithstanding, it would be critical to note that any formulation and development of alternative medicine should be done in consideration of WHO policy regarding medicinal plants [6,7,8].

It emerged that the efficacy of these plant extracts was almost comparable with that of the standard drug Dexamethasone. At each dose level, *P. africana* demonstrated better efficacy compared to *W. ugandensis* water extracts. This implies that, there would be more active compound in *P. africana* than in *W. ugandensis* though it is subject to further investigation. Efficacy was better demonstrated when the extracts were administered at higher doses, 250

and 500 mg/kg body weight while at 125 mg/kg the efficacy was far below that of reference drug.

5. CONCLUSION

This study indicated that the bark extract doses of *P. africana* and *W. ugandensis* employed in BALB/c mice had no obvious deleterious effect which to a large extent provided more information on the therapeutic safety of the herbal remedy. The two extracts had immunological effects in asthma induced BALB/c mice, seen by reduced levels of Ovalbumin specific antibodies (IgE) and BALF Eosinophil counts in extract treated groups. Nevertheless to confirm on the non-toxic nature of *P. africana* and *W. ugandensis*, the effect that various factors such as the specific parts of the plant (leaves, roots, flowers and seeds), as well as geographical variability of the plants presence should be looked into. Further, in this study crude extracts were used thus providing the need for investigations of specific chemical compounds that led to the anti-asthmatic activity. Therefore bio-aided extraction should be done to establish potential lead compounds which can be used in silico studies for synthesis of potential drugs for asthma management. Clinical evaluations of the stem bark extracts in the human asthma should be mounted to demonstrate the therapeutic efficacy seen in the murine model.

CONSENT (WHEREEVER APPLICABLE)

Not applicable

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee; the KEMRI Animal Care and Use Committee (ACUC), the Scientific Steering Committee (SSC) and the Ethical Review Committee (ERC).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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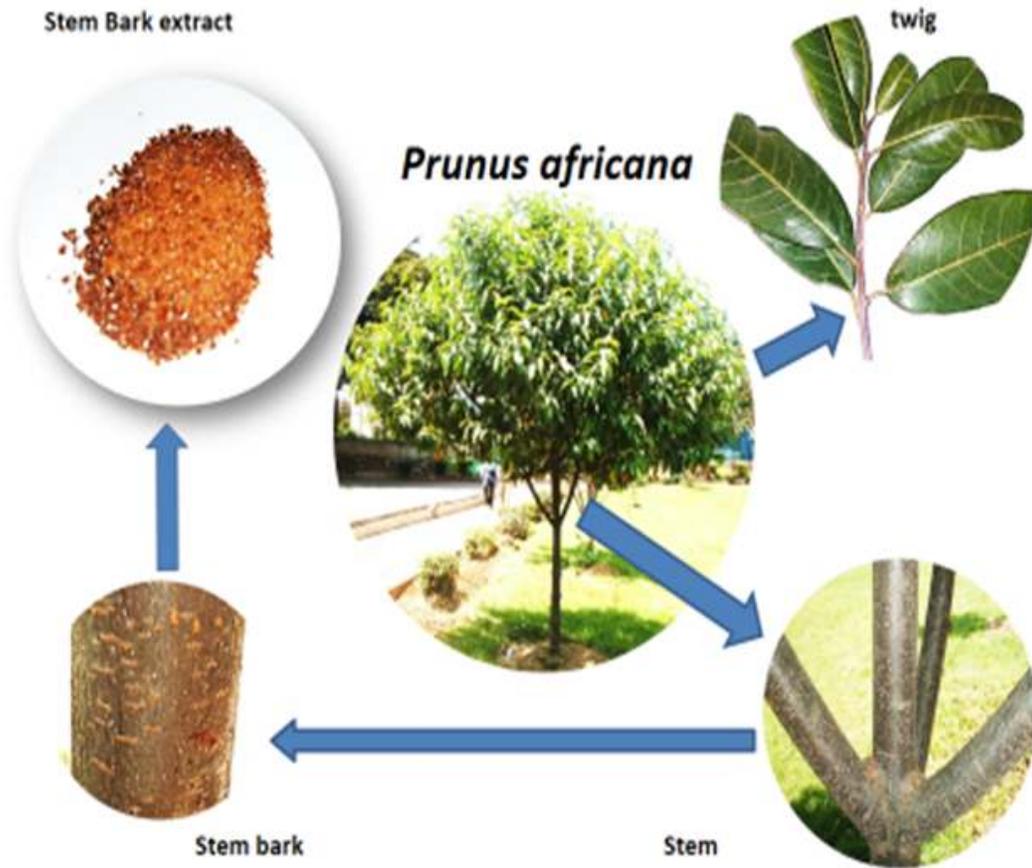
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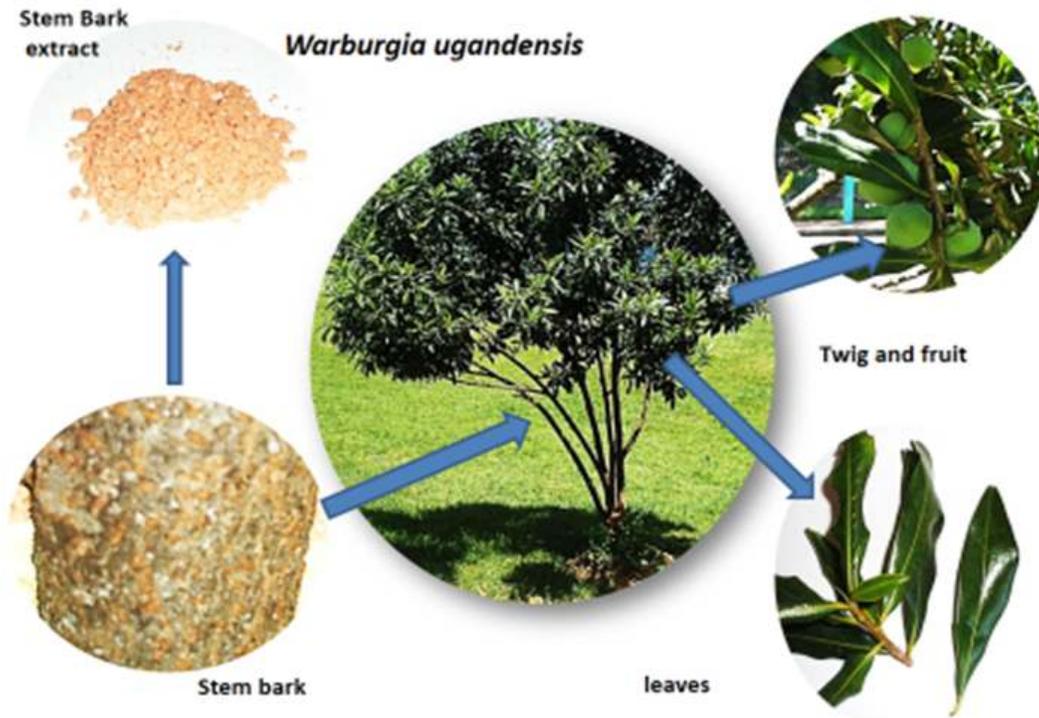
APPENDIX - I

Photographs of *Prunus africana* and its stem bark water extract



APPENDIX – II

Photographs of *Warburgia ugandensis* and its stem bark water extract



APPENDIX - III

Dose Calculation in Acute Toxicity by Lorke 1983

$$r = \sqrt[n-1]{\frac{L}{l}}$$

Key:

r = ratio

n = number of doses (5)

L = largest dose level

l = smallest dose level

$$r = \sqrt[4]{\frac{5000\text{mg/kg/day}}{500\text{mg/kg/day}}} = \sqrt[4]{10} = 1.778$$

Dose 1 = 5000mg/kg/day,

$$\text{Dose 2} = \frac{5000\text{mg/kg/day}}{1.778} = 2812.148 \text{ mg/kg/day}$$

$$\text{Dose 3} = \frac{2812.14\text{mg/kg/day}}{1.778} = 1581.6358\text{mg/kg/day}$$

$$\text{Dose 4} = \frac{1581.6358\text{mg/kg/day}}{1.778} = 889.559\text{mg/kg/day}$$

Dose 5 = 500mg/kg/day

APPENDIX - IV

Calculation of Amount of Freeze Dried Extract per Glass (250ml glass equivalent)

P. africana: 150g dry barks extracted with 1500ml/6 glasses of water yields 13.452g,

1 glass contains $\frac{13.452\text{g}}{6} = 2.242\text{g}$ of freeze dried stem bark extract.

W. ugandensis: 150g dry stem barks extracted with 1500ml/6 glasses of water yields 19.481g

1 glass contains $\frac{19.481\text{g}}{6} = 3.247\text{g}$ of freeze dried stem bark extract.

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