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Effect of Varying Concentrations of Ethanol and Lime Extracts Pre - treatments on the Aflatoxins and Chemical Quality Characteristics of Stored Irvingia Seeds

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Abstract
In this study the effects of different concentrations of ethanol and lime extracts pre- treatments on the aflatoxins contamination and quality characteristics of stored *Irvingia* seeds were investigated. The quantity of total aflatoxins produced by *Aspergillus flavus*((FR33818)) on stored *Irvingia* seeds was determined using ELISA method. *Aspergillus flavus* used for this study was identified by ITS rDNA sequence analysis by CABI identification services UK. The results showed that the aflatoxins contaminations and the overall quality characteristics of the *Irvingia* seeds were influenced by the pre-treatments applied. No aflatoxins were produced at the different concentrations of the ethanol and lime extracts used for the first two month of the storage period. However aflatoxins were produced from the third month but the concentrations in the stored *Irvingia* seeds decreased with increasing concentrations of the pre-treatments applied except for sample pre-treated with 70% ethanol that recorded no aflatoxins throughout the study period. The pre-treatments applied also significantly improved the chemical qualities of the seeds when compared with the control sample. Thus the finding of this study showed that 70% ethanol and lime extracts concentrations could be used to improve the shelf-life and other quality characteristics of stored *Irvingia* seeds.
Keywords: Aflatoxins, *Aspergillus flavus*, Ethanol, Lime extracts, Pre-treatments

1.0. Introduction

Aflatoxins are toxic secondary metabolites produced naturally in many agricultural crops by *Aspergillus flavus* and *Aspergillus parasiticus* (Dasan et al., 2016). Aflatoxins are among the most potent carcinogenic, teratogenic and mutagenic compounds in nature (Jackson and Al-Taher, 2008). They are genotoxic and carcinogenic and can cause both acute and chronic toxicity in humans (EFSA, 2007). Humans can be exposed to aflatoxins by the periodic consumption of contaminated food, contributing to an increase in nutritional deficiencies, immunosuppression and hepatocellular carcinoma (Williams et al., 2015). Aflatoxins have a wide occurrence in different kind of foods, such as spices, cereals, oils, fruits, vegetables, milk, meat (Ayhan and Ufuk, 2002). Aflatoxins interact with the basic metabolic pathways of the cell disrupting key enzyme processes including carbohydrate and lipid metabolism as well as protein synthesis (Beyhan et al., 2016; Quist et al., 2000). The complete elimination of aflatoxin in agricultural products is extremely unlikely. They are stable in foods and resistant to degradation under normal cooking procedures. Thus, the recommendation from Joint Expert Committee on Food Additives (JECFA) is that the amounts present in crops and foods should be reduced to the lowest levels that are technologically achievable (Herrman and Walker, 1997). Aflatoxins are produced primarily by the fungi *Aspergillus flavus* and *A. parasiticus* (Ebimieowei and Dorcas, 2012), and these fungi have been shown to grow on *Irvingia* kernels displayed for sales in Nigeria markets (Adebayo-Tayo et al., 2006). *A. flavus* with capability for aflatoxins productions have been more regularly isolated from *Irvingia* seeds displayed for sale in many Nigeria market (Ebimieowei and Dorcas, 2002). Chronic exposure to low or moderate amounts of aflatoxins—a more common problem than acute aflatoxicosis—can also cause liver and immune system problems in humans and animals (Cagindi and OGürhayta, 2016).

Irvingia species (*I.gabonensis and I. wombulu*) is a highly economically and nutritionally important tree native to most tropical forest in west and central Africa as well as South-East Asia (Ikhatua et al., 2010). It was identified as a high priority species for the state of knowledge reports at the non- timber forest products (NTFP) workshop held in Limbe in May 1998. This tree is especially valued for its fat and protein rich kernels as well as its rich dietary fibre (Ebimieowei and Dorcas, 2012). *Irvingia* seed is also wildly used in Nigeria as a flavouring ingredient in soups thickning because of its viscous properties (Akusu, and Kiin-Kabari, 2013). In addition to its nutritional benefits, *I. gabonensis* is highly valued for its health and medicinal benefits. For instance recent studies have revealed that the dietary fibre present in *Irvingia* kernels has the ability to reduce the hyperglycemic effects and lipid metabolism disruption caused by diabetes mellitus (Lesley, 2006). It has also been shown that kernel extract of *I. gabonensis* caused a significant reduction in body weight among obese people in Cameroon (Ngondi et al.,2005).*Irvingia* kernels and its products have also been shown to have wide

range of industrial applications. These include the use of the fat extracted from the kernel in production of margarine, soap, cosmetics and pharmaceutical products (Van, 2010; Ebimieowei and Dorcas, 2012). These uses and applications have made the market for the products of *Irvingia* kernels very robust and economically viable. However, Irvingia kernels are prone to contamination and spoilage during storage by fungi that are potentially hazardous to both human and animal health. The spoilage of *Irvingia* kernel during storage often results in changes in certain functional, chemical and organoleptic properties of the kernels (Akusu, Kiin-Kabari, 2013). These changes subsequently result in significant reduction in both the economic and nutritional value of the *Irvingia* kernel.

According to a research conducted by Tchoundjeu et al., (2005), the most common storage place for *Irvingia* seeds after sun drying is often on the platform of bamboo rack hung over a fire place in the kitchen. This is because local farmers had observed with time that sun drying was not effective in keeping the storage quality of the *Irvingia* seeds. In particular, they observed that sun dried *Irvingia* kernel was prone to fungal contamination and spoilage during storage. This often necessitates hanging the kernel over a fire place in the kitchen after sun drying.

This Spoilage is often seen as brown to black discolouration (Joseph et al., 2001). Others include blue, red, white or pink depending on the nature of the spoilage fungi.

Thus a major setback in the sales and consumption of *Irvingia* kernels is its susceptibility to post harvest spoilage fungi with its attendant health risk.

Several studies have shown that *Irvingia* seeds displayed on shelves for sales in Nigeria markets are often contaminated with spoilage fungi (Ebimieowei and Dorcas, 2012). Furthermore studies carried out by Adebayo-Tayo et al., (2006) showed that fungal contaminated *Irvingia* kernels are potentially harmful to those who consume it. In particular they observed that fungal contaminated kernels possess aflatoxin. Dorner et al., (1999) have reported that the health risk from consumption of aflatoxin include acute and chronic liver damage, liver cirrhosis, induction of tumours, neurotoxicity, immunosuppression, embryonic damage, abortion and death.

Several efforts have been made toward enhancing the keeping quality of stored *Irvingia* kernel and preventing post-harvest fungal contamination and spoilage of *Irvingia* seeds. These include roasting and grinding the kernel before storage. The paste that is obtained in this way is put in a cake tin (container) and left to dry for few hours. Once solid, the cake is removed from the container and is ready for use or stored for future use. According to their report, the duration of storage reported most frequently are 6 months to less than one year. This effort was not as successful as expected because the *Irvingia* cake was reported to have lost its physical and functional qualities after the storage period due to fungal contamination and spoilage (Tchoundjeu et al.,2005). Other researchers have reported the use of chemicals in the preservation of *Irvingia* kernel before storage. Ebimieowei and Dorcas, (2012) reported some success with 0.9% Sodium chloride (NaCl) and 3% Potassium bicarbonate ($KHCO_3$). However these treatments were not effective against some fungal species such *as Mucor spp., Aspergillus niger, Aspergillus flavus and Rhizopus stololonifer.* Ineffectiveness against *Aspergillus flavus* suggests that the risk of aflatoxin poisoning still persists after this pre-treatment. Thus despite improved effort in handling, processing and storage of *Irvingia* seed, aflatoxins contamination still remain a public health problem in the sales and consumption of *Irvingia* seed in Nigeria. Therefore the objective of this study is to determine the most effective concentrations of ethanol and lime extracts for *Irvingia* seeds preservation to avoid post-harvest aflatoxins contaminations and spoilage.

2.0. Materials and methods

2.1. Sample Collection

Irvingia gabonensis(Ogbono) fruits were purchased from local market in Agbaja Izzi Local Government Area of Ebonyi State, Nigeria. A local farmer that specialized in extraction and processing of *Irvingia* kernel was contracted to extract the kernel. The extracted kernels were subsequently sorted into grades. The kernels that met the grade "A" requirement Ladipo, (2012) were used for this study.

2.2. *Irvingia* kernel Pre-treatment

The extracted kernels were divided into three sets; the first two sets were immediately pre-treated with different concentrations of Lime extract and Ethanol while the third set was left untreated as control. Kernels (I kg) that received different pre- treatment were sealed separately in jute bags before storage.

2.3. Pre-treatment process

Different concentrations (%) of ethanol and lime (50, 55, 60, 65 and 70), 40 ml each were used to pre-treat 400g of freshly harvested *Irvingia* seeds. The different ethanol concentrations were sprayed on the seeds, sun dried and stored.

2.4. Storage Conditions

The samples were stored on shelves, indoors. Room temperature was between (29 ± 2°C) and relative humidity of 84% - 92%, during the storage period of six months.

2.5. Analysis for physicochemical quality characteristics

2.6.1. Proximate Analysis

2.6.1.1. Determination of Moisture Content

Moisture content was determined by the Gravimetric method. A measured weight of each sample (5g) was weighed into a cleaned, dried Petri dish. The dish and sample were dried in an oven at 105^{O}C for 3 h at the first instance. It was then cooled in a desiccator and reweighed. The weight was recorded while the sample was returned to the oven for further drying. The drying, cooling and weighing continued repeatedly until a constant weight was obtained. By the difference, the weight of the moisture loss was determined and expressed as a percentage.

It was calculated as shown below;

$$\% \text{ Moisture Content} = \frac{W_2 - W_3}{W_2 - W_1} \times \frac{100}{1}$$

Where; W_1 = Weight of the empty Petri dish

W_2 = Weight of the dish and sample before drying

W_3 = Weight of the dish and sample after drying to a constant weight

2.6.1.2. Determination of crude Protein

The protein content of the sample was determined using the Kjeldahl method. The total Nitrogen was determined and multiplied by the factor 6.25 to obtain the protein content. Five grams (5g) of the grounded *Irvingia* seeds was weighed into the Kjeldahl digestion flask. A tablet of Selenium catalyst was added to it. Concentrated H_2SO_4 (10 ml) was then added to the flask and digested by heating it under a fume cupboard until a clear solution was obtained. Then it was carefully transferred to a 100ml volumetric flask and made up to mark. A 100ml of the digest was mixed with equal volume of 45% NaOH solution in Kjeldahl distillation apparatus. The mixture was distilled and the distillate collected into 10ml of 4% boric acid solution containing mixed indicator methyl red bromocressol. A total of 50ml distillate was collected and titrated against 0.02N H_2SO_4. The crude protein was obtained by multiplying the nitrogen value by a factor of 6.25.

$$\% \text{ Crude Protein} = \frac{\text{Titre value} \times 50 \times 5.46}{\text{Weight of the sample}} \times \frac{100}{1}$$

2.6.1.3. Determination of Carbohydrate

The carbohydrate content of sample was determined by estimation using the arithmetic difference method. The carbohydrate content was calculated and expressed as the Nitrogen free extract as shown below:

% CHO = 100 - % (a + b + c + d + e)

Where; a = Protein

b = Ash

c = Fat

d = Crude fibre

e = Moisture content

2.6.1.4. Determination of Ash and crude fibre

The method of AOAC, (1995) was used to determine the ash and crude fibre contents of the sample.

2.6.1.5. Extractable colour measurement

The determination was carried out according to the method proposed by the American spice trade association (ASTA 1995). The ground seeds (1g) was mixed in 10ml acetone and allowed to stand for 16 h underambient temperature. The aliquot of the solution was used for the spectrophotometric measurement at 460nm. The absorbance was recorded as displayed on the spectrophotometric screen.

2.6.1.6. Determination of changes in Sliminess

The sliminess of the stored *Irvingia* kernel was determined as viscosity with the aid of a Rotary digital viscometer (NDJ – 85) China using spindle 2 at 30 rpm.The mucilage from the *Irvingia* kernels was extracted with the boiling water at 100^{o}C using *Irvingia* kernel flour to water ratio of 1:40 (W:V). The extraction was carried out by stirring the mixture. The mixture was left to cool at room temperature. The mixture was then centrifuged at 4500 rp 30m and filtered through cotton wool. The extract was transferred into a beaker and placed on the rotating spindle and the values of the viscosity of the extract from the *Irvingia* kernel displayed on the LCD screen was read in pascal per second (Pa.S).

2.6.1.7. pH determination

The pH of the samples were determined using highly sensitive digital pH meter (Montini 095, Romania).Two grams of each of the samples(ground into paste) were measured into a Cylindrical glass container containing 20ml of distilled water. The mixture was stirred and allowed to stand for about 1h. The pH was determined at

temperature of 26^{O}C to 29^{O}C by dipping the pH meter tip into the sample solution and the pH of the solution read off.

2.6.1.8. Determination of concentration of free fatty acid (FFA)

The concentration of FFA was determined using the standard analytical methods for fats and oils as recommended by American oil chemists' society (AOCS, 1995). The oil was first extracted from the ground *Irvingia* kernel using petroleum ether. One gram of the extracted oil was measured into a 250ml conical flask and 25ml of absolute ethanol (99.5% w/v) was also measured and added. Two drops of phenolphthalein indicator was added and the titration was done with 0.1M NaOH. The percentage FFA value was calculated from the equation below.

$$A = \frac{V \times M \times W}{m} \times 100$$

Where A is the % FFA
V is the volume of NaOH used (ml)
M is the molarity of the NaOH used (mol/1000ml)
W is the average molecular weight of the fatty acid (myristic acid) component in the oil
m is the mass of the extracted *Irvingia* kernel oil used.

2.7. Total viable count

Ten- fold serial dilution and pour plate method were used for microbial enumeration. Saboraud Dextrose Agar was prepared according to manufacturer's instruction (BIOTECH India) and autoclave for 15minutes at 121'C and 15psi. The prepared media was allowed to cool to about 40°C in a water bath and was then poured into sterile petri- dishes containing 1 ml aliquot of the appropriate dilutions (normal saline as diluents). The samples solutions were prepared by adding 1 g of the sample into 10 ml of normal saline. The plates were incubated for 3 d at 28 ± 2°C and colonies formed were counted and expressed in colony forming unit per gram CFU/g.

2.8. Identification of fungal isolate

Fungal isolates on SDA plate suspected to be *Aspergillus flavus* following macroscopic observation, lacto phenol blue staining and comparison with different fungal Atlases (Azi et al., 2016; Nwankegu et al., 2016) were identified to species level at Centre for Agriculture and Biotechnology International (CABI) Microbial Identification Services (United Kingdom, Bakem Lane, Egham Surrey TW20 9TY, UK) where internally transcribe spacer (ITS), partial calmodulin and transcriptional elongation factor (TEF) rDNA sequencing analyses were used for the identification.Briefly, a unique CABI reference number (IMI number 504738) was assigned to the sample.The original sample was subjected to purity check. All procedures were validated and processing undertaken in accordance with CABI's in – house method as documented in TPs 61 – 68 and TP70. Procedures involved the following steps: Molecular assays were carried out on the sample using nucleic acid as a template. A proprietary formulation [microLYSIS®-PLUS (MLP) Microzone, UK)] was subjected to the rapid heating and cooling of a thermal cycler, to lyse cells and release deoxyribonucleic acid (DNA). Following DNA extraction, polymerase chain reaction (PCR) was employed to amplify copies of the rDNA invitro. The quality of the PCR product was assessed by undertaking gel electrophoresis. PCR purification step was carried out to remove unutilized dNTPs, primers, polymerase and other PCR mixture compounds and obtained a highly purified DNA template for sequencing. This procedure also allowed concentration of low yield amplicons. Sequencing reactions were undertaken using BigDye® Terminator v3.1 kit from Applied Biosystems (Life Technologies, UK) which utilizes fluorescent labeling of the chain terminator ddNTPs, to permit sequencing. Removal of excess unincorporated dye terminators was carried out to ensure a problem-free electrophoresis of fluorescently labelled sequencing reaction products on the capillary array AB 3130 Genetic Analyzer (DS1) DyeEx™ 2.0 (Qiagen, UK). Modules containing prehydrated gel-filtration resin were optimized for clean-up of sequencing reactions containing BigDye® terminators. Dye removal was followed by suspension of the purified products in highly deionized formamide Hi-Di™ (Life Technologies, UK) to prevent rapid sample evaporation and secondary structure formation. Samples were loaded onto the AB 3130 Genetic Analyzer and sequencing undertaken to determine the order of the nucleotide bases, adenine, guanine, cytosine, and thymine in the DNA oligonucleotide. Following sequencing, identifications were undertaken by comparing the sequence obtained with those available in European Molecular Biology Laboratory (EMBL) via the European Bioinformatics Institute (EBI).

2.9. Aflatoxin analysis

Determination of total aflatoxin on the *Irvingia* samples was done by the use of Enzyme link immunosorbent assay (ELISA) Method. Extraction of the aflatoxin was donewith Tween- ethanol. The sample was first ground into fine powder. Tween- ethanol (25 ml) was added to 5 g of the sample and mixed properly. The sample solution was then centrifuged at 250 rp 3 mins. The centrifuged sample was filtered with Watman1 filter paper.

Aflatoxin conjugate (200 micro liter) was dropped in a clean mixing wall and 100 microliter of the sample analyte was added. The mixture of the aflatoxin conjugate and the sample was then transferred into antibody incubated micro-walls and incubated under dark cover at room temperature for 15 mins. This process was allowed for the antibody/antigen reaction to take place. After the incubation the solution was then washed off 5 times using deionized water and then 100 microliter of the substrate was added and allowed to stand for 5mins. Finally a stop solution was added and the result read with ELISA machine.

2.10. Data analysis
Each set of data in the experiments conducted was collected in three replicates and the analytical result was the mean of three data sets. The standard deviations (error bars) and statistical differences (5% level of significance) were analyzed by using GraphPad Prism 6® software tial version (GraphPad Software, CA, USA) and SPSS version 21 (ANOVA).

3.0. Results
3.1. Effects of ethanol and lime extracts pre-treatment on the Aflatoxins
The result showed that there was no aflatoxin contamination on the pre-treated samples for the first three and two months in ethanol and lime extracts pre-treatment respectively (Table 1). However there was aflatoxins contamination for the samples pre-treated with 50% - 65% ethanol and lime extracts starting from the fourth month of the storage period. The aflatoxins levels increased with increase in storage time but decreased with increased in ethanol and lime extracts concentrations. The aflatoxins levels of the pre-treated samples were however lower than the untreated samples(control). The *Irvingia* seeds pre-treated with 70% ethanol had no aflatoxins contamination from the beginning to the end of the storage period while the sample pre-treated 70% lime extracts had an international acceptable levelof aflatoxins (3.02 %) at the end of the storage time.

3.2. Effect of ethanol and lime extracts pre-treatment on the fungal population of the *Irvingia* samples
There was no fungal growth on the ethanol and lime extracts pre-treated samples at the end of the first month of storage (Table 2). There was fungal growth from the second month of storage and the fungal population increased with increase in storage time but decreased with increased in concentration of the ethanol and lime extracts. The fungal population of the pre-treated samples were however lower than the control sample throughout the storage period. The result of molecular identification showed 99.8% identity to ITS sequence from multiple strains of *Aspergillus flavus* (FR33818) and several strains of *A. oryzae*.

3.3. Effect of ethanol and lime extracts pre- treatment on the proximate composition of the *Irvingia* before storage
The Proximate composition (Table 3) revealed that there was decrease in moisture and fat content of the *Irvingia* seeds with increased in ethanol/lime extracts concentrations while the carbohydrate, ash, crude protein and crude fibre contents increased with increased in ethanol/lime extracts concentrations of the pre-treated samples. The moisture and fat content of the pre-treated samples were lower than the control sample while the carbohydrate, ash, crude protein and crude fibre of the pre-treated samples were higher than the control sample at the end of the storage.

3.4. Effect of ethanol and lime extracts pre-treatment on the colour of the *Irvingia* seeds
The colour of the *Irvingia* seeds pre-treated with ethanol and lime extracts remained unchanged at 0.032% and below 0.045% respectively for the first two month of the study period (Table 5). There was change in colour of the *Irvingia* seeds from the fourth month but the colour change decreased with increased in ethanol and lime extracts concentrations. The result further revealed that the colour change of the pre-treated samples were significantly lower than the control at the end of the study period.

3.5. Effect of ethanol and lime extracts pre-treatment on the pH of the *Irvingia* seeds
The pH (Fig 1) of the ethanol pre-treated samples increased (increased alkalinity) with increased ethanol concentrations while the pH of lime extracts pre-treated samples decreased with increased in lime extracts concentrations. The pH of the different pre-treated samples was stable for first two month of the storage but decreased from the fourth month of the storage. The pH of the pre-treated samples was higher than the control (untreated sample) throughout the storage period.

3.6. Effect of ethanol and lime extracts pre-treatment on Free Fatty Acid (FFA) of the *Irvingia* seeds
The percentage free fatty acid concentration (Fig 3) of the ethanol and lime extracts pre-treated samples were (0.24) and below 0.29% respectively for the first two month of the storage. The FFA level of the samples pre-treated between 50-65% ethanol and lime extracts concentrations increased from the fourth month of the storage

period. The FFA level of the sample pre-treated 70% ethanol was the same (0.24) throughout the storage period while the FFA of the sample pre-treated with 70% lime extracts was 0.29%. The FFA level of all the pre-treated samples was significantly lower than the control sample at the end of the storage period.

4.0. Discussion

Irvigia kernels are prone to postharvest fungal spoilage whilst in storage. This study revealed that the severity of the postharvest fungal spoilage of *Irvingia* seeds during in storage were influenced by the pre- treatments used. Thus the fungal populations and the concentrations of aflatoxins present in the stored *Irvingia* seeds were observed to be dependent on the concentrations of the pre – treatments used and were significantly lower than the control sample.

The ethanol pre-treatment showed that the different concentrations of ethanol pre-treatment were able to prevent aflatoxins production on the *Irvingia* seeeds for the first two months of the storage period. However aflatoxins were produced from the third month for *Irvingia* seeds pre-treated with 50 – 65% ethanol while *Irvingia* seeds pre-treated with 70% ethanol recorded no aflatoxins throughout the storage period. This implies that pre-treating *Irvingia* seeds with 70% ethanol could be very effective in the preservation of *Irvingia* seeds before storage. The effectiveness of 70% ethanol concentration in preventing post-harvest aflatoxin contamination of *Irvingia* seeds could be due to the ethanol's ability to significantly reduce the moisture content of the *Irvingia* seed thereby inhibiting the fungal growth and subsequent aflatoxin production. This is similar to the work of Adebayo-Tayo et al., (2006); Ebimieowei and Dorcas, (2012), in which they reported that the population of toxigenic fungi present on stored Irvingia seeds where influenced by the chemical treatment applied. The findings of this research also further revealed that concentrations of the detectable aflatoxins decreased with increased in ethanol concentrations. In a similar study aflatoxins B_1, B_2, G_1 and G_2 in Italian piadina/ peanut-based products/ hazelnuts and dried figs were reduced by the applications of isothiocyanates/drying (Federica et al.,2016; Nur. et al., 2016; Bulent. 2016) .The result of this finding also showed that all the concentrations of ethanol used were able to keep the aflatoxins concentrations below 10ppb which is the maximum acceptable limit of aflatoxins in food meant for domestic consumptions according to National Agency for Food and Drug Administration and Control (Ebimieowei and Dorcas, 2012).

Lime extract pre- treatment showed that the different concentrations of lime extract were able to prevent aflatoxins production on the *Irvingia* seeds within the first two month of level on the *Irvingia* seeds. The result further revealed that the different concentrations of the limit extracts used were able to keep the aflatoxins level below 10ppb within the first four month of the storage. The levels of total aflatoxin in the food commodities were generally below the maximum allowable limits (10ppb for food meant for domestic consumption) specified by the European Commission (AESAN, 2011), which is also currently being used by the National Agency for Food and Drug Administration and Control (NAFDAC), in Nigeria. This observation agrees with the findings of previous studies (Adebayo-Tayo et al., 2006; Williams et al., 2015; Ebimieowei and Dorcas, 2012; Federica, et al., 2016). However, the levels of total aflatoxins were found to be above 10ppb in the samples pre –treated with lime extract after five months of storage. This similar to the report of Ebimieowei and Dorcas, (2012) in which the level of toxigenic fungi in *Irvingia* seeds were found to be above acceptable limit after three months of storage. Lime extract has a characteristic acid nature that significantly reduced the pH of the *Irvingia* seeds thus could have been the factor in inhibiting the growth and multiplication of the toxigenic fungi and its ability to produce aflatoxins. The result showed that at 65-70% lime extract pre-treated*Irvingia* seeds could be stored beyond six month without post-harvest aflatoxins contaminations. The control sample (*Irvingia* seeds without pre – treatment) showed a total aflatoxin content of 42.10ppb at the end of the storage period. These quantities of aflatoxin in the *Irvingia* sample were above the 10ppb maximumpermissible limit in food as recommended by the National Agency Food and Drug Administration and Control (NAFDAC) (Williams et al., 2015). This invariably portend a great health risk to consumers of *Irvingia* seeds as ingestion of this level of aflatoxin in food have been directly link with liver cancer or even acute death (Dorner et al*.,* 1999).

The fungal population increased with increase in storage time for both the control and the samples pre – treated with varied concentrations of ethanol and lime extracts. There was significant difference between the fungal population of the control sample and the pre-treated samples ($P<0.05$). This implies that varied concentrations of pre-treatment applied had significant effect on the fungal populations of the *Irvingia* samples. However samples pre – treated with 70%ethanol and 70% lime extracts had the lowest fungal population while the control sample had the highest fungal population at the end of the storage period. The effectiveness of 70% ethanol and lime extract in significantly reducing the fungal populations of the *Irvingia* seeds when compared with the control could be as a result of the ethanol's ability to reduce the moisture content of the *Irvingia* seeds and the lime extracts' ability to alter the pH of seeds making growth and multiplication of the fungi cells difficult (Nwachukwu et al., 2007). The effectiveness of this concentration of ethanol and lime extract in inhibiting the multiplication of these toxigenic fungi even at a moderate storage temperature (29-33^{0}C) and relative humidity (84-92%) shows the efficacy of these pre-treatments as potent post-harvest *Irvingia* seeds preservatives. This

result is similar to the findings of Ebimieowei and Dorcas, (2012) in which they reported that the fungal populations of stored *irvingia* kernels were greatly influenced by pre – treatment with 0.9%NaCl and 3%$KHCO_3$. The high levels of total aflatoxins and fungal populations found in the control sample (*Irvingia* seeds without pre-treatments) and the samples pre-treated with less than 70% ethanol and lime extracts in this study may be as a result of high storage temperature and relative humidity as well as the moisture contents and the *Irvingia* seeds chemical composition. These factors are interrelated, as certain temperatures and moisture levels increases the activities of endogenous enzyme and microbes which in turn speed up spoilage. Studies have shown that temperature favourable for aflatoxins production is 25-30^{0}C and relative humidity of 97-99% (Simsek et al., 2012). If both temperature (20-38^{0}C) and moisture are favourable for *Aspergillus flavus*, aflatoxin can be produced within 48 h (Williams et al., 2015; Bulent, 2016).

Pre-treatments of the *Irvingia* seeds with ethanol significantly affected the chemical composition of the *Irvingia* seeds when compared with the untreated sample. This change in the chemical composition of the pre-treated Irvingia samples may be as result of reduction in the moisture content of pre-treated *Irvingia* samples which in turn reduced the fat content of all ethanol pre-treated samples. It could also be for the same reason of reduced moisture content that led to increase in protein, carbohydrate, ash and crude fibre contents of the pre-treated *Irvingia* samples compared with control. Reduction in moisture content has been observed to be a major factor in changing the chemical composition of foods (Egan et al., 1981). In research conducted by Giami et al., (1981), they reported that there was decreased in fat and increased in protein content of heat-treated *Irvingia* flour as a result of reduction in moisture content of the flour. The result of this study further showed a decreased in the proximate composition of the *Irvingia* seeds with increase in storage time. This could be as a result of increase in microbial activities in the *Irvingia* seeds with increase in storage time which in turn reduced the nutrient content of the seeds. Increased in microbial populations in stored *Irvengia* kernel have been linked with decreased in physical and chemical qualities of stored *Irvingia* seeds (Onyeike et al., 1995; Akusu and Kiin-Kabari , 2013).

The pre-treatment of the *Irvingia* seeds with different concentrations of lime extracts had similar effect on the proximate composition of the seeds as ethanol pre-treatment. The pre-treatment decreased the moisture and fat contents of the seeds but increased the carbohydrate, ash, protein as well as the fibre content of the *Irvingia* seeds (Table 4). The significant change in the proximate composition of the pre-treated *Irvingia* seeds when compared with the control sample could have been as a result of the chemical composition of the *Irvingia* seeds. Lime extracts contains among others volatile compound such as d-Limonene and β-Pinene, these compound could have helped in increasing the rate of evaporation of the *Irvingia* seeds during sun drying thus leading to decreased moisture content (María et al., 2012). The reduction in the nutritional quality of the lime extracts pre-treated seeds with increase in storage time could be as a result of increased in microbial activities in the seeds samples. However it is important to note that the nutritional quality of the control sample was significantly lower than all the lime extracts pre-treated samples and more particularly sample pre-treated with 70% lime extracts had the best nutritional quality at the end of the storage. Lime extracts contains organic acids such as malic acids, lactic acids and acetic acids and these acids have been shown to exhibits inhibitory effect against some food spoilage organisms and thus could have helped in preserving the nutritional quality of the *Irvingia*seeds (Berhow et al., 1994; Onyeike et al., 1995).

The pre-treatment of the Irvingia seeds with ethanol improved the stability of the colour of the *Irvingia* seeds for longer period of time compared to the control (Table 5). The absorbances of the pre-treated samples were significantly lower than the control sample with the colour stability increasing with increased in ethanol concentration. The better colour quality of the ethanol pre-treated samples could be as a result decreased microbial activities in the pre-treated *Irvingia* samples compared to the control. Results of similar work have shown that *Irvingia* kernels displayed on shelves for sale in Nigeria market are contaminated with spoilage fungi which often grow and impact different colours on the *Irvingia* kernel during storage (Onyeike et al., 1995;Adebayo-Tayo et al., 2006; Ladipo2012).

Pre-treatment of the *Irvingia* seeds with lime extracts altered the colour chemistry of the *Irvingia* seeds as the pre-treated samples were more coloured than the control sample at the beginning of the storage. However the colour of the pre-treated samples was more stable compared to the control samples with increase in storage time thus at the end of the storage period the lime extracts pre-treated samples were significantly less coloured than the control sample. Lime contains some chemical compounds which perhaps altered the colour of the *Irvingia* seeds at the beginning but because of the preservative quality of the lime extracts it was able to significantly preserve the seeds from microbial attacks thus ensuring better colour stability as storage lasted. *Irvingia* seeds with less spoilage have been shown to have better chemical and sensory quality (Akusu and Kiin-Kabari, 2013; Ebimieowei and Dorcas, 2012).

Sliminess is a major quality characteristics of an *Irvingia* seed as consumers of the seed prefers the seed with better sliming ability (drawability). The finding of this study showed that pre-treatment of the *Irvingia* seeds with ethanol significantly improved the rheological behavior of the seeds measured as viscosity throughout the

storage period when compared with the control sample. The sliminess increased with increased in ethanol concentration and remained stable for the two month of the storage period for the samples pre-treated with 50-65% ethanol while the sample pre-treated with 70% ethanol was stable throughout the six month of this study (Table6). Sliminess in food such as okra and *Irvingia* seed is as a result of sugar residues called exopolysacharrides/*mucilage* and proteins called glycoproteins, the mucilage's viscosity increases when heat is applied. (Cui et al., 1993; Anderson and Fireman, 1995; Anderson and Lowe, 1947; Zhang et al., 2016). Pre-treatment with ethanol increased the carbohydrate content of the *Irvingia* seeds and thus could be the reason for increased sliminess of the pre-treated samples with increasing ethanol concentrations. The decrease in sliminess with increased storage time could be as result microbial spoilage. This is similar to a work done by Akusu et al. (2013) in which they reported a general decrease in viscosity (sliminess) of ogbono flour over a storage period of six weeks as a result of microbial spoilage.

Pre-treatment of the *Irvingia* seeds with lime extracts also significantly improved the sliminess of the *Irvingia* seeds when compared with the control sample. The sliminess increased with increased in lime extracts concentrations and was stable for two month except for sample pre-treated with 70% lime extracts that was stable for the four month of the storage period. It could be attributed to increase in carbohydrate content of the *Irvingia* seeds as a result of the lime extracts pre-treatment. Lime extracts contains organic acids that with preservative ability against spoilage organisms (Berhow et al., 1994), and this could be the reason for the significant level of stability shown by the pre-treated samples as compared with the control throughout the storage period.

The significant increase in the pH (more alkalinity) of the ethanol pre-treated samples compared with the control sample could be as a result of the chemical composition of the ethanol. Ethanol contains an –OH group and that would have reacted with other chemical components of the *Irvingia* seed leading to increase in the seeds' pH. There was significant reduction in the pH of the control sample with increased in storage time compared with the pH of the pre-treated samples that were relatively stable throughout the storage period. Decreased pH in is a characteristics of food spoiled by fungi (Doyle, 2007). Fungi produce acidic metabolite when they grow in foods leading to significant decrease in the pH of the affected food. This could be the reason for the significant reduction in the pH of control sample at the end of the storage period.

The significant reduction in the pH of lime extracts pre-treated samples compared to the control sample could be as a result of the organic acids content of the lime extracts. Lime extracts contains organic acids and other volatile compounds that could significantly affect the chemical composition/pH of foods (María et al., 2012; Berhow et al., 1994). The pH of the pre-treated samples were stable throughout the first fourth month of the storage period but reduced slightly at the six month compared with control sample that recorded significant reduction in pH throughout the storage period. The significant reduction in pH shown by the control sample throughout the storage period could be as a result of microbial spoilage (Doyle 2007; Ebimieowei and Dorcas, 2012).

The Free Fatty Acid(FFA) content of both the *Irvingia* seeds pre-treated with ethanol and lime extracts increased with increased in storage time but however were significantly lower than the control sample throughout the study period. The FFA of the *Irvingia* samples pre-treated 70% ethanol and lime extracts (which was the best performing ethanol and lime extracts concentrations) rose from 0.00 to 0.24% and0.29% respectively at the end of the storage period while that of the control sample rose from 0.00 to 5.45%. The presence of high levels of FFA in food is often an indication of rancidity. Akusu et al. *(*2013) reported that the acidity and a rancid taste often begin to be noticeable in foods when the concentration of free fatty acid is about 0.5 to 1.5%. American Oil Chemists' Society, (2004) recommend that the maximum acceptable level of FFA in crude extracted oil from plant source meant for domestic consumption should be between 0.0 and 3%. This is because consumption of food high in free fatty acids has been widely reported to have direct link with cardiovascular diseases (Babalola et al., 2011; Wang et al., 2016). The present study showed that both ethanol and lime extracts pre-treated samples had FFA levels below 0.5% throughout the study period. This implied that the oil quality of the pre-treated samples were within the internationally acceptable levels throughout the study period unlike the control sample that its FFA level (5.46) was above AOCS's acceptable limit. This further suggests that ethanol and lime extracts could be a very effective substance for preservation of *Irvingia* kernel before storage.

5.0. Conclusion

The findings of this research showed that pre-treatments of *Irvingia* seeds with 70% ethanol and lime extracts significantly improved the chemical and microbial quality of the seed during storage. The pre-treatment of the seed with 70% ethanol concentration inhibited the growth of *A. flavus* and consequently prevented aflatoxins production throughout the study period. The chemical/functional quality index of the *Irvingia* seed such as the viscosity, pH, FFA, moisture and colour were significantly improved during the storage period compared to the untreated *Irvingia* seeds. This suggested that pre- treatment of *Irvingia* seed with 70% ethanol may represent

alternative approach for *Irvingia* seeds preservation not just to avoid aflatoxins contaminations but also to improve the overall organoleptic property of the seed during storage. Pre-treatment with lime extracts significantly inhibited the growth of *A.flavus* and reduced the capacity of the mould to produced aflatoxins. The FFA, viscosity, colour and moisture content and overall quality characteristics of the seed pre-treated with lime extracts were better and more stable compared with the untreated *Irvingia* seed throughout the study period. It can then be said that the used of lime extracts which is known to have no residual harmful effect on human would be a better option in the preservation of *Irvingia* seed.

It is therefore recommended that *Irvingia* seed be pre – treated with 70% ethanol and lime extracts concentrations before storage as this will not only improve the chemical quality characteristics of the *Irvingia* seed during storage but will also guarantee the health and safety of the people that consume the seed.

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Conflict of interest
Authors declared that there is no conflict of interest

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Table 1
Effect of ethanol and lime extract pre-treatments on aflatoxin level

Month		ethanol (pbb)					Lime extract (pbb)				
	Control	50	55	60	65	70	50	55	60	65	70
0	0.00 ± 0.0	00.00 ± 0.0	0.00± 0.0	0.00 ± 0.0	0.00± 0.0	0.00± 0.0	0.00± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00± 0.0	0.00± 0.0
1	6.32 ± 0.2	0.00 ± 0.0	0.00 ± 0.0	0.00± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00± 0.0	0.00± 0.0
2	10.02± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.21 ± 0.3	0.00 ± 0.0	0.00 ± 0.0	0.00± 0.0	0.00± 0.0
3	19.11± 0.2	0.00± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	6.09 ± 0.6	5.55 ± 0.8	4.61 ± 0.7	2.78± 0.1	1.82± 0.2
4	35.15± 0.1	6.85 ± 0.7	5.30 ± 1.9	3.12 ± 1.1	1.65 ± 0.4	0.00 ± 0.0	8.32 ± 0.9	6.19 ± 1.2	4.97 ± 1.3	3.08± 0.9	1.99± 0.4
5	40.16 ± 0.4	8.35± 1.2	6.26 ±1.4	5.30 ± 0.3	1.92 ± 0.8	0.00 ± 0.9	. 56± 1.1	9.36 ± 1.6	8.46 ± 0.5	5.67± 0.8	2.32± 0.7
6	42.10 ± 0.5	9.29± 1.7	8.85 ± 1.3	7.81 ± 0.9	3.32 ± 0.9	0.00 ± 0.0	6.69 ± 1.5	11.56 ± 1.6	10.96 ± 0.1	7.36± 0.6	3.02± 1.1

Values are mean of triplicate analyses ± SD

Table 2
Fungal populations of ethanol and lime extracts pre-treated *irvingia* seeds

Month		Ethanol (10^3CFU/g)					Lime extract (10^3CFU/g)				
	Control	50	55	60	65	70	50	55	60	65	70
0	0.00 ± 0.0	0.00 ± 0.0	0.00± 0.0	0.00 ± 0.0	0.00± 0.0	0.00± 0.0	0.00± 0.00	0.00 ± 0.0	0.00 ± 0.0	0.00± 0.0	0.00± 0.0
1	24.0 ± 1.2	0.00 ± 0.0	0.00 ± 0.0	0.00± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.00± 0.0	0.00± 0.0
2	46.0± 0.9	16.0 ± 0.9	12.0 ± 0.5	10.0± 0.1	8.0 ± 1.1	3.0 ± 0.6	21.0 ± 0.9	13.0 ± 1.6	11.0 ± 0.7	8.0± 0.5	5.0± 0.6
3	62.0± 1.2	23± 1.3	18.0 ± 0.7	15.0 ± 0.6	6.0 ± 0.9	5.0 ± 1.2	27.0± 0.6	18.0 ± 1.8	14.0 ± 0.8	12.0± 0.1	7.0± 0.2
4	108.0± 3.1	32.0 ± 0.7	23.0 ± 1.9	20.0 ± 1.1	9.0 ± 0.4	6.0 ± 0.8	44.0 ± 0.9	31.0 ± 2.2	29.0 ± 1.3	23.0± 0.9	12.0± 0.4
5	287.0 ± 1.4	68.0± 1.2	42.0 ±1.4	37.0 ± 0.3	19.0 ± 0.8	14.0 ± 1.4	89.0 ± 3.1	67.0 ± 1.6	52.0 ± 0.5	43.0± 0.8	25.0± 1.7
6	201.0 ± 2.5	52.0± 1.7	38.0 ± 1.3	28.0± 0.9	12.0 ± 0.9	9.0 ± 0.9	83.0 ± 1.9	61.0 ± 1.3	47.0 ± 1.1	38.0± 0.6	18.0± 1.1

Values are mean of triplicate analyses ± SD

Table 3
Proximate composition of *irvingia* seeds pre-treated with ethanol at different concentrations and time

Parameter (%)	Initial (point of pre-treatment)						After six months incubation				
	Control	50	55	60	65	70	50	55	60	6570	Control
Moisture	9.35 ± 1.1	8.56 ± 0	.9 8.36± 0.5	8.00 ± 0.4	7.38± 1.0	7.26± 1.3	10.58±1.1	10.37±1.4	9.69±1.7	9.35±1.6	8.42±1.0	11.14±1.2
Fat	55.37 ± 1.2	53.22 ±1.3	50.28 ±1.6	49.86± 1.6	48.81± 1.1	47.35±2.1	56.21±2.2	56.02±1.3	55.32± 2.2	55.04±1.4	54.48±3.1	58.39±0.9
CH_2O	23.02± 0.9	23.35 ±1.2	25.93 ± 0.5	26.03± 0.1	26.96± 1.1	27.73±0.6	20.09 ±0.9	20.19± 1.6	21.17 ±0.7	21.23±1.1	22.15±0.6	18.89±2.4
Ash	3.14± 0.2	3.55± 0.3	3.65±0.7	3.85 ±0.6	3.88±0.9	4.36±1.2	3.12±0.6	3.18 ±1.1	3.28±0.8	3.36±0.1	3.68±0.2	3.06±0.7
Protein	7.34± 3.1	9.42± 0.7	9.82± 1.9	10.20± 1.1	10.86± 0.4	10.92± 0.8	8.38 ±0.9	8.56 ±2.2	8.82±1.3	9.21±0.9	9.41± 0.4	7.12± 1.4
Fibre	1.78± 1.4	1.90± 1.2	1.96±1.4	2.06± 0.3	2.31± 0.8	2.38±1.4	1.6 2±3.1	1.68±1.6	1.72±0.5	1.81± 0.8	1.86±1.7	1.40±1.7

Values are mean of triplicate analyses ± SD
CH_2O = Carbohydrate

Table 4
Proximate composition of *irvingia* seeds pre-treated with lime extract at different concentrations and time

Parameter (%)	Initial (point of pre-treatment)						After six months incubation					
	Control	50	55	60	65	70	50	55	60	65	70	Control
Moisture	9.35 ± 1.1	9.08 ± 0.9	9.00± 0.5	8.87± 0.4	8.72± 1.0	8.32± 1.3	11.11±0.3	11.01±1.4	10.59±1.7	10.18±1.6	9.01±1.0	11.14±1.2
Fat	55.37 ± 1.2	54.42±1.3	54.02±1.6	53.47± 1.6	53.28± 1.1	53.02±2.1	56.52±2.2	56.24±1.3	56.28± 2.4	56.08±0.3	55.33±3.1	58.39±0.9
CH_2O	23.02± 0.9	22.83±1.2	23.16± 0.5	23.65± 0.1	23.76± 1.1	24.09±0.6	19.11 ±0.9	19.17± 1.6	19.39±0.7	19.78±1.1	21.27±0.6	18.89±2.4
Ash	3.14± 0.2	3.26± 0.3	3.31±0.7	3.33±0.6	3.37±0.9	4.39±1.2	3.04±0.1	3.11±1.3	3.13±0.4	3.19±0.7	3.33±0.6	3.06±0.7
Protein	7.34± 3.1	9.16± 0.7	9.19± 1.9	9.23± 1.1	9.31± 0.4	9.40± 0.8	8.62±1.9	8.86±2.4	8.99±1.5	9.08±0.8	9.12± 1.1	7.12± 1.4
Fibre	1.78± 1.4	1.25± 1.2	1.32±1.4	1.45± 0.3	1.56± 0.8	1.78±1.4	1.60±2.1	1.61±1.3	1.62±0.3	1.69± 0.2	1.74±1.1	1.40±1.7

Values are mean of triplicate analyses ± SD
CH_2O = Carbohydrate

Table 5
Effect of pre-treatment with ethanol and lime extracts on colour of the *irvingia* seeds

Month	Ethanol (460 nm absorbance)						Lime (460 nm absorbance)				
	Control	50	55	60	65	70	50	55	60	65	70
0	0.03 ± 0.0	0.03± 0.0	0.32± 0.0	0.03± 0.0	0.03± 0.0	0.03± 0.0	0.04± 0.0	0.04 ± 0.0	0.04± 0.0	0.04± 0.0	0.05± 0.0
2	0.03± 0.1	0.03± 0.1	0.03±0.1	0.03± 0.1	0.03± 0.1	0.17± 0.3	0.04± 0.0	0.04± 0.0	0.04± 0.0	0.04± 0.0	0.05± 0.1
4	1.96± 0.5	0.06± 0.2	0.60± 0.3	0.46± 0.2	0.33± 0.2	0.32 ± 0.1	0.07± 0.1	0.07± 0.1	0.05±0.0	0.05± 0.0	0.05± 0.1
6	2.03± 0.1	0.08± 0.1	0.06±0.1	0.49± 0.2	0.38± 0.1	0.33± 0.1	0.09± 0.0	0.09± 0.0	0.08±0.1	0.62± 0.2	0.48± 0.1

Values are mean of triplicate analyses ± SD

Table 6
Effect of pre-treatment with ethanol and lime extracts on sliminess (viscosity) of *irvingia* seeds

Month		Ethanol (pa.s)					Lime (pa.s)				
	Control	50	55	60	65	70	50	55	60	65	70
0	0.80 ±0.3	0.82± 0.2	0.85± 0.3	0.85± 0.3	0.86± 0.1	0.87± 0.4	0.82± 0.2	0.82± 0.2	0.82± 0.4	0.82± 0.1	0.85± 0.3
2	0.80± 0.1	0.82± 0.1	0.85±0.1	0.85± 0.3	0.85± 0.3	0.87± 0.5	0.81± 0.3	0.82± 0.2	0.82± 0.5	0.82± 0.3	0.85± 0.4
4	0.70± 0.4	0.82± 0.2	0.85± 0.3	0.85± 0.2	0.85± 0.2	0.87± 0.1	0.81± 0.1	0.82± 0.4	0.82±0.2	0.82± 0.3	0.85± 0.1
6	0.69± 0.1	0.80± 0.1	0.84±0.1	0.84± 0.2	0.85± 0.1	0.87± 0.1	0.80± 0.3	0.81± 0.1	0.81±0.1	0.85± 0.2	0.85± 0.3

Values are mean of triplicate analyses ± SD

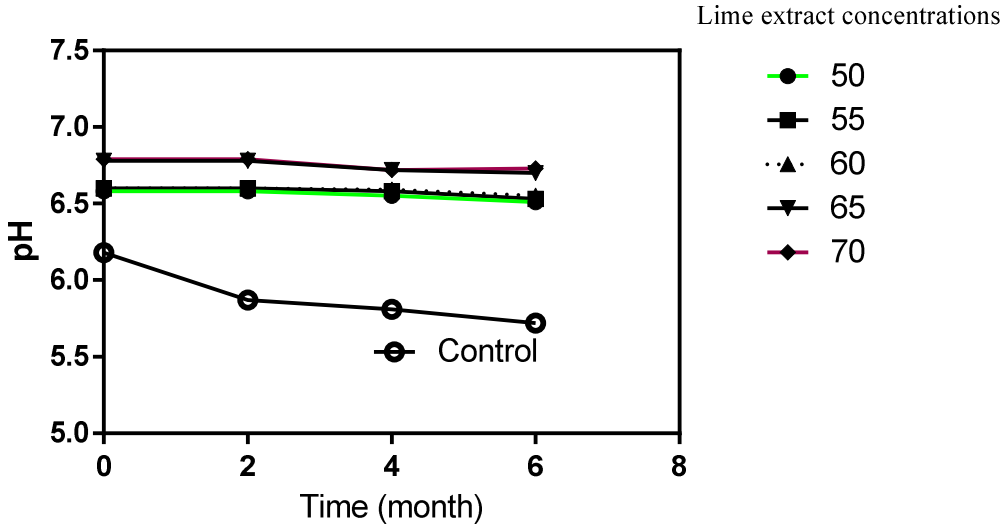

Fig.1. Effect of ethanol pre-treatment on pH of *Irvingia* seeds during six months incubation

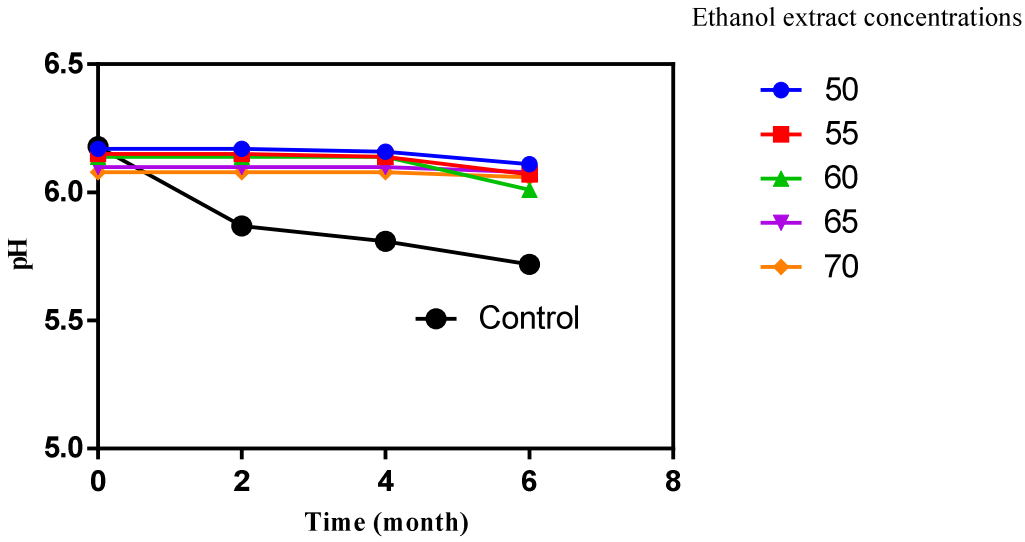

Fig.2. Effect of lime extract pre-treatment on pH of *Irvingia* seeds during six months incubation

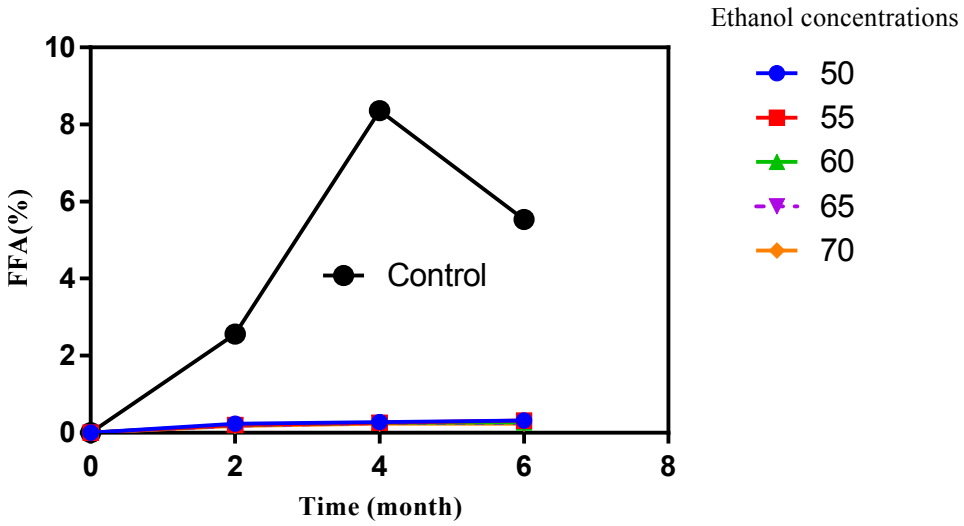

Fig.3. Effect of ethanol pre-treatment on free fatty acid (FFA)of *Irvingia* seeds during six months incubation

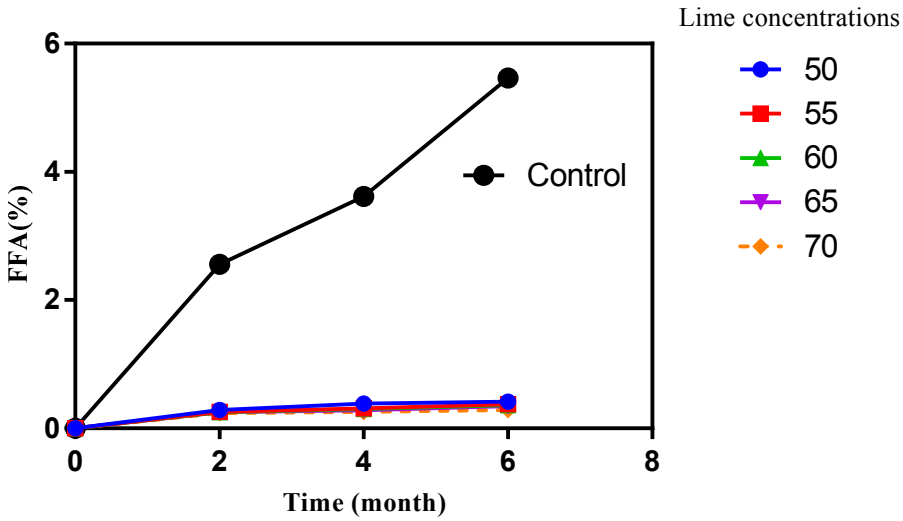

Fig.4. Effect of lime pre-treatment on free fatty acid (FFA)of *Irvingia* seeds during six months incubation