

**EVALUATION OF ANTIMICROBIAL ACTIVITY AND SAFETY OF SYRUP
PREPARED FROM AMBU ROOTS**

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ABSTRACT

Some rural communities in Tanzania use decoctions from different parts of AMBU for treatments of various health conditions. However, few studies have been conducted to ascertain their quality, efficacy and safety. This study aimed at establishing baseline information on the quality, efficacy and toxicity of syrup prepared from AMBU roots. Decoction was prepared and some of the aliquots were freeze dried into powder then dissolved in sucrose vehicle to make the final syrup. Both the decoction and herbal syrup aliquots were evaluated for stability after storage under different conditions. Antimicrobial activity and toxicity were investigated by micro broth dilution method and toxicity assay in mice respectively. Mice were randomly grouped into eleven groups of five each and administered oral doses of 0, 2.5, 25, 100, 200 and 500 mg/kg body weight of AMBU root decoction and the developed syrup. Animals were administered the drugs at an interval of eight hours every day for seven days. The results demonstrated that the minimum inhibitory concentration of the developed syrup in *Staphylococcus aureus*, *Basillus subtilis*, *Escherichia coli* and *Pseudomonus aeruginosa* were 15.6, 31.2, 15.6 and 62.5 mg/ml respectively. The shelf life for decoction and the formulated herbal syrup at room temperature was 2 and 17 weeks respectively and those kept in refrigerator was 12 and more than 18 weeks respectively. The toxicity assay revealed neither mortality nor signs of abnormality that could be associated with AMBU treatment at low oral doses. However, AMBU treatment at higher doses data revealed significant changes on body weights and liver to body mass index. The formulation of syrup has improved the quality, activity and safety as demonstrated in mice model. These findings validate the ethno-medical potential of AMBU syrup and the feasibility of its use for treatment and control of bacterial diseases.

DECLARATION

I, James Mwesongo, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution.

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DEDICATION

I dedicate this work to my mother Marry Mkunda, my wife Pendo J. Madda, my children Anna, Monica, Veronica, Samuel and Edward, who have encouraged me from the beginning to the end of this study.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standards Institute
Cr	Crude extracts dissolved in water
EPOPA	Export Promotion of Organic Products from Africa
Fo	Formulated syrup
HE	Haematoxylin and Eosin stain
HIV	Human Immuno-deficiency Virus
IBDV	Infectious Bursal Disease Virus
INT	Iodonitrotetrazolium chloride
MIC	Minimum Inhibitory Concentration
NBF	Neutral Buffered Formalin
NDV	Newcastle Disease Virus
SUA	Sokoine University of Agriculture
USP	United States Pharmacopeia
WHO	World Health Organisation

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Tanzania like any other developing country suffers from inadequate health services especially in remote rural areas (Kayombo *et al.*, 2013). Herbal remedies thus, provide an alternative option for alleviation of different health challenges in humans, animals and plants. Plants in particular are the dependable source of medicines which are used to treat diseases and infections (Daniel, 2010). Plants have been used for treatment of various illnesses in the history of human beings even before the knowledge of microbes and antibiotics (Taheri and Ownagh, 2015). Plants provide phytochemicals such as phenolic compounds, alkaloids, vitamins and minerals which have anti-oxidant and anti-tumor properties that are important in the treatment of diseases. These properties can also be used as leads for manufacture of many drugs (Prathiba raj and Manjunath, 2014).

It is also reported that about one quarter of the total drugs prescribed in industrialized countries contain one or more components derived from plants (Gurib-Fakim, 2006). In developing countries, similarly in Tanzania, most of the information on herbal medicine is still owned by the traditional healers (Kayombo *et al.*, 2013). The traditional healers usually consider their medicinal knowledge as a spiritual family heritage (Ngarivhume, *et al.*, 2015). According to Nikhat and Fazil (2013), traditional knowledge about medicinal plants has been passed from one generation to another in the form of oral knowledge. However, this practice may lead to distortion of some information due to lack of proper documentation, especially the practical aspects; details of sourcing of medicinal plants and their post harvest handling. Furthermore, it is observed that most of traditional herbal drug

preparations have not been studied for quality in terms of safety, efficacy, shelf life and characteristics which endangers the health of users.

In Tanzania, for example, there are many medicinal plants which are traditionally used for treatment of different diseases in both human and animals (Kayombo *et al.*, 2013). There are many reasons to why medicinal plants are more preferred; first, they are natural and wholesome, thus considered to have relatively less side effects (Delamare *et al.*, 2007). Another reason is that herbal drugs has definite advantages besides being cheap to manufacture; they are biodegradable and readily accessible (Mwitari *et al.*, 2013; Kayombo *et al.*, 2013). Also plants are preferred because of the presence of compounds that can be used to control mechanism by which the bacteria resist drugs (Kristian, 2012). Besides the above mentioned health benefits, herbal drugs are source of income and employment. Despite the formal recognition by the Government of Tanzania, the production and marketing of herbal drugs is still at its infancy. Processing of herbal products and retailing are two main marketing outlets which are found to exist in Tanzania (EPOPA, 2005). It is well known that the demand for herbal remedies in Tanzania is high, not only in rural communities but also in urban areas. Street vendors of herbal medicines as practiced by the Maasai who sells crude drugs; semi processed or raw herbal medicines are indicators of that scenario.

The majority of the herbal drugs sold by street vendors are not registered despite the existence of control measures done by the Tanzania Food and Drug authority (TFDA) (TFDA, 2014). For any herbal drug to be registered, pre-market evaluation should be conducted to ensure it meets quality and safety standards (TFDA, 2014). Notwithstanding all of the richness in medicinal plants, still Tanzanian market is full of expensive but well

processed herbal products from abroad countries like China, America and India. There are some few certified herbal products manufactured in Tanzania as shown in Table 1.

Table 1: Some of the herbal drugs made in Tanzania

Product	Manufacturer	Indication
NIMREX	NIMRI	Anti HIV
NIMREVIR	NIMRI	Anti HIV
MUNDEX	NIMRI	Sex activator
NIMREVIT	NIMRI	Neutraceutical
MORIZELA	NIMRI/MUHAS	Neutraceutical
TMS	NIMRI	Anti malaria
PESCAN	NIMRI	Anti diabetic
PERSIVIN	NIMRI	Anticancer
WABUGISTART	NIMRI	Bacterial d'ses
TENGESHA	NIMRI	Anti anemia
HERPCURE	NIMRI	Liver cure
NGETWA	HERBALISTS	Anti diabetic

AMBU which belongs to the family *Euphorbiaceae* (Carter, 1986) is among the medicinal plants commonly used in various parts of Tanzania for the treatment of various ailments. For instance, in Morogoro, Kaguru people of Gairo use the decoction from roots as crude drug for treatment of respiratory disorders (cough).

AMBU crude extracts have antimicrobial activity against a wide range of bacteria and fungi. Also it inhibits multiplication of some viruses such as Newcastle Disease (ND) virus, Infectious Bursal Disease virus (IBDV) and Fowl Pox disease virus (Mabiki *et al.*, 2013a,b). Nevertheless, the use of this AMBU drug is not scientifically validated and the safety, shelf life and efficacy are not yet known.

1.2 Problem Statement

AMBU root decoction has been used by some communities for treatment of various diseases in both human and animals with successful results (Mabiki *et al.*, 2011; Bichang'a, 2009); in spite of being condemned as a poisonous plant with no therapeutic value (Maroy, 2012). Despite this successful exploitation, AMBU root decoction is of poor quality characterized by bad odor, short shelf life and bad taste. Such limitations are due to lack of scientific data on quality, dosage, safety and lack of standard formulations. This has led to poor dose estimations during administration which may lead to acute or chronic toxicity that renders the decoction unacceptable and un-commercialisable. Pre-market evaluation of herbal product is necessary to ensure quality and safety standards (TFDA, 2014; Rasch and Ponti, 2015). Therefore, the present study aimed at evaluation of herbal syrup formulated from AMBU roots for its potential in the treatment of infectious diseases.

1.3 Objectives

1.3.1 Overall objective

To investigate the quality of syrup prepared from AMBU roots by studying its characteristics, antimicrobial activity and toxicity.

1.3.2 Specific objectives

- i. To establish the characteristic and stability of the herbal syrup formulated from AMBU roots.
- ii. To determine the antimicrobial activity of AMBU root syrup against selected bacteria of medical importance.
- iii. To evaluate acute toxicity of the formulated syrup from AMBU roots using mice model.

1.4 Significance of the Study

This study will establish baseline data on characteristics, toxicity and antimicrobial activity of syrup formulation from AMBU roots decoction. These results will contribute to scientific knowledge on bio-prospecting and value addition of natural products. Stability studies are highly recommended to trace depletion in quality of syrup over a specified period of time. Toxicity studies are also compulsory for promising herbal drug safety.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Herbal Medicine Overview

Herbal medicine is popularly known as complementary alternative medicine (Tanwar *et al.*, 2014). The herbal drugs are believed to have lesser side effects because the transformation enzymes can easily act on them more rapidly than the chemical analogues (Dewick, 2002; Patrick, 2013; Ola *et al.*, 2013). The uses of herbal remedies remain relevant in the primary health care especially in developing countries due to easy access and for economic reasons (Afolabi *et al.*, 2012; Mabiki *et al.*, 2013a; Rutebemberwa *et al.*, 2013). Herbal drugs are also popular in developed countries because are relatively safe compared to many synthetic drugs (Taheri and Ownagh, 2015). Diverse plant species are used in traditional medicine in various parts of Tanzania. AMBU is among those medicinal plants commonly used by some rural communities of Tanzania (Neuwinger, 2004).

2.2 The use of AMBU in Ttraditional Medicine

AMBU is a plant which belongs to *Eurphorbiaceae* family that is commonly found in East Africa. In Tanzania, the plant is found in different parts such as Lushoto in Tanga, and Mkata in Morogoro (Carter, 1986). *Eurphorbiaceae* plants are poisonous and are believed to have no therapeutic value (Maroy, 2012). However, AMBU have been used for treatment of diseases in human and animals in many parts of Tanzania without any reported of side effects (Rukunga, *et al.*, 1990; Mabiki *et al.*, 2011).

2.3 Chemical Characterization of Extracts from AMBU

Preliminary chemical characterization of pharmacologically active compounds of AMBU enabled isolation of glucose present in the aqueous extracts of its leaves that is responsible

for inhibition of the electrically induced contractions of guinea pig ileum (Rukunga *et al.*, 1990). Phytochemical screening of the dichloromethane extracts indicates that it is composed of two main triterpenoids that best correspond with Lanosterol and Cycloartenol. Other minor compounds recognized through chromatographic analysis include phytol, ergostadiol, hentriacontane, sitastriol acetate, lupeol and hopenone (Mabiki *et al.*, 2013c). The aqueous extract of the leaves and stems of AMBU have a positive reaction for tannins, triterpenoids and coumarins. Petroleum ether extracts contain carotenoids, steroids, triterpenoids, volatile oils and glucosides (Neuwinger, 1994).

2.4 Importance of Herbal Drugs Stability and Shelf Life Assessment

Stability and shelf life assessment is among the WHO guidelines for standard herbal formulation (WHO, 2000). The stability testing is meant to provide confirmation of how the quality of a finished product varies with time under the influence of a variety of environmental factors such as temperature, humidity and light (Gupta *et al.*, 2011). Thakur *et al.* (2011) and Nikhat and Fazil (2013) assert that plant products like many other organic compounds remain stable for a particular period after which it begins to spoil. Additionally, they insist that during storage, herbal products are often prone to deterioration that can lead to loss of activity or even production of toxic metabolites (Thakur *et al.*, 2011; Nikhat and Fazil, 2013). It has also been reported that active principles in medicinal plants undergo fluctuation periodically due to seasonal factors (Soni *et al.*, 2014). Thus, the assessment of stability and shelf life is necessary in order to determine the quality, efficacy, safety and increase acceptability of the herbal formulation. It is further upheld that the understanding of the problems associated with herbal product stability give the answer of dealing with the shelf life issues. Likewise, modifications such as addition of preservatives to conventional herbal formulations can contract with the stability troubles to a bulky coverage. For instance, Ngarivhume *et al.* (2015) reveal that

some of the plant parts used by traditional healers to treat some diseases such as malaria are stored as dried powders in closed bottles that are soaked in hot or cold water and the water extract is taken as the active medicine. Therefore, it is important to have standard procedure for getting ready made herbal formulations instead of struggling for quick preparation after the problems have occurred. Thus improvement of herbal remedies is of crucial importance for health welfare and income generation.

2.5 Antimicrobial Properties of AMBU Extracts

Different studies have shown that extracts from AMBU have antimicrobial activities against a wide range of microorganisms including *S. aureus* ATCC 25923, *B. subtilis* local isolate, *Streptococcus* spp., *E. coli* ATCC 25925, and *Salmonella typhimurium* local isolates (Mabiki *et al.*, 2009) and *M. tuberculosis* (Bichang'a, 2009). The water extracts from the leaves and stems of AMBU has been reported to have antimolluscicidal activity (Kloos *et al.*, 1987). Organic solvent extracts from AMBU have demonstrated antimicrobial activity against some fungi including *Candida albicans* and *Aspergylus niger* (Mabiki, 2013).

2.6 Reports on AMBU Root Extract Toxicity

The toxicological aspects about AMBU and many other herbal drugs have been abandoned. This is because of the reasons that they have been used as drugs for long time without observed side effects (Mabiki *et al.*, 2011). However, according to the WHO guidelines on toxicity of herbal remedies, even in cases of drugs used over a long period, chronic toxicological risks may have occurred without being recognized (WHO, 2000). Thus assessment of safety is very pertinent. Although AMBU is among plants used in poisonous fishing (Neuwinger, 2004), Nyigo *et al.* (2015) contend that extracts from dried plant parts of AMBU under the short term use do not cause any adverse effects. They

maintain that different extracts from dried leaves and root barks when applied on skin of animals have no adverse effect both externally and internally. Another report by Nyigo *et al.* (2016) concludes that AMBU extracts exhibit low to non-significant activity against ticks. This conclusion was based experimental results in which activity in adults and larvae were less than 50%. Moreover, the experience as reported by the local people that AMBU is very corrosive and toxic has been validated by brine shrimp lethality test using the extracts from AMBU which also confirms an indicator for both bioactive and anticancer (Mabiki *et al.*, 2013a).

2.7 Importance of the Liver in Drug Toxicity

As well known the liver is the most important organ in detoxification (Larson, 2016). As Patrick (2013) articulates, the absorption of the drug by the intestine, goes to the liver through the hepatic vessel for detoxification (first pass effect) using the major metabolic and digestive functions before distribution to other parts of the body. Based on this phenomenon, the liver is the first target organ to be assessed for signs of toxicity. Some reported herbal drugs that are associated with liver damage include *Atractylis gummifera* (Stickel *et al.*, 2000; Haller *et al.*, 2002). In the American herbal drug market, drug and herbal-drug induced liver injury is an issue of importance in terms of clinical, research and drug development especially the significance of pre-clinical assays (Rasch and Ponti, 2015). Not only herbal drugs but also dietary supplements can be associated with liver injury (Navaro *et al.*, 2015; Zheng and Navarro, 2016). Therefore, toxicity studies cannot be neglected in assurance of drug safety. Assessment of the abnormalities in the liver may lead to understanding of the potential for herbal drug – induced hepatic injury because several parameters such as relative organ to body weights and histopathological changes can be expressed (Rasch and Ponti, 2015). Thus in the present study liver was selected for histopathological investigation for any damage that could be due to AMBU extracts treatment.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Design

This study involved field sample identification and collection; laboratory - based *In vitro* and *In vivo* randomized experimental designs.

3.2 Sample Collection and Preparation of Decoction

3.2.1 Plant collection and processing

AMBU roots were collected from Gairo district in Kibedya village. Elevation 37 M 0271266, UTM 9326650 and voucher specimen were brought to Sokoine University of Agriculture for confirmatory identification by botanist from the Faculty of Forestry. The roots were collected by digging the ground using hoe and cutting using bush knife carefully. The processing was carried out at the Faculty of Science and freeze drying at the Department of Food Technology, Nutrition and Consumer Sciences laboratory.

3.2.2 Preparation of decoction

The crude drug decoction was prepared according to the United States Pharmacopeia (USP) standard procedures as described by Patel *et al.* (2012) with some modification adhering to the traditional healer's procedures. The roots were partially treated by hot firewood ash, cleaned and cut into small pieces of approximately one centimeter. The chopped AMBU roots (125 g) were mixed with one liter of water then reduced to one fourth of the origin volume by boiling. The decoction was cooled and assessed immediately for physical chemical parameters for comparison with the formulated syrup. Then some aliquots of the decoction were freeze dried into powder that was used for final syrup preparation.

3.2.3 Preparation of the vehicle

Although the simple syrup was prepared according to the United States Pharmacopoeia guide lines, there were slight modifications based on the nature of the decoction bitterness. The amount of the sucrose was reduced to one fourth of that in the formula. Sucrose (166.7 g) was dissolved into 1 L distilled water, which was used as vehicle for the final syrup preparation.

3.2.4 Preparation of the final syrup

To prepare the final syrup, the dry powder was dissolved into the vehicle prepared in section 3.2.3 to make concentration of 500 mg/ml, followed by addition of 0.5% vanilla and 0.2% sodium benzoate as flavouring and preservative agents, respectively. The final syrup like the decoction was also assessed immediately for physical chemical parameters.

3.3 Characterization of the Formulated Herbal Syrup

3.3.1 Physical chemical parameters

The herbal syrup was evaluated for physical appearance (colour, odour and taste), pH and specific gravity with slight modification according to the USP standard procedures as described by Patel *et al.* (2012).

3.3.1.1 Colour examination

The decoction and the syrup were examined for colour by observation using naked eyes. Five millilitres of each were taken into watch glasses and placed against white background in white tube light. The conclusion of colour was reached after a separate blinded observation by three ladies.

3.3.1.2 Odour examination

Two millilitres of decoction and syrup separately were smelled. The time interval among two smelling was kept at 5 minutes to nullify the effect of previous smelling.

3.3.1.3 Taste examination

A pinch (150 μ l) of decoction and final syrup was taken and examined for its taste on taste buds of the tongue. There were three different people each tasting the decoction and then the syrup.

3.3.2 Determination of pH

To determine the pH, digital pH meter, model HANNA HI 9811 - 5 USA SN 08682177 was used to measure the pH for each sample in a duplicate.

3.3.3 Determination of turbidity/homogeneity

Five millilitres of each sample was taken into clean glass test tube and visually assessed for turbidity. The sample was left to stand for half an hour and then the assessment was repeated.

3.3.4 Determination of density

The density was calculated from the mass and volume of the sample in triplicate and the mean value was taken. Using analytical balance, the weight of empty clean and dry 10 ml volumetric flask was recorded. The flask was then filled with the sample up to the mark and reweighing to get its weight with sample. The exact weight of the sample was obtained through elimination of the weight of empty flask from the weight of the flask filled with the sample and the density was obtained by the formula; $\text{Density} = \text{Mass} / \text{Volume}$.

3.3.5 Shelf life assessment

The shelf life was assessed on weekly bases for 18 weeks by repeating the evaluation of physicochemical parameters (section 3.3.1 to 3.3.4.) for samples kept in different storage conditions.

3.4 Determination of Antimicrobial Activity of Syrup from AMBU roots

The determination of antimicrobial activity aimed at knowing the minimum inhibitory concentration (MIC) of the test drug. This was performed with modification of micro-plate dilution method as stipulated in the standard guideline of Clinical and Laboratory Standards Institute (CLSI) and the antimicrobial activity was evaluated in the presence of standard drug Gentamicin (CLSI, 2012). *p*-Iodonitrotetrazolium chloride salt (INT) was used as microbial growth indicator.

3.4.1 Bacterial strains

The microorganisms in the study were selected as representatives of Gram negative and Gram positive bacteria. Reference strains, (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, and *Pseudomonas aeruginosa* ATCC 27853) were obtained from the Microbiology Department of Muhimbili National Hospital. Mueller - Hinton agar (DIFCO, LOT. 2144259) was used for the activation of the bacteria while the Mueller–Hinton Broth (DIFCO, LOT. 108953) was used for the antibacterial assays.

3.4.2 Antimicrobial susceptibility testing

Micro dilution method was used with slight modification as described by Bakele *et al.* (2015). That is, 0.1 ml of broth was put in each well of a micro titer plate, followed by 0.1 ml of the drug, then serial dilution from this ratio of 1:2 to 1:1024 drug to media,

respectively. The standard 0.5 McFarland (known to form 1.5×10^8 CFU/ml) was prepared by taking two to four colonies in normal saline solution following standard procedure. Then, 0.01 ml of 1.5×10^8 CFU/ml of the test bacterial suspension were added to the wells containing serially diluted drug. The standard drug gentamicin was serially diluted the same way as the test drug. The MIC of samples was detected after 18 hr. incubation at 37 °C, following addition (40 µL) of 0.2 mg/ml INT. The minimum inhibitory concentration was defined as the sample concentration at which no colour developed following addition of the INT.

3.4.3 Evaluation of the toxicity of AMBU in albino mice

This experiment aimed at establishing baseline data on the toxicity of syrup prepared from AMBU roots in albino mice (*Mus musculus*). Albino mice were obtained from the animal's house of the College of Veterinary Medicine and Medical Sciences, Sokoine University of Agriculture. The animals were allowed to acclimatize at the experimental cages for one week before they were assigned to treatment. The experiment was carried out with slightly modification, based on the toxicity testing standard guidelines for herbal drugs (WHO, 2000). Mice were grouped and randomly administered orally with dosages of 0, 2.5, 25, 100, 200 and 500 mg/kg body weight of AMBU root aqueous extract and the developed syrup at time interval of eight hours for seven days. Mortality, signs of toxicity, body weight, food consumption, and gross findings were observed for post treatment of both AMBU extract and syrup separately. At the end of the study, all the animals in all groups were sacrificed humanly and the vital organs to body mass index were compared with values from the vehicle control group.

The toxicity study was carried out using fifty five (55) randomly distributed male and female mice weighing 20 to 30 g, arranged into 11 groups as shown in Table 2.

Table 2: Toxicity set up of different oral doses of decoction and syrup

No.of animals	Cr/Fo Dose (mg/kg)	Description
5	Vehicle	Control group
5	2.5	Approximately similar to human dose where the plant was obtained
5	25	10 times greater than human dose
5	100	40 times greater than human dose
5	200	80 times greater than human dose
5	500	200 times greater than human dose

Cr = crude drug, Fo = the formulated syrup

**Figure 1: Oral administration of AMBU Root Syrup to Albino mice**

3.4.6 Observation of clinical signs

The mice were observed for clinical signs before and after dosing at least three times a day. Food and water consumption and animal behavior were monitored. Body weights were measured at the beginning, immediately before treatment (day 0) and at the end of the experiment (day seven).

3.4.7 Post mortem observation

Animals were sacrificed under anaesthesia. Dissections were performed and internal organs including liver, lung, heart, kidney, spleen, gonads and gastrointestinal tract were observed for abnormalities related to AMBU treatment in comparison to the controls. The organs were weighed and fixed in 10% NBF (neutral buffered formalin) until further analysis.

3.4.8 Histopathological observation

Livers of animals were taken, fixed for histopathological processing and subsequent examination by light microscopy. Fixation of tissue was allowed to proceed for a minimum of 24 h before dehydrating in ascending series of ethanol, cleared in xylene then specimens were processed to wax embedded blocks using a manual processor and according to standard protocols (Feist *et al.*, 2015). Sections were cut at 4 – 6 microns thick by a manually rotary microtome (Baird and Tatlock (London) LTD.), mounted on glass slides, dried and stained with haematoxylin and eosin (HE) stains (Cardiff *et al.*, 2014). The prepared liver sections were examined using light microscope (Olympus Model: CX41RF) for abnormalities compared to the sections from the control group.

3.4.9 Statistical analysis

Data were recorded and stored in excel program and analysed to get the Mean and Standard deviation, as well as the standard error of the mean. Further analysis by one way ANOVA test was done using SPSS version 16.0 and a P-value of less than 0.05 was considered to be a significant difference. Multiple comparison tests for different dose groups were conducted.

CHAPTER FOUR

4.0 RESULTS

The present study results indicated that the formulated AMBU syrup has more stable characteristics than that of the decoction under the similar environmental factors. The comparative characters of the decoction and the developed syrup are depicted in Tables 3 and 4. The syrup has shown good antimicrobial activity against the selected standard bacterial strains (Table 6). The toxicity study in mice model, both AMBU decoction and the formulated syrup results revealed a dose dependent trend in which there was significant changes in animals received higher doses compared with the control group.

4.1 Characteristics of the Herbal Decoction

Evaluation of decoction was made immediately after preparations and on weekly basis for 18 weeks. The characteristics of fresh decoction especially the odour was quite different from that of the deteriorated decoction as shown in Table 3. The fresh decoction had strong foul herbal smell. On storage at room temperature turned into alcoholic product smell at the second week and followed by rotten herb smell (nasty smell) during the fourth week.

Table 3: Physicochemical parameters of the decoction

Parameter	Observed value at start	Observed value at week 18
Colour	Brownish – grey	Pale brown
Odour	Herbal – unpleasant	Nasty unpleasant smell
Taste	Bitter	Bitter
Density	0.9988	0.993
pH	5.4	4.2

4.1.2 Characteristics of the developed herbal syrup

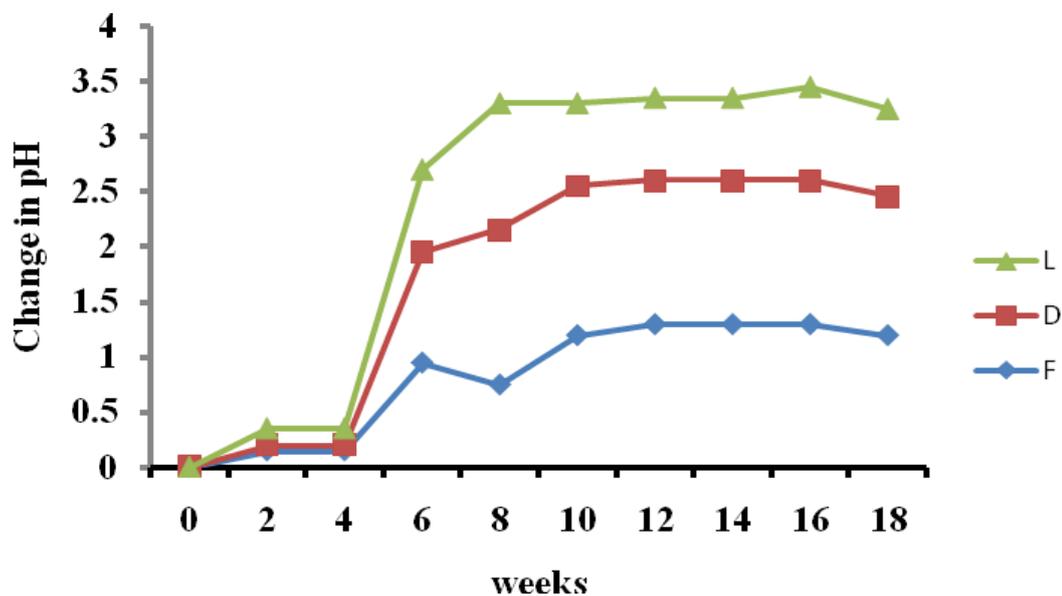
Evaluation of the formulated AMBU root syrup revealed good elegance and palatability. The study results of stability of the final syrup aliquots stored in refrigerator revealed no changes in all the tested physicochemical parameters except slightly change of the pH and relative density. Other physical chemical parameters are as shown in Table 4. The pH and relative density drop was almost the same compared to that of decoction.

Table 4: Physicochemical parameters of final syrup

Parameter	Observed value at start	Observed value at week 18
Colour	Brownish – grey	Brownish – grey
Odour	Pleasant	Pleasant
Taste	Sweet	Sweet
Density	1.0742	1.0644
pH	5.7	4.5

4.1.3 Comparative changes in pH of decoction and syrup

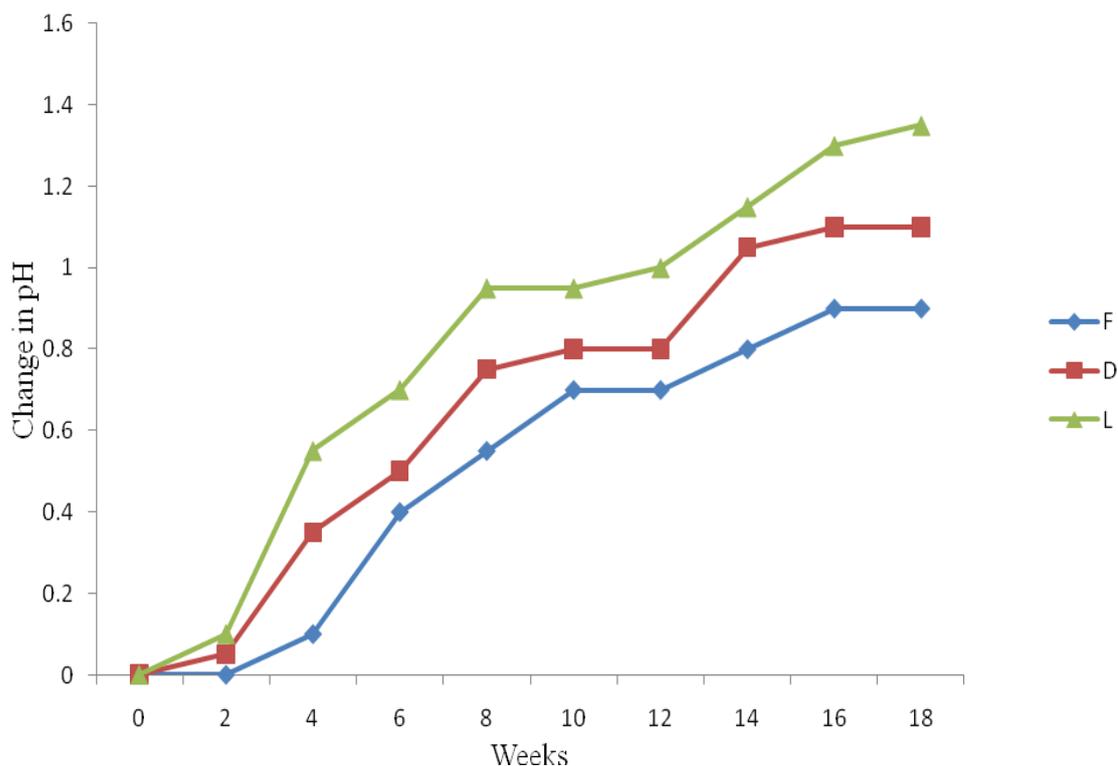
The pH of decoction from all aliquots under all storage conditions showed sudden changes at week four and then slight changes were seen until week 18 (Figure 2). This indicates that the decoction stability was relatively lower compared to that of the syrup (Figure 3) in which changes in pH were gradual.



L = Change in pH for aliquots of decoction stored at room temperature exposed to light,
D = Change in pH for aliquots of decoction stored at room temperature in darkness,
F = Change in pH for aliquots of decoction stored in refrigerator.

Figure 2: Change in pH of decoction against time

The results for all decoction aliquots showed sudden changes of pH from the second week and reached the maximum at week 6. It was clearly observed that light and temperature had effects on decoction storage in which high temperature and exposure to light increase the rate of deterioration. Thus, refrigeration was the best way of storage followed by darkness storage condition (Figure 2).



L = Change in pH for aliquots of syrup stored at room temperature exposed to light,
 D = Change in pH for aliquots of syrup stored at room temperature in darkness,
 F = Change in pH for aliquots of syrup stored in refrigerator.

Figure 3: Change in pH of Syrup against time

The results for all aliquots of the formulated syrup revealed a gradually changes of pH in which the maximum change was in week 18. Also refrigeration demonstrated the best condition of storage compared to room temperature. Therefore, the results indicated clearly that light and high temperature had effects on AMBU root syrup storage as shown in Figure 3.

4.1.4 Determined shelf life for decoction and syrup

The shelf life study results revealed that the developed syrup remained fresh for more than 18 weeks for aliquots kept in refrigerator and 17 weeks for samples kept at room temperature with exposure to light and darkness, respectively (Table 5). Odour was the most important among the physicochemical parameters tested. On storage at room

temperature, all decoction aliquots stored in the darkness and those exposed to light smelled alcoholic and produced gas during the second week and subsequent during the fourth week changed to rotten smell. Decoction aliquots kept in refrigerator changed to alcoholic smell and gas during the 12th week. Syrup aliquot kept in refrigerator retained its original smell (no change) beyond 18 weeks while those stored at room temperature produced alcoholic smell and gas during the eighteenth week. Both the decoction and syrup were homogeneous, but on standing for 45 minutes clear brownish solution was separated and thin layer grey sediment noted at the bottom.

Table 5: Shelf life for decoction and the developed syrup

Sample	Storage Condition	Shelf life (weeks)	Other observation
Decoction	L	2	Alcoholic smell, pale clear
Decoction	D	2	Alcoholic smell, pale clear
Decoction	F	12	Alcoholic smell, pale clear
Syrup	L	17	Alcoholic smell
Syrup	D	17	Alcoholic smell
Syrup	F	>18	Fresh, pleasant smell

L = storage condition at room temperature exposed to light, D = storage at room temperature in darkness, F = storage in refrigerator.

4.2 Antimicrobial Susceptibility Test

The results for the antibacterial assays as determined by broth micro-dilution method are summarized in Table 6. The results indicate that the tested herbal syrup displayed broad spectra of antibacterial activities against both Gram positive and Gram negative bacteria.

Table 6: Antimicrobial Activity of AMBU Syrup

Species	MIC(mg/ml)	Gentamicin ($\mu\text{g/ml}$)
<i>Bacillus subtilis</i>	31.25	6.25
<i>Staphylococcus aureus</i>	15.62	3.125
<i>Pseudomonas aeruginosa</i>	62.5	12.5
<i>Escherichia coli</i>	15.62	6.25

Staphylococcus aureus and *Escherichia coli* were the most susceptible compared to *Bacillus subtilis* and *Pseudomonas aeruginosa*. The formulated herbal syrup activity against *Pseudomonas aeruginosa* had a minimum inhibitory concentration (MIC) of 62.5 mg/ml showed the most resistant. Also, it has shown high resistance to the control as well with MIC 12.5 $\mu\text{g/ml}$ compared to other bacterial strains. Generally, the activities of syrup from AMBU roots have shown a comparable trend with the control (Table 6).

4.3 Toxicity of Syrup and Decoction in Mice

The results have shown that there was no mortality, clinical signs and gross findings related to AMBU treatments at lower oral doses. However, AMBU treatments at higher doses have shown no mortality but significant differences ($P \leq 0.05$) in body weight and liver to body mass index between the treated animals in comparison with the control group (Figure 4 and 5). Post-mortem findings in some few animals, congestion spots of lung were identified. These were considered as accidental findings and they were not considered to be AMBU treatment related abnormal gross. This was due to the fact that the congestion spots were not dose dependent lesions and were also observed in mice in the control group.

4.3.1 Mortalities

There were no unscheduled mortality for all groups treated with syrup and decoction observed. All animals (100%) survived until the termination of the experiment.

4.3.2 Clinical signs

In this study, there were dose dependent AMBU extract-treatment abnormal clinical signs observed during observation period. The control group and animals received lower AMBU doses were normal in feeding and active. Weight loss in some of the animals that received higher doses was noted and also they were hiding at the conner of cages, reduced feeding, rough coat and weakness.

4.3.3 Post mortem observations

There were no AMBU treatment related abnormalities observed as compared with the control group. Conversely, accidental congestion of some lung tissues, which were not dose dependent features were observed in both treated animals and the control group. All other internal organs were apparently normal.

4.3.4 Body weight changes and organ to body mass index of the treated mice

The toxicity study results reveal a dose depended trend effects on body weights and organ to body mass index of some of the principle organs. The body weight results for both AMBU decoction and the formulated syrup treated groups compared with the control group are shown in Figure 4.

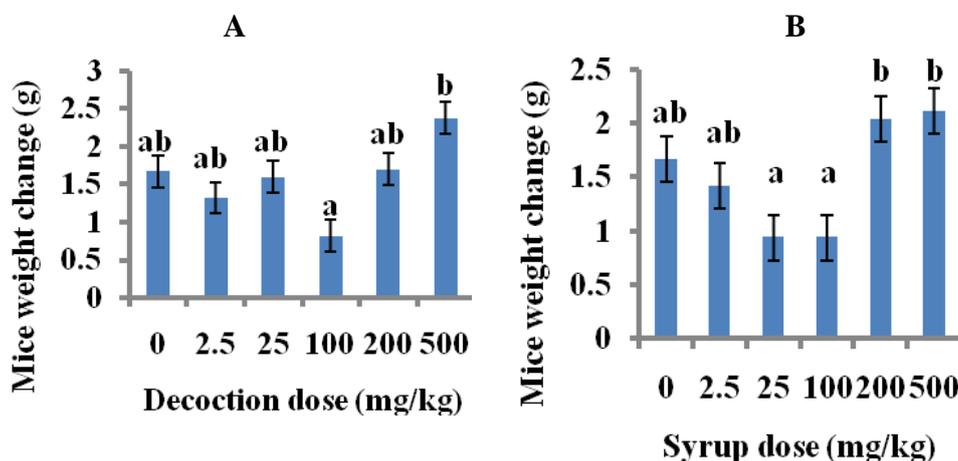


Figure 4: Mice weight changes against decoction and syrup at different doses

Comparisons for body weights between time of exposure and sacrifice of animals demonstrated comparable changes ($P > 0.05$) for groups that received 2.5 and 25 mg/kg with animals in the control group. A significant difference ($P \leq 0.05$) was observed in animals that received 100 mg/kg of both AMBU decoction and syrup in which they had the least body weight difference followed by those that received 200 mg/kg. The groups that received 500 mg/kg had the highest body weight difference where the difference with other groups was statistically significant ($P \leq 0.05$) (Figure 4). The formulated syrup have demonstrated more effects on body weights compared to the decoction in which animals receiving 25 mg/kg had similar response to that receiving 100 mg/kg. Also the group receiving 200 mg/kg had similar response to that receiving 500 mg/ml (Figure 4 B). Thus, the results have shown that AMBU treatment at higher doses has effects on mice body weights.

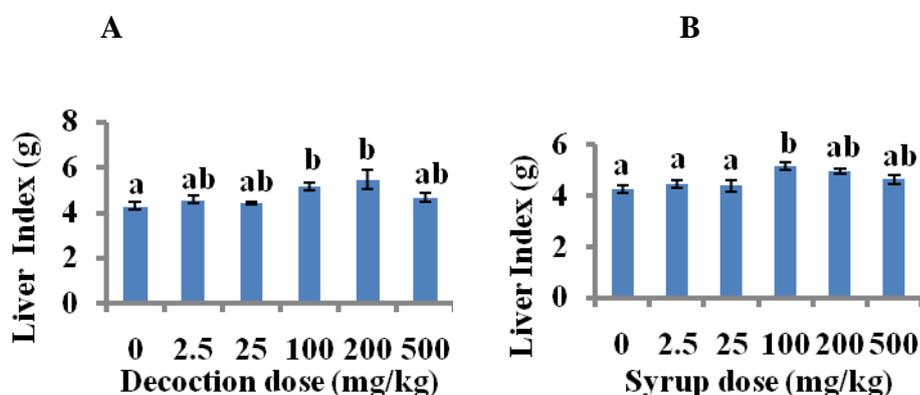


Figure 5: Relative Liver to body mass index

A similar trend was observed in the liver to body mass index in which there was no statistically significant difference ($P > 0.05$) between the control group and the animals treated with 2.5 and 25 mg/kg of both AMBU decoction and the formulated syrup (Figure 5). On the other hand a statistically significant difference ($P \leq 0.05$) was shown between the liver to body mass index of animals treated with higher AMBU doses and the control group. Also is an indication that higher doses of AMBU have effects on the mice liver.

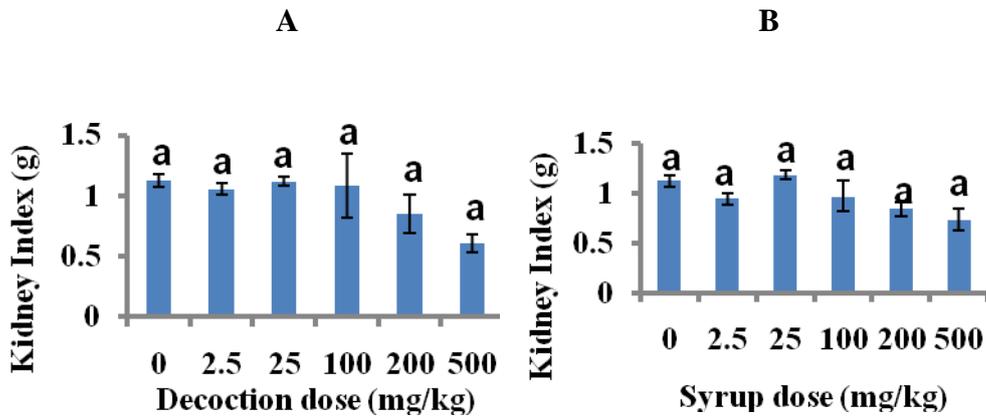


Figure 6: Relative Kidney to body mass index

The organ to body mass index of kidney (Figure 6), spleen, heart and lungs was comparable ($P > 0.05$) between mice in treated and control group. The results revealed that apart from the liver, there was no any damage that could be due to AMBU treatment on other internal organs.

4.3.5 Histopathological changes in liver sections

Generally no significant changes on the histopathological findings of the liver were observed in all dosing groups tested compared to that of vehicle control. Histological examination of liver sections from all groups including control group, revealed numerous Kupffer cells in sinusoidal spaces, hepatocytes cytoplasmic vacuolation and congestion in hepatic blood vessels (Figure 7). Those were referred as accidental features because they were not dose dependent features that could be evidence for AMBU treatment results. Thus, the histopathological findings reveal that there was no damage that could be caused by AMBU treatment to the liver of mice.

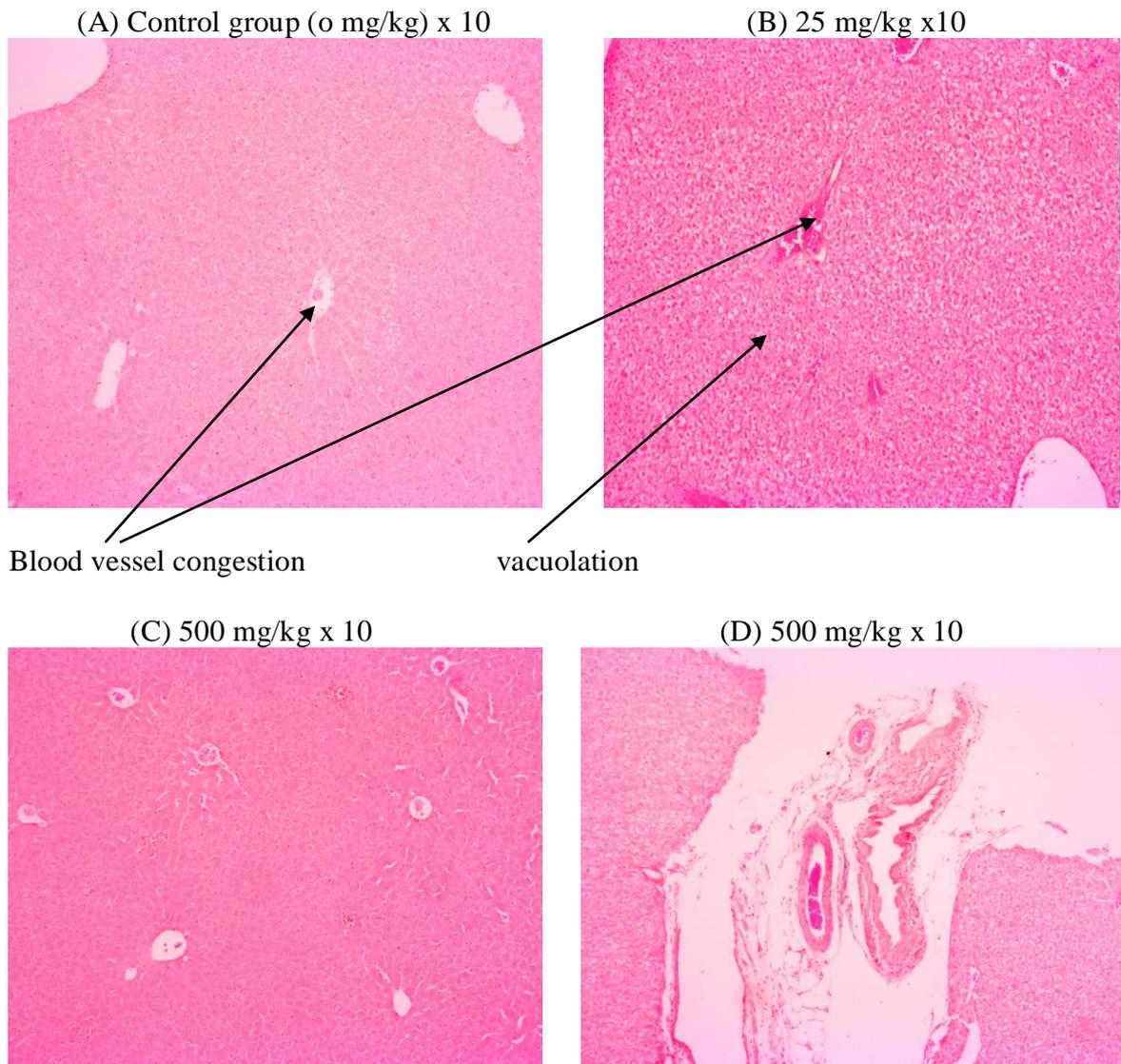


Figure 7: Liver section (HE) after treatment with AMBU root extract

Figure 7 shows the histopathological structures of the liver from the control group and AMBU treated groups. They were normal with exception some congestion in hepatic blood vessels observed. They were randomly detected throughout the whole experimental groups including that animals from the vehicle control group, and most of these sporadic histopathological findings do not show any dose-dependent frequencies encountered.

CHAPTER FIVE

5.0 DISCUSSIONS

5.1 Introduction

This study aimed to establish the baseline data of syrup prepared from AMBU roots by studying its characteristics, antimicrobial activity and toxicity. It is the first study to develop syrup from the traditionally used AMBU root decoction, assessing its characteristics, antimicrobial activity and toxicity in mice model. The formulated syrup has better quality as compared to the decoction in parameters including taste, odour as well as improved shelf life (Table 3, 4 & 5). The improvement has been achieved through addition of additives according to the standard procedures in which sweetener masks bitterness, deodorant and preservative improves the odour and shelf life, respectively. As it is guaranteed, sodium benzoate inhibits decompositions by microbial growth or undesirable chemical changes (Pongsavee, 2015).

The samples were opened during assessment period on weekly basis and that could cause gas either escape from or be introduced into storage containers. Consequently, there were possibilities of contamination by microbes as usually, when the drug is in use and there is frequent opening and closing. Deterioration was primarily judged as any deviation from the original state of the test sample. In this investigation, the first alteration noted during the second week in decoction kept at room temperature which was the alcoholic smell and gas formation. This implies that fermentation process likely had taken place in the decoction sample. Fermentation is well known as the biological process which converts sugars such as glucose, sucrose and fructose into cellular energy, resulting to production of ethanol and carbon dioxide as by-products (Robinson *et al.*, 1999). There are a number of microorganisms that ferment sugars, especially yeasts and some bacteria. Thus, additives

to the formulated syrup have made its value relatively higher than that of decoction because of improved shelf life, sweet taste and pleasant smell.

5.2 Herbal Drugs Quality and Shelf Life Assessment

Throughout the world, various medicinal plants and herbal drugs have been widely used as primary treatment agents or supplements for treating various human ailments (Ola *et al.*, 2013). Herbal drugs are usually multi-constituents and some may react to each other under environmental factors, hence form inactive products with either poor efficacy or form toxic compounds with adverse effects to users (Thakur *et al.*, 2011; Nikhat and Fazil, 2013). Thus, it is important to determine their shelf life. In this experiment aliquots of both the decoction and the formulated syrup were stored at environments with different temperature, light and relative humidity. This was based on the reality that communities especially in rural areas store their drugs at relatively higher temperature than that of refrigerator. But there is argument that temperature rise increases the rate of chemical reaction, light and air have effects on oxidation and reduction of some active ingredients hence can create substantial variability (Thakur *et al.*, 2011). Storage of samples at high temperature and light exposure aimed at accelerating reaction of fragile compounds which change the physicochemical parameters of the test herbal formulations. In the present results demonstrated clearly the effects of temperature and light on both the decoction and the formulated syrup.

5.2.1 Effect of Temperature on Odour of Decoction and Syrup

In the present investigation odour was the most important parameter in shelf life assessment of both the decoction and the formulated herbal syrup aliquots stored under different environments. There was obvious difference between the changes in smell of decoction test samples kept under refrigeration and those kept at room temperature. Those

changes were understandable due to effects of temperature on chemical reaction in which temperature rise was proportional to speed of the reaction (Thakur *et al.*, 2011). The smell of decoction kept under refrigeration changed into alcoholic smell after week 12; while that of the formulated syrup remained unchanged for more than 18 weeks. Similarly, the smell of decoction kept at room temperature changed into alcoholic during the second week. On the other hand, the formulated syrup kept under the same room environment remained stable until week 18 when started to produce alcoholic smell. The alcoholic smell followed by rotten herb smell in the fourth week indicates that the reaction of constituents took place, thus producing alcohol like products and subsequent decomposition to rotten herbal products.

5.2.2 Effect of temperature on decoction and syrup pH change

The change in pH of decoction stored in refrigerator was slightly lower compared to those stored at room temperature (Figure 2). From week four decoction pH changes were exponentially to the maximum change, unlike that of syrup which changed gradually reaching the maximum at week 18. Additives especially preservatives improved the shelf-life of the formulated syrup by controlling prompt pH changes. However, both decoction and the formulated syrup pH changes were not significant ($P > 0.05$) with regard to the acceptable limit (3.6 to 6) (WHO, 2000). In comparing with other previous studies, such as the designed and developed poly herbal syrup had pH of 4.25 (Swain and Nayak, 2013) and herbal syrup which had pH of 4.5 (Sandhyaran and Praveen, 2014).

5.2.3 Effect of light on decoction and syrup pH change

Some natural compounds such as polyphenols affect stability of products. This is possible because polyphenols are very sensitive to light (Munin and Edwards, 2011). The findings demonstrated clearly the effect of light in both the decoction and the formulated syrup

aliquots stored at different environments (Figures 2 and 3). This applauds the phenomenon of photo degradation which according to Gmurek *et al.* (2015), light radiation may destroy natural coloured organic compounds dissolved in water. Syrup aliquots stored at room temperature, exposed to light have shown relatively higher pH changes followed by those stored at room temperature but in darkness and least changes for those refrigerated. It is an indication that there were some photophobic constituents in both the decoction and the syrup that increased reactions on exposure to light.

5.3 The Assessed Antimicrobial Susceptibility

The results for the antibacterial assays reveal that the tested syrup displayed broad spectra of antibacterial activities against both Gram positive and Gram negative bacteria. *Bacillus subtilis* and *Staphylococcus aureus* were selected in this study as representative for Gram positive bacteria, while *Escherichia coli* and *Pseudomonas auruginosa* for Gram negative bacteria. In comparing the minimum inhibitory concentration (MIC) of the formulated syrup with that of the control (gentamicin), a clear comparable trend was demonstrated. A dose dependent trend which was when AMBU syrup MIC increases, that of the control increases as well depending on the test bacterial strain's resistance (Table 6). The efficiency of AMBU extracts was demonstrated while in crude form, hopeful it could do better if it was in a purified form.

Apart from these results, previous studies have also shown that extracts from different parts of AMBU has antimicrobial activities against wide range of causative agents for both human and animal diseases. Among the bacteria inhibited by AMBU extracts are *Mycobacterium tuberculosis* (Bichang'a, 2009), *Salmonella typhymurium* and some viruses; New Castle virus, Infectious Bursal Disease Virus and Fowl Pox Virus (Mabiki *et al.*, 2013a, b). Thus, the studied AMBU root syrup has the potential to be used for the

management of some bacterial and viral infections. AMBU decoction has been reported in the treatment of coughing related to tuberculosis. Antimicrobial studies against *Mycobacterium tuberculosis* have revealed good activity with minimum inhibitory concentration of 2 mg/ml (Bichang'a, 2009), thus validates also the traditional use and the potential of AMBU in the treatment of tuberculosis.

5.4 The Toxicity of Syrup from AMBU Root Aqueous Extracts

Although, AMBU root decoction has been used by some communities for treatment of various illnesses and research had been done to investigate its effects (Bichang'a 2009; Mabiki *et al.*, 2013a, b), no studies reported about its safety. Thus, this study was conducted to get baseline data on the quality, efficacy and safety of its syrup. The existing results for AMBU treatment demonstrated no mortality in mice. Similarly, no significant changes in body weight, organ to body mass ratio for animals treated with lower doses (Figures 4, 5 and 6). Also no liver histological changes have been detected as an evidence for the damage in the liver and other body organs for the mice (Figure 7). However, significant changes ($P \leq 0.05$) in body weights and liver to body mass index of animals from groups that received 100 mg/kg body weight and above were observed. The results indicated that the formulated syrup is relatively safe when administered orally at doses below 100 mg/kg body weight but had side effects with higher doses.

These toxicity properties could be of pharmacological value with regard to the antimicrobial activity results shown above that lower doses are relatively safe to the animals but effective to the bacteria (Table 6). Also the present study results reveal that AMBU treatments with higher concentrations have a decrease effect on mice body weights but no histopathological evidence of liver damage. Based on previous studies, chemical characterization of extracts from AMBU have shown diverse pharmacological

active compounds including carotenoids, steroids, ergostadiol, lupeol, triterpenoids and glucosides (Neuwinger, 1994; Mabiki *et al.*, 2013 c). Thus, body weight drop can be due to some of these active compounds on cholesterol biosynthesis that is responsible for obesity in animals. Some plants such as *Commiphora swynnertonii* which have some similar active compounds previously reported to reduce animal body weight as well as blood cholesterol levels (Mdegela *et al.*, 2017).

Generally, this study is in agreement with the traditional and ethno-botanical studies that the use of AMBU in the treatments of both animal and human diseases is safe when used orally at low doses (Bichang'a, 2009; Mabiki *et al.*, 2011). The results in this study have revealed that AMBU root decoction when administered orally up to concentration of 100 mg/kg body weight at an interval of eight hours for seven days has relatively low toxicity in mice model. Similarly, Nyigo *et al.* (2015) report that extracts from dried AMBU parts when applied on skin of animals under short term use do not cause adverse effects both externally and internally. Also, the toxicity studies of AMBU extracts to larvae and adult ticks demonstrated less than 50% (Nyigo *et al.*, 2016). However, AMBU fresh milky sap is well known to be highly toxic as used in poisonous fish (Neuwinger, 2004). Therefore, this study have validated the traditional preparation of the decoction in which treatment with fire during the preparation destroy the highly toxic compounds of the plant to ensure its safety.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This study has established for the first time the baseline data about the quality, efficacy and toxicity of the herbal syrup prepared from AMBU roots. Based on the toxicity results and indigenous information that AMBU has been used for long time without observed side effects, it can also be concluded that AMBU root aqueous extracts administered orally with doses lower than 100 mg/kg have relatively low toxic effects on mice model and is likely to be safe in humans. The results obtained in this study also suggest that the lethal oral dose of AMBU root extracts were considered to be over 500 mg/kg body weight given three times a day, for seven days in mice. Treatment by high doses of AMBU extracts have decrease effects on mice body weights with relatively low side effects.

6.2 Recommendations

Stability, toxicity and shelf life studies are essential to each formulation to ensure that the herbal product is safe and effective throughout its time of storage. Based on the results, further stability studies are highly recommended because are used as the quick tests to trace depletion in quality of syrup over a specified period of time and storage condition. Further toxicity studies should focus at the concentration between 100 and 200 mg/kg body weight. Antimicrobial activities against some more bacterial strains and other causatives agents of human and animal diseases are as well recommended. Furthermore, isolation and purification of active ingredients from AMBU root decoction is highly recommended for more herbal drug improvements. Chronic toxicity studies and effects of AMBU extracts on body weights and cholesterol levels are also recommended. Further

studies are recommended to be carried out seasonally to confirm these preliminary observations alongside with seasonal variations.

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