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HYDROCARBON DEGRADING POTENTIALS OF BACTERIAL ISOLATES FROM PETROLEUM REFINERY SLUDGE

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ABSTRACT
Petroleum contain fractions that are potentially toxic, carcinogenic and or mutagenic. Thus the intrinsic biodegradability of petroleum hydrocarbon and distribution in the environment of competent catabolic microorganisms are critical to the implementation of bioremediation strategies for the clean-up of petroleum polluted environments towards ecosystem and public health preservation. In the present study, the petroleum degrading potentials of bacteria species isolated from petroleum refinery sludge was evaluated. The bacterial species were isolated and identified using standard microbiological techniques. The petroleum degrading potentials of isolates was determined by a 14-day enrichment assay using basal salt medium (BSM) supplemented with 2% Escravos light crude oil (ELCO) and assessed by gas chromatography (GC). The petroleum degrading isolates were identified as *Micrococcus* sp., *Bacillus* sp., *Arthrobacter* sp., *Bacillus* sp., *Staphylococcus* sp. and *Streptomyces* sp. The mean bacterial counts during enrichment assay ranged between 0.95×10^1 cfu/ml and 9.7×10^1 cfu/ml. *Staphylococcus* sp. exhibited the highest (96.20%) total petroleum hydrocarbon (TPH) degrading potential; while *Micrococcus* sp. showed the least (35.42%) TPH degrading capacity. The isolates were also remarkable in the degradation of polycyclic aromatic hydrocarbon (PAH), removing between 4 and 10 PAH compounds after treatment in the following order (highest to lowest): *Staphylococcus* sp. >*Streptomyces* sp. >*Arthrobacter* sp. >*Bacillus* sp. >*Micrococcus* sp. >*Bacillus* sp. The study demonstrated that the bacterial isolates were remarkable petroleum degraders with great potentials for deployment as candidates in bioremediation of petroleum polluted sites in the Niger Delta region of Nigeria.

KEYWORDS: Petroleum sludge; Biodegradation; TPH; PAH; Bacteria

INTRODUCTION
Petroleum hydrocarbon remains the principal source of energy globally. Its mining, transport, storage and use for industrial and allied purposes has resulted in numerous occasions of environmental pollution with severe consequences on the ecosystem and public health (Das and Chadran, 2011). Due to the environmental and public health impact of petroleum pollution, various treatment options have been

proffered to deal with the challenge over the years. Treatment options include physical, chemical and biological methods/approaches. The physiochemical treatments options include incineration, thermal desorption, chemical oxidation, immobilization and solvent extraction (Liu *et al*., 2010). In general, such treatments are more expensive, energy intensive and unsustainable with respect to their environmental impact (Battikhi, 2014). The biological alternative, otherwise called bioremediation is more reliable, simple and cheap. Bioremediation is a technology that harnesses the metabolic capacity of microorganisms to enzymatically convert toxic chemical contaminants in the environment into harmless or less harmful products. The technology can be applied as green technologies, as it offers an environmentally friendly and cost effective response to environmental oil pollution (Chikere *et al*., 2012). Bioremediation makes use of the catabolic activities of indigenous hydrocarbon utilizing microorganisms (especially bacteria) to decontaminate oil-polluted environments (Mahmoud *et al*., 2009). Hence, knowledge of indigenous oil-degrading bacteria is critical to the search for agents that can enhance the self-purification/cleaning capacity of polluted environments.

Oil sludge is a thick, viscous mixture of sediments, water, oil and high hydrocarbon concentration often encountered during crude oil refining (Prakash *et al*., 2005). A major constituent of oil sludge is polycyclic aromatic hydrocarbons (PAH). PAHs are a group of hydrophobic organic compounds composed of two or more fused aromatic rings in their chemical structure (Hesham *et al*., 2014). An investigation into PAH concentrations in some Niger Delta ecosystems revealed elevated values of these priority pollutants in the studied environments (Ezemonye and Ezemonye, 2005). The petroleum fractions (PAHs) are reported to possess toxic, carcinogenic and mutagenic properties, thus making them be of serious environmental and public health concerns. (Hesham *et al*., 2014). The genotoxicity of PAHs to many microbes and other organisms, implies that microorganisms that are able to survive in petroleum sludge (containing high concentrations of PAH) have evolved capacity not only tolerate toxicity of the compound, but also for effective hydrocarbon degradation. This assertion was corroborated by the observation of Hesham *et al*. (2014) who reported that microbial degradation of petroleum pollutant was the most dominant and significant process for removing PAHs from the environment.

Although a number of studies (Prakash *et al*., 2005; Battikhi, 2014; Musa *et al*., 2015; Obi *et al*., 2016) have reported PAH degrading bacteria isolates from oil sludge around the world, there is dearth of information on the PAH degrading potentials of bacterial strains isolated from oil sludge of the Warri Refinery and Petrochemical Company (WRPC), Ekpan, Delta State, Nigeria. It is therefore imperative to screen WRPC refinery sludge for bacterial strains that can degrade PAH fractions of petroleum as potential candidates for bioremediation of petroleum polluted environments especially in the Niger

Delta region of Nigeria. Thus, the aim of this study was to determine the catabolic potentials of bacterial isolates from sludge of the WRPC refinery wastewater effluent in the biodegradation of target hydrocarbon.

MATERIALS AND METHODS

Sample Collection

Crude oil sludge samples were collected weekly in sterile one litre bottles from the wastewater effluent of the Warri Refenary and Pectrochehemical Company (WRPC), Ekpan, Nigeria between December, 2015 and January, 2016. The refinery is located within latitude 5º31.0'0.12''and longittude 5º45.0'0.00'' in the Niger Delta region of Nigeria. Samples were transported in cooler boxes containing ice to the Benson Idahosa University Microbiology laboratory for analysis. All samples were analysed within 24 hrs of sample collection.

Isolation and Identification of Petroleum-degrading Bacteria

The enrichment culture technique described by Odjadjare *et al*. (2008) was used (with slight modification) for the isolation of petroleum degrading bacteria, using Escravos Light Crude Oil (ELCO) as the sole source of carbon and energy. Basal Salt Medium (BSM) composition was as follows (g/L): K_2HPO_4 (1.5), NH_4NO_3 (1.0), $MgSO_4 \cdot 7H_2O$ (0.5), $CaCl_2$ (0.2), NaCl (30), KCl (0.3) and $FeCl_3$ (0.02). Five (5) grams of each sludge sample was transferred into a 250 ml flask containing 50 ml of sterile BSM broth supplemented with 2% sterile ELCO, sterilized separately in a universal bottle at 121°C for 15 mins before being aseptically added to the BSM. Control was set up comprising BSM broth supplemented with 2% ELCO without the sludge sample. The cultures were incubated on a rotary shaker at 150 rpm and 29±2 °C for 3–7 days. Aliquots of the enriched cultures were inoculated onto BSM agar supplemented with 2% ELCO and incubated at 29±2 °C for 3 to 7 days. Selected pure isolates were identified by their cultural, morphological, and biochemical characteristics with the aid of Bergey's manual determinative bacteriology (Holt *et al*., 1994).

Determination of Petroleum Degradation Potentials of Isolates

The biodegradation potentials and growth profile of the bacterial isolates were determined by inoculating 1.0 ml of standardized (OD 0.1 at wavelength of 540 nm) 24 hrs culture of each selected isolate into a 250 ml Erlenmeyer flask containing 50 ml of BSM broth (pH 7.2) supplemented with 2% ELCO as earlier described. A control flask containing only ELCO without inoculum, was also set up. The culture was incubated on a rotary shaker at 150 rpm and room temperature (29±2°C) for 14 days. At each sampling interval (48 hrs), viable bacterial count analysis was done by standard pour plate techniques (Seeley and Van Denmark, 1981). The residual total petroleum hydrocarbon (TPH) and polycyclic aromatic hydrocarbon (PAH) fractions were determined by gas chromatography (GC).

Gas Chromatography Analysis

Extraction of Residual Hydrocarbon

Thirty millilitre (30 ml) of each bacterial treated sample and control was

measured into a separating funnels. Acetone (15ml) and dichloromethane (DCM) (15ml) were added in ratio 1:1 to content of the funnel. The separating funnel was corked and agitated vigorously for 1 to 2 mins with periodic venting to release excess pressure. The organic layer was allowed to separate from the water phase for a minimum of 5 mins by opening the tap of the separating funnel. The solvent extracted was shaken vigorously and the emulsion interface between layers was more than one-third the size of the solvent layer. Mechanical techniques like stirring, filtration etc. were employed to complete the phase separation. The separated solvent was collected in a round-bottom flask and concentrated to about 2 ml. Methylene chloride was exchanged with hexane by adding 10 ml of hexane to the round-bottom flask and re-concentrated to 2 ml final volume. The extract was then transferred to a Teflon-lined screw-cap vial, labelled and refrigerated for further analysis. A blank was extracted with each batch of samples under the same conditions as for the samples, with the use of reagent water.

Determination of Residual Hydrocarbon

The residual petroleum was determined by injecting 2 µL of hydrocarbon extract onto an Agilent model Hewlett Packard 5890 series 2 gas chromatograph (GC) equipped with flame ionization detector (FID) and an HP-5 capillary column (J&W Scientific 25 m × 0.32 mm × 0.52 µm). The carrier gas was helium, set at a flow rate of 6.5 ml/min with an injector temperature of 225^{o}C. The initial oven temperature was 45 ^{o}C held for 3 mins, followed by a ramp at 12 ^{o}C/min to 225 ^{o}C where it was held for 7 mins. The total run time was 25 mins. Pure alkane standards were used to identify individual petroleum hydrocarbons and to calibrate the response of the GC at each sampling point.

Statistical Analysis

Means and standard deviations of values recorded in this study were derived using Microsoft Excel. One-way analysis of variance (one-way ANOVA) was carried out using SPSS 17.0 statistical software. Values were adjudged to be significant at 95% confidence interval ($P< 0.05$).

RESULTS

Bacterial Identity and Density during Enrichment Assay

Six strains of bacterial species (*Micrococcus, Bacillus* (two strains), *Arthrobacter, Staphylococcus and Streptomyces* spp.) were isolated and identified using their morphological, cultural and biochemical characteristics (Table 1). Bacterial cell density during enrichment assay on BSM supplemented with 2% ELCO ranged from 0.95×10^1 cfu/ml (*Streptomyces* sp.) to 9.7×10^1cfu/ml (*Bacillus* sp.) (Figure 1). The counts varied significantly ($P<0.05$) with bacterial strains.

Table 1. Morphological, cultural and biochemical characteristics of selected bacterial isolates

	Morphology	Gram Reaction	Biochemical Reactions									Sugar Fermentation Test							Probable Identity
			Catalase	Coagulase	Oxidase	Starch Hydrolysis	Citrate	Hydrogen Sulphide	Methyl Red	Urease	Vosges Proskauer	Glucose	Lactose	Sucrose	Mannitol	Xylulose	Fructose	Galactose	
	Cocci	+	+	-	+	+	-	-	+	+/-	-	+	-	-	-	-	AG	A	*Micrococcus sp.*
	Rod	+	-	+	+	-	+	-	+	+	+	AG	-	-	A	A	A		*Bacillus sp.*
	Short rods	+	+	-	-	+	+	-	+	+	+	AG	-	-	AG	AG	AG	A	*Arthrobacter sp.*
	Short rods	+	-	-	+	+	-	-	-	+	+	A	-	AG	-	-	-	AG	*Bacillus sp.*
	Cocci	+	+	+	+	-		+	-	+	+/-	A	-	A	AG	AG	AG	A	*Staphylococcus sp.*
	Rods	+	+	-	-	+	-	-	-	+	+	-	-	-	-	A	AG	G	*Streptomyces sp.*

Key: + = positive - = negative A = Acid production AG = Acid and gas production

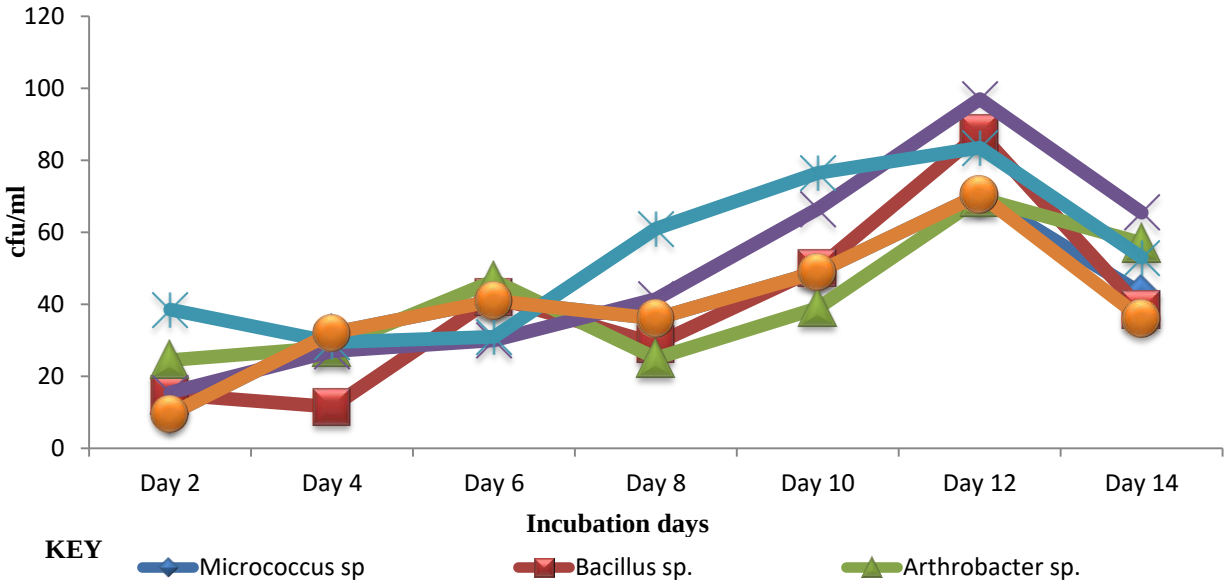


Fig. 1: Bacterial growth patterns in BSM supplemented with 2% Escravos light crude oil during enrichment assay

Determination of Petroleum Degrading Potentials of Isolates

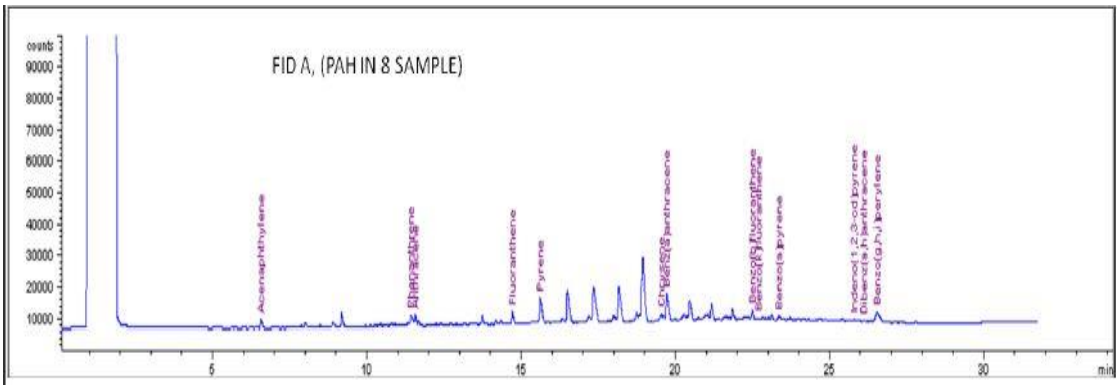
Gas chromatography analysis after the 14-day enrichment assay showed that abiotic factors accounted for loss of 46.87% of the total petroleum hydrocarbon (TPH) fraction of ELCO (Table 2). The percentage biodegradation of TPH of the ELCO ranged from 35.42% (Micrococcus sp.) to 96.20 % (*Staphylococcus* sp.). Others were 51.29%, 67.58%, 72.66% and 83.98% for *Bacillus* sp., *Bacillus* sp., *Arthrobacter* sp. and Streptomyces sp. respectively. A comparison of chromatograms of the two controls (Figure 2a (raw crude oil; Sample 8) and Figure 2b (uninoculated treated crude oil; Sample 7)) indicated that abiotic factors resulted in the removal of phenathrene and dibenz (a,h) anthracene from the raw crude (ELCO) after treatment. However, increased concentrations of indeno (1,2,3 – cd) pyrene was also observed after the treatment.

The PAH degrading potentials of the bacterial isolates were determined by comparing the chromatogram of each isolate with that of control 2. Figure 3 shows that treatment with *Micrococcus* sp. (Sample 1) resulted in the degradation of six (6) PAH compounds: acenaphthylene, phenathrene, indeno (1, 2, 3- cd) pyrene, dibenz (a,h) anthracene and benzo (g, h, i) perylene. Treatment with *Bacillus* sp. (sample 2) resulted in the removal of acenaphthylene, phenathrene, benzo(a) pyrene, indeno(1,2,3 - cd) pyrene, dibenz (a, h) anthracene and benzo (g, h, i) perylene (7 PAH compounds) (Figure 4). While *Arthrobacter* sp. (sample 3) degraded seven (7) PAH compounds including acenaphthylene, phenathrene, anthracene, fluoranthene, indeno (1,2,3 –cd) pyrene, dibenz (a, h) anthracene and benzo (g, h, i) perylene (Figure 5). However, the bacterial treatment also resulted in the introduction of fluorine into the sample (Figure 5).

Table 2. Rate of degradation of TPH components of ELCO after 14-day enrichment

Isolates	Residual TPH (mg/l)	Percentage Undegraded (%)	Percentage Degraded (%)
Micrococcus sp.	9.0195	64.57	35.42
Bacillus sp.	6.8041	48.71	51.29
Arthrobacter sp.	3.8183	27.34	72.66
Bacillus sp.	4.5288	32.42	67.58
Staphylococcus sp.	0.5306	3.80	96.20
Streptomyces sp.	2.2376	16.02	83.98
Control 1 (uninoculated treatment)	13.9674	53.13	46.87*
Control 2 (raw crude)	26.2908	Not applicable	Not applicable

a



Retention time (mins)

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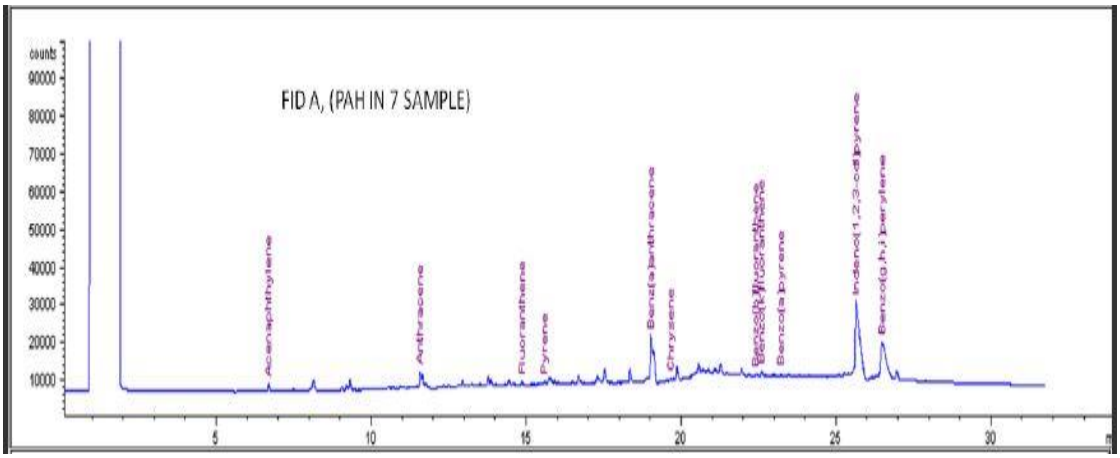

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Fig. 2: Chromatogram of PAH fractions of Controls 1 and 2

(a) Chromatogram of PAH component of raw (untreated) ELCO (control 2)

(b) Chromatogram of PAH components of uninoculated ELCO after enrichment assay (Control 1).

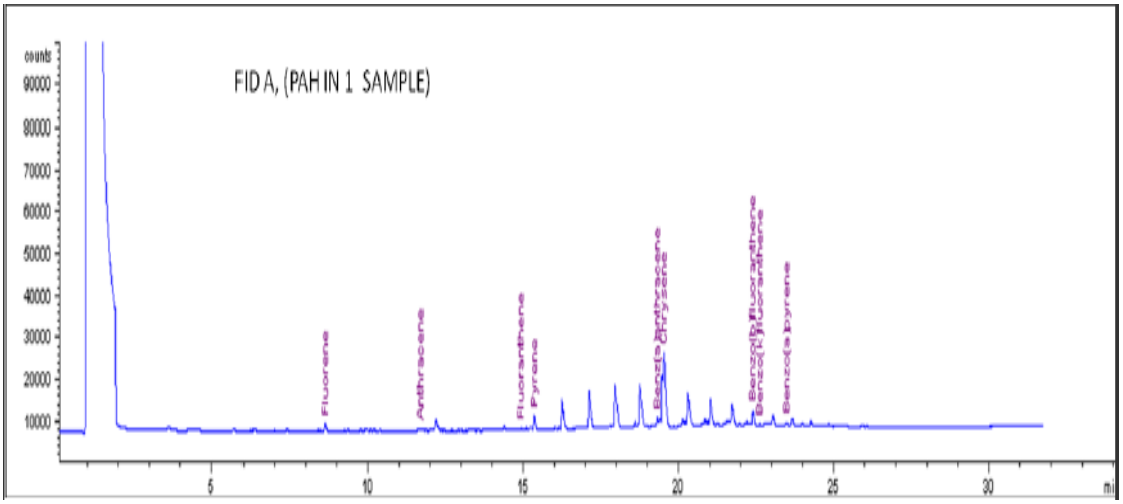


Fig. 3: Chromatogram of the PAH fraction of crude oil substrate after treatment with *Micrococcus* sp.

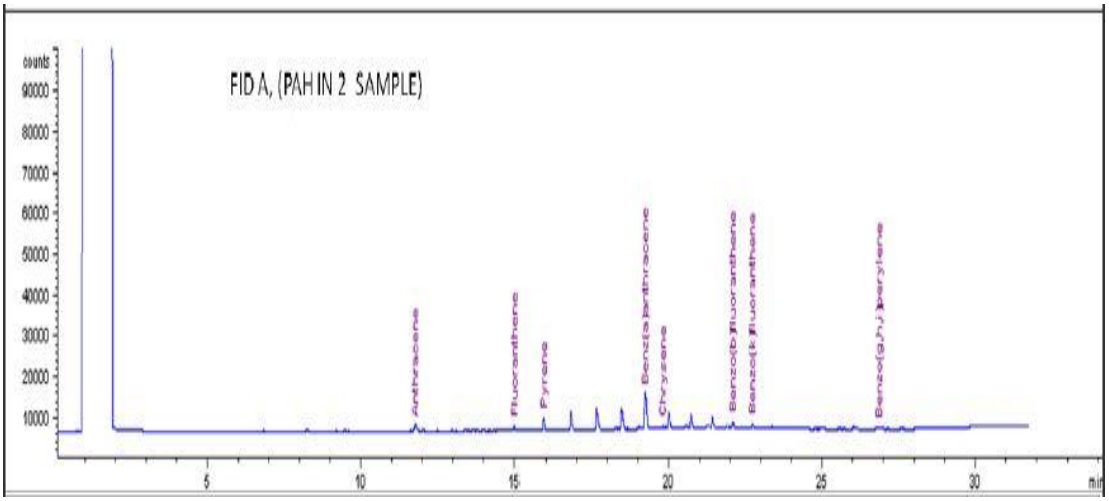
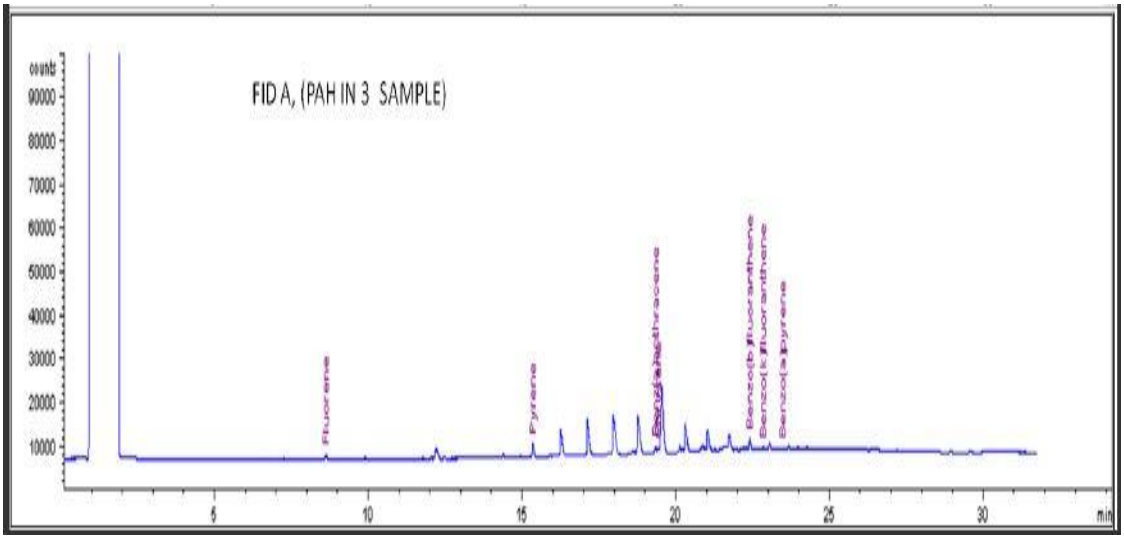


Fig. 4: Chromatogram of the PAH fraction of crude oil substrate after treatment with *Bacillus* sp.



Retention time (mins)

Fig. 5: Chromatogram of the PAH fraction of crude oil substrate after treatment with *Arthrobacter* sp.

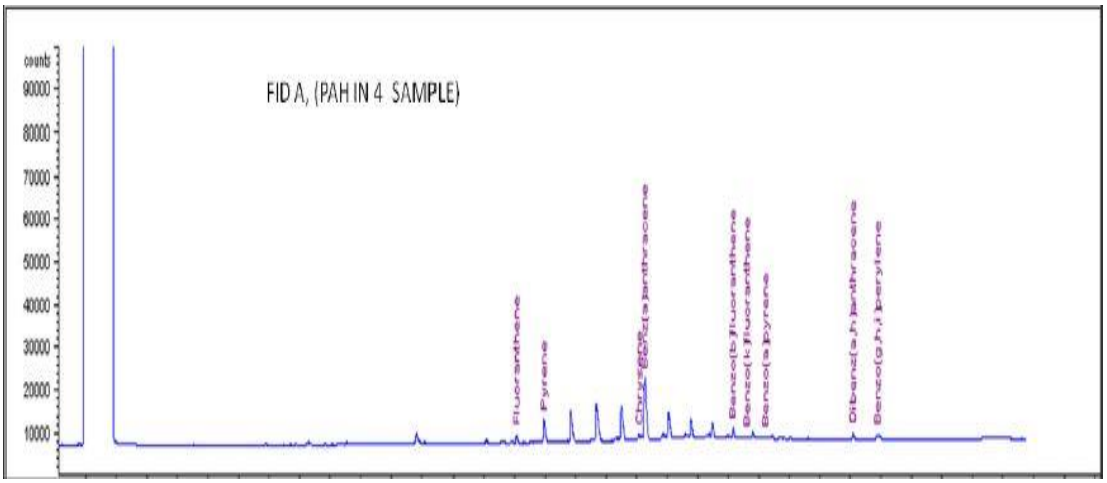
ELCO treatment with *Bacillus* sp. (Sample 4) resulted in the degradation of four (4) PAH compounds including acenaphthylene, phenathrene, anthracene and indeno (1,2,3 – cd) pyrene (Figure 6); whereas increased concentrations of fluoranthene and pyrene were observed after treatment with the isolate (Figure 6). *Staphylococcus* sp. (sample 5) degraded ten (10) PAH compounds: acenaphthylene, anthracene, fluoranthene, pyrene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, indeno (1,2,3–cd) pyrene and dibenz (a, h) anthracene after treatment (Figure 7). However, the bacterial strain caused the introduction of naphthalene and fluorine after the treatment (Figure 7). Treatment with *Streptomyces* sp. (Sample 6) resulted in the degradation of eight (8) PAH compounds including anthracene, fluoranthene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, indeno(1,2,3–cd)-pyrene and dibenz (a, h) anthracene (Figure 8). However, the treatment also resulted in the formation of fluorine in the sample (Figure 8).

Discussion

The current study was initiated to evaluate the catabolic potentials of bacterial isolates from petroleum refinery sludge. Isolates similar to those observed in this study have been previously reported in petroleum refinery sludge (Nkwelang *et al*., 2008; Mansur *et al.*, 2014, Musa *et al*., 2015; Ubani *et al.*, 2016). The bacterial cell density during enrichment were remarkably lower than that reported by Odjadjare *et al.* (2008) who observed a range of 1.76×10^8 cfu/ml to 4.26×10^{11}. The higher concentration of crude oil supplement (2%) in the current study compared to the 1% used by Odjadjare

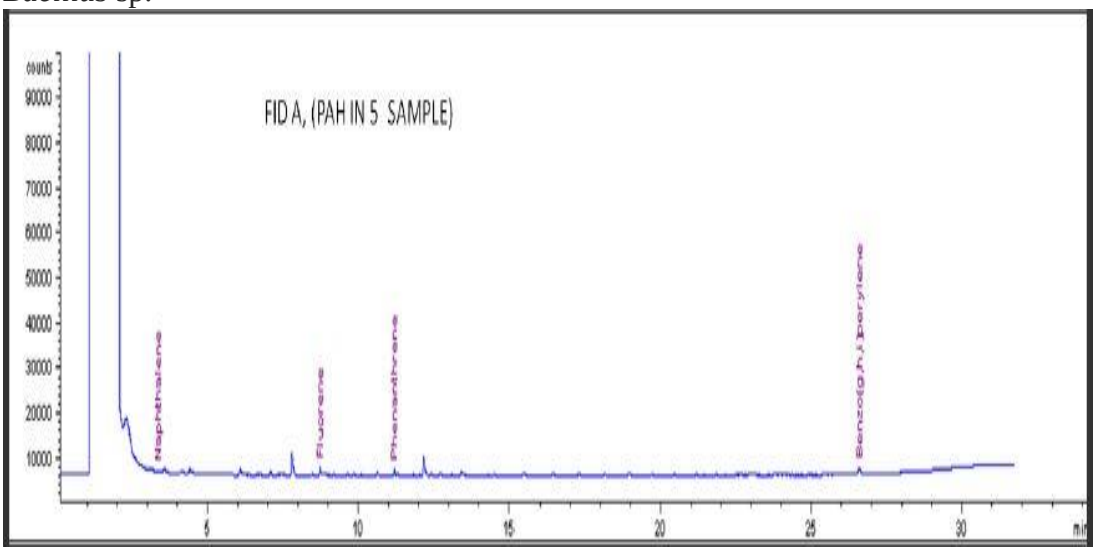
et al. (2008) could be responsible for the observed difference in cell density. High concentrations of petroleum have been reported to be toxic to microbial cells (Das and Chadran, 2011); hence the relatively higher concentration could have affected the growth potentials of the isolates.

The percentage (46.87%) of TPH fraction in ELCO lost to abiotic factors in this study is relatively higher than those reported by Atlas (1999; 30%), Sakalle and Rajkumar (2008; 15%) and Roy *et al*. (2014; 5%); but lower than that (75%) observed by Chikere *et al*. (2012).



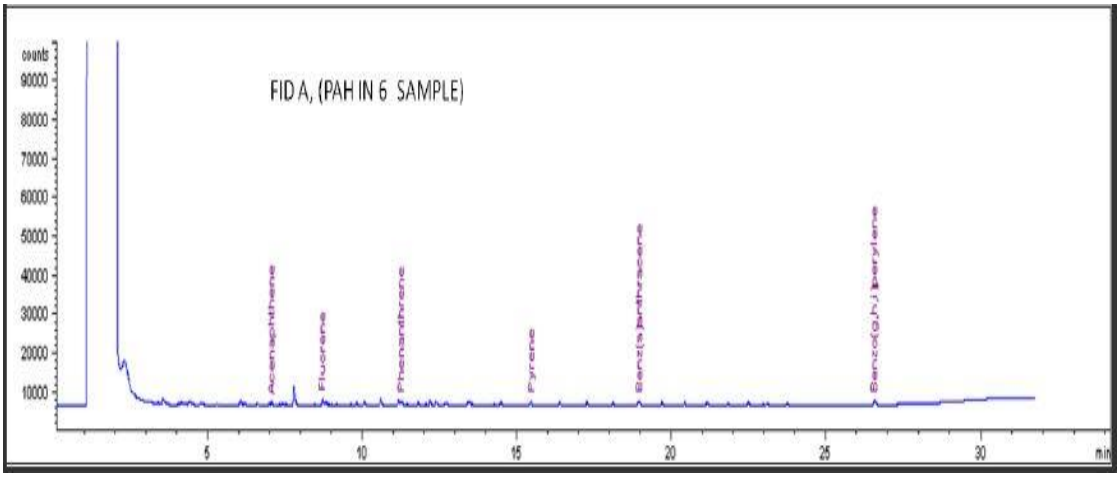
Retention time (mins)

Fig. 6: Chromatogram of the PAH fraction of crude oil substrate after treatment with *Bacillus* sp.



Retention time (mins)

Fig. 7: Chromatogram of the PAH fraction of crude oil substrate after treatments with *Staphylococcus* sp.



Retention time (mins)

Fig. 8: Chromatogram of the PAH fraction of crude oil substrate after treatments with *Streptomyces* sp.

The TPH biodegradation rates in this study were higher than those reported by Yerushalmi *et al.* (2003) (41.3% to 58.9%), but similar to rates (28.71% to 99.01%) observed by Odjadjare *et al.* (2008). The observation suggests that a good number of the test bacterial isolates in this study had remarkable petroleum degrading potentials. The difference in observed rates of biodegradation could be a function of the temperature at which the experiments were conducted. Whereas enrichment assay in the current study and that of Odjadjare *et al.* (2008) were carried out at about 30 ^{o}C, those of Yerushalmi *et al.* (2003) proceeded at 10 ^{o}C. Temperature had previously been reported to be an important factor in determining the rate of biodegradation of petroleum (Trapp *et al*., 2001).

Staphylococcus species had the best (96.20%) TPH degrading potential in this study (Table 2). The observation is consistent with the reports of Matvyeyeva *et al*. (2014) who asserted that *Staphylococcus aureus* was one of the highest biosurfactant producing bacterial strains isolated from petroleum refinery effluent in Nigeria. However, contrary to the observation of this study, Sarma and Sarma (2010) reported *Staphylococcus* strains with relatively poor (17.39%) petroleum degrading potentials.

In agreement with the observation of this study Ferradji *et al.* (2014) reported *Streptomyces* species with high petroleum degrading capacity. A relatively lower (57%) petroleum degrading potential was observed by Oaikhena *et al.* (2016) for *Streptomyces* species. *Arthrobacter sp*. in this study was also observed to be a relatively good TPH degrader (72.66%) contrary to the observation of Odjadjare *et al*. (2008) who reported ELCO degrading potentials of 37.62% by the organism. Strains of *Bacillus* spp. isolated in this study showed petroleum degrading potentials of 51.29% and 67.58%; rates that were lower than those (89.22%)

reported by Omotayo *et al*. (2012) but higher than the observation (28.27%) of Odjadjare *et al.* (2008). Although, Omotayo *et al*. (2012) reported that *Micrococcus varians* was the isolate with highest (93.01%) petroleum degrading capacity amongst bacterial isolates from soil composts in Lagos, Nigeria, observation from the current study suggests that *Micrococcus* sp. was the least TPH degrading isolate with degradation potential as low as 35.42%. However, the observation of this study is consistent with the report of Wolinska *et al*. (2016) who reported degradation rate as low as 27%; while Odjadjare *et al.* (2008) reported a slightly higher (54.95%) degradation potential for *Micrococcus* sp. isolated from the Niger Delta region of Nigeria.

The bacterial isolates in this study demonstrated remarkable potentials for the degradation of PAH fractions of ELCO; removing between 4 to 10 PAH compounds after treatment with the isolates (compare Figures 2, 3 to 8). The PAH degradation potentials of the isolates was in the following order (based on number of PAH compounds removed (highest to lowest)): *Staphylococcus* sp. >*Streptomyces* sp. >*Arthrobacter* sp. >*Bacillus* sp. (sample 2) >*Micrococcus* sp. >*Bacillus* sp. (sample 4). The observation of this study is consistent with the report of Nikitha *et al*. (2017) who observed considerable PAH degrading potentials for *Staphylococcus*, *Arthrobacter* and *Bacillus* spp. Juhasz and Naidu (2000) also observed that *Streptomyces*, *Arthrobacter* and *Bacillus* spp. exhibited good PAH degrading potentials; while PAH degrading capacity of *Micrococcus* species were documented elsewhere (John *et al*., 2012; Kafilzadeh *et al*., 2012).

The observation of this study with regards to bacterial degradation of PAH is quite remarkable considering the fact that many of the PAH compounds degraded by the bacterial treatments are listed as priority environmental pollutants by the United States Environmental Protection Agency (USEPA) (www.epa.gov/sites/production/files/2015-09/documents/priority-pollutant-list-epa.pdf). PAHs are usually considered as high risk environmental pollutants due to their high mutagenic and carcinogenic potentials when exposed to living organisms within the environment (Kafilzadeh *et al*., 2012). Biodegradation using microorganism is usually the preferred and major route of PAH removal from contaminated environments because of its cost effectiveness and capacity to transform the complex pollutants to simpler forms; or completely mineralize the contaminants into harmless substances such as carbon (iv) oxide, water, inorganic compounds, and cell protein (John *et al*., 2012; Nikitha *et al*., 2017). Thus this study demonstrated that the bacterial isolates investigated were good petroleum degraders that could serve as important candidates for bioremediation of hydrocarbon (especially PAH) polluted environments.

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EFFICACY OF THREE SELECTED BOTANICALS IN THE CONTROL OF *BOTRYTIS CINEREA* ASSOCIATED WITH DAMPING OFF IN *CITRULLUS LANATUS* (THUNB.) MATSUM AND NAKAI

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ABSTRACT
The use of plant extracts in controlling plant diseases has witnessed appreciable success; such was exploited in this study when efficacy of extracts from *Azadirachta indica*, *Calotropis procera* and *Anacardium occidentale*, each at different concentrations; 30 mg ml^{-1}, 60 mg ml^{-1}, 90 mg ml^{-1} and 0 mg ml^{-1} (Control), were examined *in vitro* to control *Botrytis cinerea* that causes damping off disease of *Citrullus lanatus* var. 'lanatus'. Result showed that the mean inhibition in colony diameter at 90 mg ml^{-1} ranged from 0.26-2.26 (*A. indica*), 0.43-3.30 (*C. procera*) and 0.13-2.50 (*A. occidentale*) compared respectively to 0.5-4.36, 0.57-4.33 and 0.43-4.10 in their 0 mg ml^{-1} concentration. The 90 mg ml^{-1} concentration that has the least fungal growth evident at different days in all extract used was significantly different ($p \leq 0.05$) from the 0 mg ml^{-1}. Furthermore, percentage inhibition in *A. occidentale* (37.50%) and *A. indica* (43.33%) were slightly significant ($p \leq 0.05$) at 90 mg ml^{-1} concentration but was significant ($p \leq 0.05$) in *C. procera* (17.50%). From this study, it can therefore be recommended that field trial of these medicinal plants on the control of damping off disease caused by *B. cinerea* can be achieved as the concentration of the extracts used here increases. Also, the use of these extracts should be inculcated as part of cultural control measure, since they are easy, simple and cost-effective in making.

KEYWORDS: Damping off, Disease control, Fungicidal, Phytochemicals, Methanolic extract, Soil-borne fungi

INTRODUCTION
Watermelon (*Citrullus lanatus* var. 'lanatus') is a tender, warm season vegetable belonging to the family Cucurbitaceae (Renner and Chomicki, 2017). It is enjoyed by many people across the world as fresh fruit. It is highly nutritious and thirst-quenching that contains vitamins A and C in the form of disease-fighting beta-carotene (Adesanya *et al*., 2011). Watermelon is rich in carotenoids, some of the carotenoids of which include lycopene, phytofluene, phytoene, beta-carotene, lutein and neurosporene (Pinto *et al*., 2011). Potassium is also available in it, which is believed to help, in the control of blood pressure, and possibly prevention of stroke (Darin, 2003). Citrulline is a non-essential amino acid first identified from the juice of

watermelon. Citrulline is used in the nitric oxide system in humans and has antioxidant and vasodilatation roles (Adesanya *et al*., 2011). Watermelon production has been low in spite of its values (FAOSTAT, 2018).

Plants and their metabolites are beneficial in fungal biomass production (Adebola *et al*., 2018). Fungi in turn causes diseases such as *Fusarium* wilt, powdery mildew, downy mildew, gummy stem blight, damping-off, etc., that can result in significant loss in plant yield, including watermelon (Michael *et al*., 2017). Mary (2011) found that damping-off is caused by several soil-borne fungi or fungus-like organisms commonly found in soils that may or may not have been previously cultivated. It is most severe when temperatures, light and other environmental conditions are unfavourable for seedling growth (Mary, 2011). It is commonly caused by several soil borne pathogens like: *Rhizoctonia solani, Thielaviopsis basicola, Pythium* spp., *Sclerotinia minor, Verticillium dahlia, Botrytis cinerea,* etc. (Mary, 2011). Damping-off caused by *Botrytis cinerea* is a filamentous, heterothallic ascomycete soil borne fungi (Ratna *et al*., 2015). Seedlings are attacked either before they emerge (called pre-emergence damping off) or after (called post-emergence damping off).

The use of botanicals instead of chemical fungicides is one of the recent approaches for plant disease control. These plants contain some organic compounds which produce definite physiological action. Victor and Chidi (2009) listed these compounds to include; tannins, alkaloids, terpenoids, steroids and flavonoids. They also included alkaloids, coumarin, flavonoids, saponnins and volatile constituents of the essential oils as being allelopathic agents.

Efforts have been made in controlling wide range of seed borne pathogens by different botanicals (Adebola *et al*., 2018). A few researches have been geared to evaluate the efficacy of botanicals against soil borne pathogens (Monaim *et al*., 2011; Mahmood and Muhammad, 2013).

In view of the above, this study was embarked on to evaluate the efficacy of extracts from *Azadirachta indica*, *Calotropis procera* and *Anacardium occidentale* in controlling mycelial growth of *Botrytis cinerea;* causal agent of damping off disease in watermelon.

MATERIALS AND METHODS

Experimental Site and Design

This experiment was carried out at the Botanical Garden and at the Department of Plant Biology laboratory, Federal University of Technology, Minna. The experiment was laid in a Completely Randomized Design with three replicates.

Collection of Materials

At the Botanical Garden, the healthy and matured leaves of *Calotropis procera* (Sodom apple), *Azadirachta indica* (Neem plant) and *Anacardium occidentale* (Cashew plant) were aseptically collected. Rhizosphere sample of watermelon showing symptoms of damping off were collected aseptically.

Plant Preparation and Extraction Procedures

Fresh leaves of *Calotropis procera*, *Anacardium occidentale* and *Azadirachta indica* were washed with sodium hypochloride and rinsed with clean water. They were dried at room temperature for 10-12 days (Cashew and Neem) and for 14-16 days (Sodom

apple). The dried materials were homogenized into powdered form. The powder of *C. procera*, *A. occidentale* and *A. indica* (50g each) were heated in 250ml methanol (in a ratio of 1:4) using a Soxhlet extractor for 4 hrs at temperature not exceeding the boiling point of the solvent. The extract was filtered using Whattman filter paper (No. 1), concentrated in vacuum, dried at 45°C for ethanol removal, and the extracts were kept in sterile bottles under refrigerated conditions until use (Jensen, 2007).

Isolation of Fungi

From the collected rhizosphere of *C. lanatus* showing symptoms of damping off, soil sample of it, about 1.0g, was obtained to make sample suspension when aseptically mixed with 10ml of distilled water. This was mixed well for 15 minutes and was serially diluted to 10^{-6}. Five (5) ml was pipetted into plates with PDA medium, swerved slightly and incubated at 27±2°C for 3-7 days (Nazir *et al*., 2007). After the incubation period, subculturing was done so as to have pure culture of *B. cinerea* (Jonathan *et al*., 2017).

Identification of the Fungi

Identification of *B. cinerea* was based on morphological examination (length, width colour and texture of the colony, the presence or absence of aerial mycelium) and microscopic examination was carried out using compound microscope (Ratna *et al*., 2015).

Pathogenicity Test

Pathogenicity of the isolated fungi was established by testing for their ability to induce damping off in healthy watermelon. Pathogenicity test was performed using a cork borer of 5mm to place *Botrytis cinerea* culture onto the surface of the soil around a healthy potted watermelon plant. Inoculated plants were incubated in humid sterile plastic pots (Adebola and Amadi, 2012).

Phytochemical Analysis on the Extracts

A preliminary phytochemical screening of the plant extracts were carried out for the presence of phytochemical compounds using methods described by Hassan (2006) and Prashanth *et al*. (2011).

Antifungal Activity of the Plant Extracts

This was done using direct plate method. The leaf extract of each plant was incorporated into replicated plates after dilution in 100ml of water at the following concentrations: 30 mg ml^{-1}, 60 mg ml^{-1}, 90 mg ml^{-1} and 0 mg ml^{-1} (Control). The plates were allowed to solidify. Isolates of *B. cinerea* were inoculated into the solidified medium using mycelia disc of 5mm disc per plate. The antifungal activity of the extracts was determined by measuring the mycelia growth of *B. cinerea* on each of the plate by drawing two perpendicular lines which meets at a right angle at the centre of the plate. The plates are then incubated at 27±2ºC for 7 days. After each day, the diameter of the growth was measured using a meter rule. Antifungal action of the extracts was calculated using the formula below;

$$\text{Growth inhibition (\%)} = \frac{\text{Colony diameter of (Control-Treatment)}}{\text{Colony Diameter of Control}} \times 100$$

Data Analysis

Statistical analyses of inhibition of radial growth was subjected to one way analysis of variance (ANOVA) using Statistical Package for Social Science (SPSS) version 17.0 and means was separated according to Duncan's Multiple Range Test (DMRT) at 5% probability level.

RESULTS

Plate1a shows the appearance of *B. cinerea* cultured for 7 days on a PDA plate. The fungus appears as pale brown in colour. Plate1b shows the photomicroscopy of the fungus when examined under light microscope. The mycelium was observed to have a set of loculated hyphae that are septate and hyaline. Conidiophore (Cd) of *B. cinerea* that branched at the apical region appears like a tree-like structure. At the terminal end of the branched conidiophore were observed globose vesicles where conidia (C) are grouped.

Symptoms of post emergence damping off were observed after six days of inoculation on healthy watermelon during pathogenicity test conducted *in vitro*. Symptoms observed include leaf blight and stem rot as shown in Plate 1c.

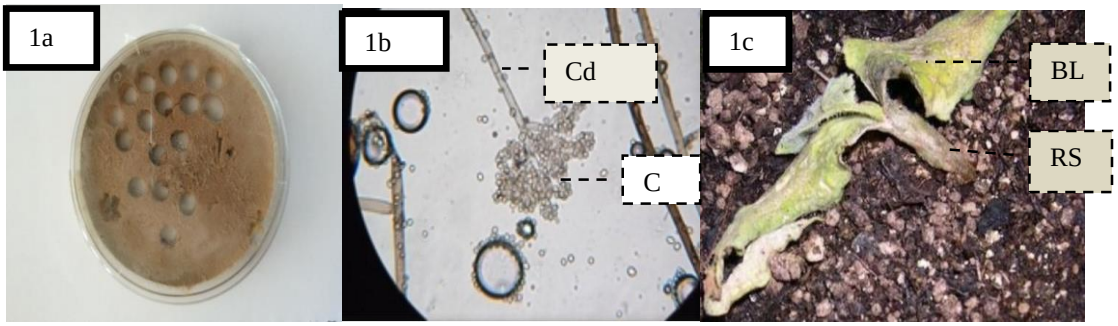

Plate 1a: Macroscopic appearance of *B. cinerea* on PDA after 7days incubation. Plate 1b: Photomicrography appearance of *B. cinerea* showing conidiophore (Cd) bearing the conidia (C). Plate 1c: Potted watermelon seedling showing symptoms of post emergence damping off; Rotten Stem (RS) and Blight Leaves (BL)

Phytochemical Screening

Phytochemical analysis (Table 1) revealed the presence of the alkaloid, saponin, tannin, anthocyanin, phenol and flavonoid in the four plant extracts screened. Alkaloids, saponin, tannins and flavonoids were found in all the plant extracts while *C. papaya* and *A. indica* did not contain steroid and phenols; and only steroid was not found in *A. occidentale.*

Table 1: Phytochemical constituent of the methanolic leaf extract of *Azadirachta indica, Anacardium occidentale* and *Calotropis procera*

Phytochemical constituents	*A. indica*	*A. occidentale*	*C. procera*
Tannin	+	+	+
Flavonoid	+	+	+
Saponin	+	+	+
Steroid	_	_	_
Alkaloid	+	+	+
Terpenes	+	+	+
Phenols	_	+	_

Note: + = Present, _ = Absent

Table 2: Effects of methanolic leaf extract of *Azadirachta indica* at varying concentrations on mycelial growth of *Botrytis cinerea*

$mgml^{-1}$	Day 1(mm)	Day 2(mm)	Day 3(mm)	Day 4(mm)	Day 5(mm)	Day 6(mm)	Day 7(mm)
30	0.00 ± 0.00	0.23±0.03 [b]	0.96±0.06 [c]	1.70±0.10 [c]	2.46±0.20 [c]	3.46±0.0.29 [c]	3.76±0.14 [c]
60	0.00 ± 0.00	0.16±0.03[b]	0.50±0.00 [b]	1.00±0.05 [b]	1.86±0.06 [b]	2.66±0.12 [b]	3.16±0.12 [b]
90	0.00 ± 0.00	0.00±0.00[a]	0.26±0.03 [a]	0.50±0.00 [a]	1.10±0.10 [a]	1.43±0.09 [a]	2.26±0.14 [a]
0	0.00 ± 0.00	0.50±0.01 [c]	1.60±0.02 [d]	2.58±0.02 [d]	3.23±0.11 [d]	4.05±0.03 [d]	4.36±0.27 [d]

Values are mean ± SEM of 3 determinations. Values along the same column with different superscripts are significantly different ($p \leq 0.05$).

Effects of methanolic leaf extract of Azadirachta indica on mycelial growth of Botrytis cinerea

The effect of the methanolic leaf extract of *A. indica a*gainst *B. cinerea* was presented in Table 2. It shows that at day one (1), the different concentration used have 0.00% effect on the fungal mycelia growth. At day two (2), there was no significant difference ($p \leq 0.05$) between the fungal growth of the 30 mg ml^{-1} (0.23±0.03) and 60 mg ml^{-1} (0.16±0.03) although, there was significant difference ($p \leq 0.05$) between the treatment 90 mg ml^{-1} (0.00±0.00) that was observed to have no fungal growth. The control treatment (0.50±0.01) however, has the highest fungal growth. At day three (3), there was much significant difference ($p \leq 0.05$) between the concentrations of 90 mg ml^{-1} (0.26±0.03) and the control (1.60±0.02), compared to the concentrations of 30 mg ml^{-1} (0.96±0.06) and 60 mg ml^{-1} (0.50±0.00) that has slight significant differences ($p \leq 0.05$).

At day four (4), there was significant difference ($p \leq 0.05$) between concentration of 90 mg ml^{-1} (0.50±0.00) and the control (2.58±0.02), while there was no significant difference ($p \leq 0.05$) between the concentrations of 30 mg ml^{-1} (1.70±0.10) and 60 mg ml^{-1} (1.00±0.05). At day five (5), the concentrations of 30 mg ml^{-1} (2.46±0.20) and 60 mg ml^{-1} (1.86±0.06) shows little significant difference ($p \leq 0.05$), as the concentration of 90 mg ml^{-1} (1.10±0.10) is significantly different ($p \leq 0.05$) from the control (3.23±0.11). At day six (6), there was great significant difference ($p \leq 0.05$) between 90 mg ml^{-1} (1.43±0.09) and the control (4.05±0.03) concentrations, and there was a slight significant difference

($p \leq 0.05$) between the concentrations of 30 mg ml^{-1} (3.46±0.29) and 60 mg ml^{-1} (2.66±0.12). At day seven (7), there was significant difference ($p \leq 0.05$) between the concentrations of 30 mg ml^{-1} (3.76±0.14) and 60 mg ml^{-1} (3.16±0.12), the concentration 90 mg ml^{-1} (2.26±0.14) that has the least fungal growth was significantly different ($p \leq 0.05$) from the control (4.36±0.27) that was observed to have the highest growth.

Table3: Effects of methanolic leaf extract of *Calotropis procera* at varying concentrations on mycelial growth of *Botrytis cinerea*

mg ml^{-1}	Day 1(mm)	Day 2(mm)	Day 3(mm)	Day 4(mm)	Day 5(mm)	Day 6(mm)	Day 7(mm)
30	0.00 ± 0.00	0.83±0.12^{b}	1.83±0.03^{c}	3.03±0.26^{c}	3.53±0.06^{c}	3.93±0.03^{b}	4.00±0.00^{b}
60	0.00 ± 0.00	0.80±0.10^{b}	1.16±0.03^{ab}	2.73±0.08^{bc}	3.23±0.08^{ab}	3.73±0.08^{b}	3.90±0.05^{b}
90	0.00 ± 0.00	0.43±0.06^{a}	0.90±0.20^{a}	1.80±0.15^{a}	2.50±0.05^{a}	3.00±0.05^{a}	3.30±0.11^{a}
0	0.00 ± 0.00	0.57±0.064^{ab}	1.43±0.16^{bc}	2.24±0.32^{ab}	3.13±0.18^{b}	3.78±0.29^{b}	4.33±0.29^{b}

Values are mean ± SEM of 3 determinations. Values along the same column with different superscripts are significantly different ($p \leq 0.05$).

Effects of methanolic leaf extract of Calotropis procera on mycelial growth of Botrytis cinerea

The result in Table 3 shows effect of the methanolic leaf extract of *Calotropis procera* against *Botrytis cinerea.* At day one (1), record shows that the different concentration used have 0.00% effect on the fungal mycelia growth. Day two (2), there was no significant difference ($p \leq 0.05$) in fungal growth between the concentrations of the 30 mg ml^{-1} (0.83±0.12) and 60 mg ml^{-1} (0.80±0.10). Also there was no significant difference ($p \leq 0.05$) between the concentration of 90 mg ml^{-1} (0.43±0.06) and the control (0.57±0.064) treatment. Day three (3), there was significant difference ($p \leq 0.05$) between the concentrations of 90 mg ml^{-1} (0.90±0.20) and the control (1.43±0.16), however, the concentrations of 30 mg ml^{-1} (1.83±0.03) and 60 mg ml^{-1} (1.16±0.03) shows slight significant differences ($p \leq 0.05$).

At day four (4), there was no significant difference ($p \leq 0.05$) between the concentrations of 30 mg ml^{-1} (3.03±0.26) and 60 mg ml^{-1} (2.73±0.08), and there was little significant difference ($p \leq 0.05$) between the concentration of 90 mg ml^{-1} (1.80±0.15) and the control (2.24±0.32). Day five (5), the concentrations of 30 mg ml^{-1} (3.53±0.06) and 60 mg ml^{-1} (3.23±0.08) shows significant difference ($p \leq 0.05$), as the concentration of 90 mg ml^{-1} (2.50±0.05) shows high significant difference ($p \leq 0.05$) from the control group (3.13±0.18). Day six (6), there was slight significant difference ($p \leq 0.05$) between concentration 90 mg ml^{-1} (3.00±0.05) and the control group (3.78±0.29), and there was no significant difference ($p \leq 0.05$) between the concentrations of 30 mg ml^{-1} (3.93±0.03) and 60 mg ml^{-1} (3.73±0.08). Day seven (7), there was no significant difference ($p \leq 0.05$) at all between the concentrations of 30 mg ml^{-1} (4.00±0.00) and 60 mg ml^{-1}

(3.90±0.05), the concentration 90 mg ml^{-1} (3.30±0.11) was significantly different ($p \leq 0.05$) from the control (4.33±0.29) which has the highest growth.

Table 4: Effects of methanolic leaf extract of *Anacardium occidentale* at varying concentrations on mycelial growth of *Botrytis cinerea*

mg ml^{-1}	Day 1(mm)	Day 2(mm)	Day 3(mm)	Day 4(mm)	Day 5(mm)	Day 6(mm)	Day 7(mm)
30	0.00 ± 0.00	1.00±0.05 ^{c}	1.80±0.05 ^{d}	2.10±0.05 ^{d}	2.60±0.15 ^{b}	3.10±0.10 ^{c}	3.80±0.11 ^{c}
60	0.00 ± 0.00	0.50±0.00 ^{b}	0.56±0.03 ^{b}	0.90±0.05 ^{b}	1.26±0.08 ^{a}	2.46±0.06 ^{b}	3.23±0.03 ^{b}
90	0.00 ± 0.00	0.13±0.03^{a}	0.30±0.10 ^{a}	0.53±0.03 ^{a}	0.76±0.12 ^{a}	1.53±0.12 ^{a}	2.50±0.15 ^{a}
0	0.00 ± 0.00	0.43±0.02 ^{b}	1.42±0.01 ^{c}	1.91±0.01 ^{c}	2.72±0.01 ^{b}	3.36±0.12 ^{c}	4.10±0.05 ^{c}

Values are mean ± SEM of 3 determinations. Values along the same column with different superscripts are significantly different ($p \leq 0.05$).

Effects of methanolic leaf extract of Anacardium occidentale on mycelial growth of Botrytis cinerea

The result in Table 4 shows the inhibitory effect of the aqueous leaf extract of *Anarcadium occidentale* against *Botrytis cinerea*. At day one (1), record shows that the different concentration used have 0.00% effect on the fungal mycelia growth. Day two (2), there was little significant difference ($p \leq 0.05$) in fungal growth between the concentrations of the 30 mg ml^{-1} (1.00±0.05) and 60 mg ml^{-1} (0.50±0.00). Also little significant difference ($p \leq 0.05$) was observed between the concentration of 90 mg ml^{-1} (0.13±0.03) and the control treatment (0.43±0.02). Day three (3), there was significant difference ($p \leq 0.05$) between the concentrations of 30 mg ml^{-1} (1.80±0.05) and that of 60 mg ml^{-1} (0.56±0.03). The concentration of 90 mg ml^{-1} (0.30±0.10) was significantly different ($p \leq 0.05$) from the control (1.42±0.01).

At day four (4), there was significant difference ($p \leq 0.05$) between the concentrations of 30 mg ml^{-1} (2.10±0.05) and 60 mg ml^{-1} (0.90±0.05). Also, there was significant difference ($p \leq 0.05$) between the concentration of 90 mg ml^{-1} (0.53±0.03) and the control (1.91±0.01). Day five (5), the concentrations of 30 mg ml^{-1} (2.60±0.15) and 60 mg ml^{-1} (1.26±0.08) shows much significant difference ($p \leq 0.05$); just as the concentration of 90 mg ml^{-1} (0.76±0.12) shows significant difference ($p \leq 0.05$) with the control (2.72±0.01). Day six (6), the concentration of 30 mg ml^{-1} (3.10±0.10) was significantly different ($p \leq 0.05$) from that of 60 mg ml^{-1} (2.46±0.06). Also, the concentration of 90 mg ml^{-1} (1.53±0.12) shows significant difference ($p \leq 0.05$) with the control (3.36±0.12). Day seven (7), there was little significant difference ($p \leq 0.05$) between the concentrations of 30 mg ml^{-1} (3.80±0.11) and 60 mg ml^{-1} (3.23±0.03). The concentration 90 mg ml^{-1} (2.50±0.15) having the least fungal growth was significantly different ($p \leq 0.05$) from the control (4.10±0.05) which has the highest growth.

Table 5: Effects of tested plant extracts on percentage mycelia growth inhibition of *B. cinerea*

mg ml^{-1}	*A. indica (%)*	*C. procera (%)*	*A. occidentale (%)*
30	5.88±1.34 [b]	0.00±0.00 [a]	5.00±0.03 [b]
60	20.83±2.45 [c]	2.50±2.34 [b]	19.16±2.75 [c]
90	43.33±4.56 [d]	17.50±2.56 [c]	37.50±4.56 [d]
0	0.00±0.00[a]	0.00±0.00 [a]	0.00±0.00 [a]

Values along the same column with different superscripts are significantly different ($p \leq 0.05$).

Percentage mycelial growth inhibition of Botrytis cinerea by Anarcadium occidentale, Azadirachta indica and Calotropis procera leaf extracts

The result in Table 5 present the percentage mycelial growth inhibition of *Botrytis cinerea* by*Anacardium occidentale, Azadirachta indica* and *Calotropis procera* leaf extracts. The methanolic leaf extract of *A.indica* at 90 mg ml^{-1} concentration significantly ($p \leq 0.05$) prevents the fungal mycelial growth by 43.33% when compared with the 30 mg ml^{-1} concentration of the leaf extract that inhibited the mycelial growth by 5.88%. The fungal growth in the methanolic leaf extract of *C. procera* showed no inhibition at all at the concentration of 30 mg ml^{-1} compared to the concentration of 90 mg ml^{-1} which showed about 17.50% inhibition. The methanolic extract of *A. occidentale* showed potential of inhibition at the concentration of 90 mg ml^{-1} as it inhibited the mycelial growth by 37.50% but showed low inhibition to be only 5% at the concentration of 30 mg ml^{-1}.

DISCUSSIONS

The descriptive characteristics of *B. cinerea* observed in this study were in agreement with those described by Ratna *et al*. (2015). These features could be indicative of a typical ascomycete fungus.

Symptoms observed in this finding were in accordance with those reported by Michael (2014). These which were observed few days after inoculation with a healthy watermelon seedling may be indications that post emergence damping off do exist in this plant.

The presence of methanolic compounds in the extracts used conforms to previous findings (Qian and Nihorimbere, 2004; Adebola *et al*. (2018). Presence of these phytochemicals could be an indication of their fungicidal activity.

Records on daily growth of *Botrytis cinerea* showed that the methanolic plant extract acted as an inhibitory constituent to the amended media. The inhibitory effect of the methanolic leaf extracts of the plants may be due to the presence of some phytochemicals like tannins, saponin, flavonoid, and alkaloids etc., that have antimicrobial properties. This can be supported by painstaking studies that have been carried out on different phytochemicals of plants (Tasleem *et al.*, 2009; Adebola *et al.,* 2018).

The *in vitro* control of *B. cinerea* using plant extracts obtained from leaves of *A. indica, C. procera* and *A. occidentale* showed that they all significantly reduced the pathogenic

activities of the fungus from the second day. This agrees with previously reported findings (Goyal and Mathur, 2011; El-Hawary *et al.*, 2013; Omojate *et al*., 2014; Aderiye *et al.*, 2015; Manoorkar *et al.*, 2015).Perhaps it could be that these extracts were unable to arrest the physiology of *B. cinerea* not until the second day when there are noticeable vegetative growths by this fungus.

The use of low-cost, readily available and phytotoxic approaches are needed when exclusion of pathogen and disease management are issue of concern. Independent researchers have studied the phytochemical properties of leaves and stem of these plants. Their result showed that these plants are potentially fungicidal (El-Hawary *et al.*, 2013, Manoorkar *et al.*, 2015; Adebola *et al.,* 2018).

CONCLUSIONS

This study has shown the antifungal activities of the methanolic leaf extract of three medicinal plants. The efficacy of the methanolic leaf extract of these plants increased with increase in concentration; 90 mg ml^{-1}concentration showed the highest level of inhibition. The methanolic leaf extract of *Azadirachta indica, Anacardium occidentale, Calotropis procera* in that order, proved to be effective in the inhibition of *Botrytis cinerea* at the three concentrations. Since the plants are easy to find and prepare, they can be used as substitute to chemical fungicide in the control of damping off disease in watermelon.

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BACTERIAL FLORA AND SENSORY CHARACTERISTICS OF FRESH AND SALTED *VERNONIA AMYGDALINA* LEAVES

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ABSTRACT
In this study, the microbial flora and sensory characteristics of fresh and salted *Vernonia amygdalina* leaves were investigated. The leaves were subjected to light salt, light brine, light brine and vinegar and heavy salt treatments for two weeks. Compared with the fresh leaves, all treatments gave leaves with lower pH and bacterial counts. Heavy-salted and light brine and vinegar treated leaves had the lowest pH and bacterial counts, and gave products containing *Lactobacillus* and *Streptococcus*, but devoid of *Staphylococcus spp*. In addition, they gave leaves with the best organoleptic characteristics, indicating that they were the most effective. The use of salting as a process for the development of value-added products from *V. amygalina* leaves is discussed.

KEYWORDS: *Vernonia amygdalina* leaves, salting, bacterial counts, microbial flora, probiotics

INTRODUCTION

Using simple technologies, it is possible to alter some nutritional and organoleptic characteristics of fruit and vegetable matrices by modifying them in a controlled way, such as salting and pH modification (Jones and Etchells, 1944; Battock and Azam-Ali, 1998). Their content of beneficial nutrients, such as minerals and vitamins makes them ideal substrates for the growth of desirable bacteria, which by fermentation would in turn, elaborate flavour compounds, decompose antinutrients and increase the availability of nutrients, and improve their palatability (Teucher *et al*., 2004; Gibson *et al.*, 2006). The salting or brining of vegetables offers tremendous possibilities both for their commercial and home preservation, and enrichment (Axtell *et al*., 2008; Oboh and Madojemu, 2010; Oboh *et al*, 2013, Oboh and Madojemu, 2016). In the process the salt exerts a selective action on the naturally occurring organisms to promote a desirable fermentation. Salt tolerant microorganisms use as their nutritive material, the soluble constituents that diffuse out of the vegetable as a result of the action of the salt on vegetable tissue. These fermentative organisms bring about the production of various compounds,

principally lactic acid, but also acetic acid, alcohols, and considerable amounts of gas. The production of a sufficient amount of acid makes the medium unsuitable for the growth of food spoilage bacteria. In addition, the acid and other microbial metabolites alter the flavour of the food (Battock and Azam-Ali, 1998; Galgano *et al.*, 2015). Compounds (prebiotics) and organisms (probiotics) in fermented foods can cause desirable changes in the composition and/or activity of the gastrointestinal microbiota resulting in health benefits (Perdigon *et al*., 1987; FAO, 2007; Galgano *et al.*, 2015).

Vernonia amygdalina Del., variously known as bitter leaf (English), oriwo (Edo), ewuro (Yoruba), shikawa (Hausa), and olubu (Igbo), is a tropical shrub, 1-3 m in height with petiole and leaf of about 6 mm in diameter, and elliptic in shape (Igile *et al*., 1995). The leaves are dark green in colour, with a characteristic smell and a bitter taste. The species is indigenous to tropical Africa where it is found wild or cultivated (Bosch *et al*., 2005). The primary use of the plant is as a source of green leafy vegetable for culinary application. The leaves are eaten, after crushing and washing thoroughly to remove the bitterness (Mayhew and Penny, 1998). It has various secondary uses – in folk medicine (all parts of the plant), as an ornamental, for forage (the leaves), for wood fuel (stems and branches) (Mayhew and Penny, 1998; Bosch *et al.*, 2005). Medicinal applications of the plant include the use of the leaf extract as a laxative (Gill, 1992; Awe *et al.*, 1999) antihelmitic and antimalarial (Abosi and Raseroka, 2003; Iwalokun, 2008). The antithrombotic and hypoglycemic effects of the leaf extract, and its hypolipidaemic effect in diabetic-hyperlipidaemic (Akah and Okafor, 1992; Nwanjo, 2005) and normoglycaemic-hyperlipidaemic rats (Adaramoye *et al.*, 2008; Oboh and Enobhayisobo, 2009) have been reported. The leaf extract also exhibits antimicrobial (Akinpelu, 1999; Oboh and Masodje, 2009; Mboto *et al.*, 2009) and anti-tumourigenic properties (Izevbigie *et al*., 2004).

Other studies have been undertaken in order to increase knowledge of the composition and utilisation of *V. amygdalina* leaves in food and nutrition. These include nutrient composition and antimicrobial activity (Oboh and Masodje, 2009), the effect of salting and drying on the organoleptic characteristics and nutrient composition (Oboh and Madojemu, 2010), the effect of salting on the phytochemicals, fungal flora and nutrient composition (Oboh *et al*., 2013), and the fractionation of *V. amygdalina* and analysis of the leaf fractions (Oboh *et al*., 2016). It was found that *V. amygdalina* leaves are rich in minerals, vitamins and phytochemicals, and contain modest amounts of proteins and lipids, all of which are moderately or highly retained in the dried and salted products (Oboh and Madojemu, 2010; Oboh *et al*., 2013), and the fibrous and protein fractions of the leaves (Oboh *et al*., 2016). Fructo- and galacto-oligosaccharide prebiotics were found in the aqueous extract, and prebiotic effects of this extract on the gastrointestinal tract of an animal model were observed, including protection of

the animals against some pathogens (Ezeonu *at al*., 2013, 2016).

This study was undertaken to investigate the effects of salting and brining on the bacterial flora of *V. amygdalina* leaves. The leaves were subjected to four treatments – heavy salting, light salting, light brining, and light brine and vinegar treatment for 14 days, and their bacterial flora and sensory characteristics were examined. The use of salting for the preservation, as well as the development of novel food products from the leaves is discussed.

MATERIALS AND METHODS

Materials

Vernonia amygdalina Leaves

V. amygdalina leaves were harvested from the vegetable garden of the Faculty of Agriculture and Agricultural Technology, from stands identified by staff of the Department of Crop Science of the faculty. Whole leaves were used for salt treatments.

Reagents

Sodium chloride and nutrient agar were from Merck, Darmstadt, Germany. White vinegar (5% acidity) was from Magic Time, Doral, Florida, USA.

Methods

To investigate the effect of salting on the bacterial flora of *V. amygdalina* leaves, a study was conducted using the following treatments:

Heavy Salting

V. amygdalina leaves were gently rinsed to remove dirt, and the water drained. Salt (37.5 g) and leaves (150.0 g) were mixed well and filled into a plastic bucket. The mixture was covered with two layers of muslin cloth and a pressure plate and weight were placed on it. Brine made of salt (37.5 g) dissolved in water (150.0 ml) was added until the pressure plate was slightly submerged. The buckets were stored in a cool, dry and shaded place for two weeks (James and Kuipers, 2003), at ambient temperature of 31.0-32.5^{o}C.

Light Salting

Rinsed and drained *V. amygdalina* leaves (150.0 g) were mixed well with dry salt (3.75 g), filled into a plastic bucket and packed tightly. The mixture was covered with two layers of muslin cloth and a pressure plate and weight were placed on it. The bucket was stored in a cool, dry and shaded place for two weeks (James and Kuipers, 2003), at ambient temperature of 31.0-32.5^{o}C.

Light Brining

To rinsed and drained *V. amygdalina* leaves (150.0 g) was added light brine (3.75 g salt dissolved in 150ml water). The brine was added to the vegetable in layers (i.e.by starting with a layer of vegetables and the addition of brine, followed by another layer of vegetables and addition of more brine, and so on) in a plastic bucket, packing tightly. The mixture was covered with two layers of muslin cloth and a pressure plate and weight were placed on it. The bucket was stored in a cool, dry and shaded place for two weeks (James and Kuipers, 2003), at ambient temperature of 31.0-32.5^{o}C.

Light Brine +Vinegar

Light brine and vinegar was made by mixing salt (7.5 g), vinegar (7.5 ml) and water (150.0 ml). This was added to washed and drained *V. amygdalina* leaves (150.0 g) as described above for light brining. The mixture was covered with two layers of muslin cloth and a pressure plate and weight were placed

on it. The set up was stored in a cool dry and shaded place for two weeks, at ambient temperature of 31.0-32.5^{o}C.

Sensory Evaluation

A sensory evaluation of the fresh and salted leaves was undertaken as follows (IFT, 1981). A description of colour, taste, odour, and texture of the formulations, based on sensory perception was carried out by an untrained panel of six judges (3 male and 3 female) selected from the final year biochemistry class of Benson Idahosa University, who apparently had no defect in their ability to perceive the characteristics examined. Labelled samples of the fresh and salted leaves were placed in glass petri dishes on a table covered with white cardboard which was placed in a well- lit and ventilated room. Prior to inspection of samples panellists were provided with a sheet containing the following descriptions of characteristics and were asked to record those which were closest to their observations (Table 1).

Table 1: Descriptive sensory evaluation sheet

Colour	Yellow-green	Lemon-green	Light green	Dull dark green	Bright dark green
Taste	Mildly salty and slightly bitter	Mildly salty and bitter	Salty, bitter and tangy	Very salty, very bitter, and tangy	Very bitter and very salty
Smell	Rotten, very offensive	Hydrogen sulphide-like, offensive	Mildly hydrogen sulphide –like, not offensive	Faint, not offensive smell.	Fresh, leafy, aromatic; attractive.
Texture	Degraded and in pieces, slimy	Limp, with smooth surface	Less firm than the fresh leaves, with a less rough surface	Less firm than the fresh leaves; rough surface	Firm, with rough surface

Examination of samples was undertaken by one panellist at a time. Subsequent open discussion by the panellists of their observations (moderated by a leader chosen by the group) resulted in the final description of the fresh and salted leaves.

Analytical Procedure

The pH of the distilled and deionized water extract of the vegetable was measured at an ambient temperature of 27.0^{o}C using a previously calibrated pH meter (Jenway Model 3505, Camlab, UK).

Microbiological Analysis

Total Viable Microbial Counts

These were estimated using the pour plate method. A ten-fold serial dilution of samples was made (i.e. 1 ml of the fresh or treated *V. amygdalina* leaf extract was made up to 10 ml with sterile distilled water in test tubes). Enumeration of microorganisms in the extract was done by inoculating 1ml aliquots of the diluted sample into labelled Petri dishes followed by pouring of the nutrient agar. The serial dilutions of the extracts were done up to 10^{-2}. The plates were allowed to solidify, inverted, and then incubated at an ambient temperature of 28.0^{o}C for 24 hours after which the colonies were counted (Cheesebrough, 2000).

Isolation of Microorganisms

Discrete bacteria colonies were isolated from nutrient agar plates and

further sub-culturing on nutrient agar plates was carried out in order to obtain pure colonies; these were inoculated and stored in slants at 4.0^{o}C for further laboratory analysis (Cheesebrough, 2000).

Identification of Microbial Isolates

Pure bacteria isolates were identified using morphological (microscopic) and biochemical characteristics as described by Cheesebrough (2000).

RESULTS AND DISCUSSION

The acidity of foods is very important because it influences the kind of spoilage that may occur and hence the way that foods are processed. Most bacteria grow readily at a pH near 7, when a food is neutral. Their growth is more easily inhibited at lower pH of about 4 to 6 (Eyabi, 2001; Rose, 1975). Table 2 shows the pH values of *V. amygdalina* leaves subjected to different salting procedures.

Table 2: Effect of salting on the pH of *V. amygdalina* leaves

Leaves	***V. amygdalina***
Fresh (day 1)	5.83
Treatments	
Light brining	5.70
Light salting	5.65
Heavy salting	5.43
Light brine + vinegar	5.09

The fresh *V. amygdalina* leaves had a pH of 5.83. This decreased to 5.70 for the light brined, 5.65 for the light salted, 5.43 for the heavy salted, and 5.09 for the light brine and vinegar treated leaves. Treatment with the combination of salt and vinegar resulted in the highest decrease, followed by treatment with concentrated salt solution (i.e. heavy salting). The decrease in the pH of the leaves indicates increase in acidity resulting from fermentation and/or addition of vinegar. Thus, salting created the right environment for the growth of acid-producing bacteria.

The effect of preservation treatments on the bacterial count of *V. amygdalina* leaves is shown in Table 3.

Table 3: Effect of salting on the bacterial count of *V. amygdalina* leaves

Leaves	**Bacterial count (cfu/ml)**	**% Decrease[b] (relative to bacterial count on day 3)**
Fresh (day 1)	0	-
On day 3	40 x 10^2	-
Treatments[a]		
Heavy salting	10 x 10^2	75.0
Light salting + vinegar	8 x 10^2	80.0
Light brining	16 x 10^2	60.0
Light salting	19 x 10^2	52.5

[a] Treatments of fresh leaves were for 14 days at the end of which bacterial counts were taken.
[b] %Decrease = 100 – (bacterial count of the treated leaves /bacterial count of untreated leaves on day 3 x 100).

Freshly harvested leaves were bacteria-free, due to their antibacterial activity (Oboh and Masodje, 2009). This activity diminished due to post-

mortem loss of physiological activity and subsequent breakdown of plant tissue resulting in loss of resistance to bacterial infection. Bacterial count of untreated *V. amygdalina* leaves on the third day was 40 x 10^2 cfu/ml. Compared with the 3-day old untreated leaves, the treatments decreased the microbial load. The highest decrease (80.0%) resulted from light brine + vinegar treatment, followed by heavy salting, which resulted in a 75.0% decrease. The other treatments were less effective, resulting in 60% and 52.2% decrease for light brining and light salting respectively.

Bacterial genera associated with fresh and salted *V. amygdalina* leaves are shown in Table 4.

Table 4: Bacterial genera observed in fresh and salted *V. amygdalina* leaves

Leaves	***Streptococcus spp.***	***Staphylococcus spp.***	***Lactobacillus spp.***
Fresh	-[a]	-	-
On day 3	+[b]	+	-
Treatments			
Heavy salting	+	-	+
Light brine and vinegar	+	-	+
Light brining	-	+	+
Light salting	-	+	+

[a]-: Absent. [b]+: Present

The fresh leaves yielded no bacterial growth. In the absence of any preservation treatment, strains of *Streptococcus* and *Staphylococcus* were observed on the third day. Only *Streptococcus* and *Lactobacillus* strains were observed in leaves preserved in concentrated salt solution and light brine + vinegar, indicating that these treatments ensured a desirable microbial succession, by creating an environment suitable for the growth of these organisms but unfavourable for the growth of *Staphylococcus. Lactobacillus* strains that produce bacteriocin active against *E. coli, Staphylococcus aureus, and Bacillus cereus* have been isolated from carrots (Joshi *et al*., 2006), and the absence of *Staphylococcus* in the products of these treatments was probably due to the lower pH (relative to the fresh leaves), the high salt concentration (of the heavy-salted leaves), and/or the presence of bacteriocin. However, the presence of bacteriocins was not examined in this study. *Lactobacillus* and *Staphylococcus* were present in the light brine and light salt treated leaves. These treatments were however, unsuitable for the growth of *Streptococcus.* Strains of *Staphylococcus* are pathogenic and are implicated in food spoilage. Compared with the products of heavy salting and light brine and vinegar treatment, the absence of the acid-producing *Streptococcus* resulted in higher pH (Table 2) and the presence of

Staphylococcus indicated poor preservation of the leaves (Table 5).

Previous authors (Oboh *et al*., 2013) have reported the presence of toxigenic fungi in fresh and salted *V. amygdalina* leaves. *Aspergillus flavus* was found in fresh and salted leaves. *Fusarium*, which was not detected in the fresh leaves, was found in the light brine and vinegar treated samples. Aflatoxin was detected in both fresh and preserved leaves; compared with the former, preservation in concentrated salt solution caused a 93.33% decrease, and in light brine and vinegar, a 64.17% decrease in the fungal count of the leaves (Oboh *et al*., 2013). Thus apart from the elimination of *Staphylococcus*, decrease in total bacterial count, and selection for *Lactobacillus* and *Streptococcus* observed in this study, both treatments may also cause a considerable reduction in total (including toxigenic) fungal count.

Probiotics are considered beneficial when they form part of the diet of humans (Saavedra *et al*., 1994; Sloan, 2004; Granato *et al*., 2010; Galgano *et al*., 2015). Microbes are used as probiotics including bacteria, yeast, and mould. The genera and species that have been used are *Lactobacillus, Streptococcus, Leuconostocc, Pediococcus, Propionibacterium, Enterococcus, Bifidobacterium, Bacillus, Saccharomyces cerevisiae, Candida pintolopessi, Aspergillus niger and Aspergillus oryzae* (Galgano *et al*., 2015). Lactic acid bacteria are considered a major group of probiotic bacteria and are commonly used in both humans and animals (Soccol *et al*., 2010). The dairy sector, which is strongly linked to probiotics, is a large functional food market, and about 78% of current probiotic sales in the world are delivered through yoghurt. However, an increased demand for non-dairy probiotic products comes from the ongoing trend of vegetarianism and possible adverse health effect of consumption of dairy products due to milk cholesterol content, and lactose intolerance (Granato, 2010). The bacterial succession which resulted in the appearance of *Lactobacillus* and *Streptococcus* suggests that heavy salt and light brine and vinegar treatments of *V. amygdalina* leaves could produce a non-dairy matrix suitable for the growth of probiotic strains of these organisms.

The production of acid (indicated by decrease in pH), caused the decrease in microbial count (compared with the fresh leaves) by making the medium unsuitable for the growth of food spoilage bacteria. Added acid, in the form of vinegar also contributed to the antibacterial effect of light salt and vinegar treatment, which though of low salt concentration, gave the product with the lowest pH. Previous authors (Oboh and Madojemu, 2010, Oboh *et al*., 2013) observed an osmotic effect on the leaves by the high salt concentration employed in the heavy-salting process, which caused a decrease in their moisture content. Thus for the heavy-salted leaves, the presence of acid in the medium (produced by the halophilic organisms present), the considerable dehydration of the leaves, and the salinity of the medium, acted in a cooperative manner to prevent the growth of spoilage organisms, and ensure the preservation of the leaves.

The acid and other microbial metabolites produced as a result of the

treatments altered the flavour of the leaves. The sensory characteristics of fresh and fermented *V. amygdalina* leaves (Table 5) were similar to those observed in previous studies (Oboh and Madojemu, 2010, Oboh *et al*., 2013). The heavy-salted and light brine and vinegar treated leaves were the closest in appearance to the fresh leaves. The latter smelled like *ogiri*, a condiment made in Nigeria by the fermentation of melon (*Citrulus lanatus*) seeds (Achi, 2005). They were slightly salty, with a mildly bitter taste, with a hint of tanginess. These characteristics indicate that they would be suitable for inclusion in salads. Heavy-salted leaves had a very salty and very bitter taste; except for their salty taste, they resembled the fresh leaves. In culinary applications, their high salt content would determine the extent of their utilisation. However, *Aspergillus flavus* has been detected in both heavy salted and light brine and vinegar treated leaves, and also *Fusarium* in the latter (Oboh et al, 2013). Thus the level of aflatoxin in the leaves from both treatments and of fumonisin in the light brine and vinegar treated leaves must be determined and found to be safe before they can be recommended for use as food. The light salt and light brine treatments gave less salty, partially debittered leaves, but because of their contamination with *Staphylococcus* they are unsuitable for food application.

Table 5. Characteristics of fresh and salted *V. amygdalina* leaves

Characteristics	**Treatments**				
	Fresh	**Light brine**	**Light salt**	**Light brine + vinegar**	**Heavy salt**
Colour	Dark green	Dull dark green	Dull dark green	Dull dark green	Dark green
Taste	Very bitter	Slightly salty and slightly bitter.	Slightly salty and slightly bitter.	Slightly salty; bitter with a hint of tanginess	Very bitter and very salty.
Smell	Fresh leafy, aromatic smell	Slight but not offensive odour. Smells like *ogiri.*	Slight but not offensive odour. Smells like *ogiri.*	Slight but not offensive odour.	Fresh leafy aromatic smell.
Texture	Firm, with slightly rough surface	Less firm and rough than the fresh leaves.	Slightly more firm than product of light brining. Less rough than the fresh leaves.	Retained more of the original structure of the fresh leaves than products from light salt, or light brine treatment. Smooth feel.	Similar to the fresh leaves- firm and slightly rough leaf surface.

CONCLUSION

The results of this study indicate that heavy salting, and light salt and vinegar treatments of *V. amygdalina* leaves offer several benefits- their preservation, development of flavour, and the possibility of their use as a matrix for the growth of probiotics. These benefits offer opportunities for product and process development, resulting in the addition of value to the produce.

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MICROBIAL LOAD AND PHYSICO-CHEMICAL PROPERTIES OF SOIL IN NATIONAL CEREAL RESEARCH INSTITUTE RICE FIELD, BADEGGI, NIGERIA

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ABSTRACT
This study was carried out to evaluate the microbial load and pyhsico-chemical properties of the soil in National Cereal Research Institute rice field, Badeggi, Nigeria. The soil samples collected from fadama, hydromorphic and uncultivated field of NCRI were serially diluted and plated on Potato Dextrose Agar and Nutrient Agar. The incubation was done at 27^{o}C and the associated bacteria and fungi were isolated and enumerated. The physico-chemical properties of the soil were also done. A total of seven (7) bacteria; *Echericha coli, Staphylococcus aureus, Enterobacter aerogenes, Chromobacterium* sp. *Pseudomonas aureginosa, Micrococcus luteus*, and *Bacillus cereus* were isolated and identified. In Fadama, *Chromobactreium* sp. has the highest percentage frequency (21.89%), *Micrococcus luteus* in hydromorphic (24.99%) and *Pseudomona aerugenosa* (23.79%) in uncultivated soil. A total of eight fungi belonging to five general were isolated, identified and characterized to be *Aspergillus niger, Ppenicillium* sp., *Fusarium* sp., *Rhizopus* sp., *Aspergillus flavus, Neurospora sitophilia, Mucor* sp. and *Aspergillus fumigatus.* In Fadama, *Aspergillus niger* has the highest percentage frequency (24.28%) while *Aspergillus flavus* has the highest percentage frequency (23.70% and 25.07%) in hydromorphic and uncultivated field respectively. The moisture content of Fadama and hydromorphic are 8.80$\pm$0.01 and 8.00$\pm$0.01 respectively which were significantly different ($p<0.05$)from Uncultivated soil(3.00$\pm$0.01) The results on mineral content of the three soils revealed that the organic carbon, phosphorus, exchange acidity, sand and clay were not significantly different ($p>0.05$) however, the percentage Nitrogen content were significantly different ($p<0.05$) with Fadama soil having the highest percentage of (0.43%) from hydromorphic soil and uncultivated soil which were not significantly different. The calcium, potassium and sodium were significantly different ($p<0.05$).Therefore, The analysis showed that soil microbes and its physico-chemical properties play very vital role in soil nutrient in rice cultivated field as the uncultivated soil showed less microbial activities and physic-chemical properties hence low nutrients.

KEYWORDS: Fadama, Hydromorphic, Uncultivated, Bacteria, Fungi, Rice field

INTRODUCTION
Rice (*Oryza sativa*) is one of the daily staple food crops in Nigeria. As the main sources of energy, rice forms the foundation of diet for many people. It is also the major source of protein to

those who cannot afford to buy meat, fish or other protein foods (Chandi and Sogi, 2006). Furthermore, rice contains vitamins, minerals, dietary fiber, low fat and also has no cholesterol (FAO, 2006; Ibukun, 2008). However, the optimum rice production is hampered by soil nutrient availability and pathogens. It is therefore pertinent to identify the effects of microbes in soil and the physio-chemical properties on the nutritional content of the soil *vis - a -vis* the production of rice (Bateman *et al*. (2000).

Soil is a complex mixture of organic and inorganic material. Soil is a product of rock weathering by agents of denudation and has physical, chemical and biological components. Soil is essentially a suitable medium for the growth and development of plants. To a great extent, soil quality determines the nature of the plant ecosystems and capacity of land to support animal life (Marinara *et al*., 2000). Results of physical and chemical tests provide information about the capacity of soil to supply the required mineral nutrients. The status of available microbes in soils and their relationship with various physico-chemical properties have been attempted by several investigators (Krishnadas, 2009).

The role of soil microbes in ecological processes and their functionality relates to 90% of all biological functions in the soil including water dynamics, nutrients cycling and disease suppression (Ritz and Young, 2004; and Hoorman *et al.*, 2011). They are a major decomposer in the ecosystem They also allow better assimilation and absorption of nutrients by plants through mycorrhizal association alter the soils natural micro flora, have a strong bearing on vegetation and correlated soil profile (Puangsombat *et al*., 2010; Sharma *et al*., 2011). According to Hoorman *et al.* (2011), the ability of Microbes to utilize nutrients such as carbon, nitrogen and phosphorus in the soil allow plants to increase their nutrients uptake capacity. The deficiency of microbes in soils has become a major constraint to productivity, stability and sustainability of soils, since soils with low microbial activity cause low growth and development in plant (Bell and Dell, 2008). This research was therefore carried out to evaluate the status of the available microbial population and the physico-chemical properties in rice field of National Cereal Research Institute (NCRI), Badeggi, Nigeria.

MATERIAL AND METHODS

Study Area

The research was carried out at National Cereal Research Institute (NCRI), Badeggi, Niger State, Nigeria (9.0568° N, 6.1434° E) rice field. The NCRI Badeggi is one of the fifteen (15) commodities Agricultural Research Institute in Nigeria under the aegis of the Federal Ministry of Agriculture and Rural Development.

Collection of Soil Samples

The soil samples were obtained from Fadama, Hydromorphic and Uncultivated (control) fields at NCRI, Badeggi Niger State, Nigeria. Samples were collected from cleared soil surface into sterile polythene bags with soil augar at the depth of 15cm. The samples were taken to the NCRI central services laboratory for all the analysis.

Preparation of Media

Potato Dextrose Agar (PDA)

Thirty nine (39) gram of PDA (Hi-media) was suspended in 1000ml distilled water and heated to dissolve the powder completely, the medium was sterilized by autoclaving at 121^{O}C for 15minutes (Aremu *et al.,* 2013).

Nutrient Agar

Nutrient agar was prepared by dissolving 28g of dehydrated nutrient agar powder into 1 liter of distilled water. It was heated to homogenize it and then sterilized by autoclaving at 121°C for 15 minutes. The Nutrient agar was used for isolation of bacteria (Aremu *et al.,* 2013).

Isolation and Identification of Bacteria

Twenty five grams (25) of samples was mixed thoroughly with 225ml of Buffer peptone water (BPW) and serially diluted to about 10 dilution factor. Already prepared nutrient agar of about 15ml (kept at about 45± 1^{o}C in a water bath) was poured into already sterilized petri dishes and allowed to solidify. 0.1ml from the seventh dilution tube was poured onto the surface of the solidified media (NA) spread evenly on the surface of the media in a petri dish and incubated at 32 ± 1^{o}C for 24 to 48hrs. After incubation, the bacterial colonies were counted and recorded. Streaking was done to get pure cultures of the different bacterial colonies. From pure culture isolated, biochemical tests (gram staining, catalase reaction, glucose fermentation, oxides test) were carried out to identify the bacteria.

Isolation of Fungi

Serial dilution technique was used. Ten (10g) of each sample was aseptically transferred into sterile distilled water in test tubes. It was shaken properly to allow even distribution of microorganisms present in the sample. Dilution factors 10^{-1} and 10^{-2} were used as stock solution. 1ml of each dilution was aseptically taken from the suspension and transferred into sterile Petri dishes. 10ml of PDA was pour into the petri dish with 1ml chlorophenicol. The plates were swirled gently to allow even distribution of the sample. Incubation was done at room temperature 28±2^{o}C for 24hrs. Subcultures were made until pure cultures were obtained. Identification of isolated fungi was done using their morphological features and mycological monograghs (Onion *et al.,* 1985. Cannon and Kirk, 2007; Adebola and Amadi, 2012).

Physico-Chemical Parameter of Soil Samples

Physical Properties

The colour of the soil sample was determined using munsel colour chart. Soil texture was determined by feel method (AOAC, 2009). Soil moisture was determined by weighing method**.**

Chemical Properties

The soil sample was air dried for about 24hrs, crushed gently and sieve with 2mm sieve before using for analysis. Soil pH was measured using digital pH meter, organic carbon was determined by dichromate titration method and nitrogen content was determined by dichromate titration method and nitrogen content was measured by micro-kjeldahl digestion method. Available phosphorus was determined by bray no 1 extraction method. Atomic absorption spectrophotometer was used to measure the calcium and mangnesium content in the extract, while sodium and potassium

was determined by flame photometer method. The effective cation exchange capacity was evaluated by the summation method (AOAC, 2009).

Analysis of Data

All data generated were subjected to analysis of variance (ANOVA) and New Dunca Multiple range test was used to separate the means.

RESULTS

A total of seven (7) bacteria; *E. coli, Staphylococcus aureus, Enterobacter aerogenes, Chromobacterium* sp., *Pseudomonas aureginosa, Micrococcus luteus* and *Bacillus cereus* were isolated and identified.

Table 1: Biochemical Characteristics and Microscopic Identification of Bacterial Isolates

Biochemical test	Isolated Bacteria						
	E.coli	*S.aureus*	*M.* sp	*B. cereus*	*C.* sp	*E. aerogenes*	*P. aeruginosa*
KOH Solubility	+	_	_	_	+	+	+
Gram staining	_	+	+	+	_	_	_
Shapes	rods	coccus	coccus	rods	rods	rods	rods
Catalase test	+	+	+	+	+	+	+
Mannitol	+	_	_	_	+	+	_
Glucose	+	+	_	+	+	_	_
Lactose	+	+	_	_	_	+	_
Indole rxn	+	_	_	_	_	_	_
Spore stain	_	_	_	+	_	_	_
Oxidase test	_	_	+	_	+	_	+
Citrate test	_	_	_	+	_	+	+

+ =Positive - =Negative

While a total of eight fungi belonging to five general were isolated and identified to be *Aspergillus niger, Penicillium* spp., *Fusarium* spp., *Rhizopus* spp., *Aspergillus flavus, Neurospora sitophilia, Mucor* spp. and *Aspergillus fumigatus* (Table 2).

Table 2: Morphological Properties and Microscopic Identification of Fungal Isolates

Macroscopic characteristic	Texture	Microscopic characteristics	Organisms
Whitish to yellowish pigment that latertum, brownish red on the reversed side	Velvety	Septate hyphae, unbranched conidiophores from the foot of species	*Aspergillus niger*
Dark green colony with white ring powdery that grows moderately. The reverse side is cream	Powdery	Septate hyphae, branched conidiophores with secondary branches metulas. Sterigmata bears round conidia in chain	*Penicillium* sp.
Orange colony with white ring at the edge that is orange in the reversed	Floppy	Sepate hyphae, side shaped macroconidia, conidiophores bears conidia containing conidiospores	*Fusarium* sp.
Creamy colony that later turns black	Powdery	Aseptate hyphae, unbranched, sporangiophores arose from foot of rhizoid. Scattered spores which submerged in agar	*Rhizopus* sp.
Light green colony with white to cream	Velvety	Sepate hyphae, unbranched conidiophores scanty sterigmata	*Aspergillus flavus*
Orange to yellow colony that turns dark brown in complete dark	Fluffy	Sepate hyphae and unbranched sporangiophores	*Neurospora sitophilia*
Grayish white colony that later turns black	Powdery	Non-septate hyphae, unbranched sporangiopores with smooth appearance	*Mucor* sp.
White colony that turns dark green with white edge	Floppy	Conidiophores is one direction only and unbranched	*Aspergillus fumigatus*

In Fadama soil, seven bacteria were isolated out of which *chromobacterium* sp. has the highest percentage frequency (21.89%) followed by *Micrococcus luteus* (19.93%). *E.coli* has the least percentage (6.72%). In Hydromorphic soil seven bacteria were isolated out of which *Micrococcus luteus* has the largest percentage frequency (24.99%) followed by *Chromobacterium* sp. (18.70%). *E. coli* has the least percentage frequency (4.32%). In uncultivated soil, the same number of bacteria were isolated out of which *Pseudomonas aureginosa* has the highest percentage frequency (23.79%)followed by *Bacillus cereus*(19.00), *Staphylococcus aureus* (5.84%) and *Chromobacterium* sp. (18.7%).The results were significantly different ($p<0.05$) from their respective uncultivated soil (Table 3).

Table 3: Frequency of Bacterial Isolates from Rice field Soil

	Frequency of Bacterial in Soil Plots (%)		
Isolated Bacteria	Fadama	Hydromorphic	Uncultivated
E. coli	6.72^{a}	4.32^{a}	9.23^{b}
Bacillus cereus	15.84^{c}	15.89^{c}	19.00^{d}
Micrococcus luteus	19.93^{d}	24.99^{d}	18.39^{d}
Pseudomonas aeruginosa	16.52^{c}	17.35^{c}	23.79^{e}
Staphylococcus aureus	7.12^{a}	8.75^{b}	5.84^{a}
Chromobacterium spp	21.89^{d}	18.70^{c}	13.24^{c}
Enterobacter aerogenes	11.98^{b}	10.00^{b}	10.42^{b}

Mean in column followed by the same superscript are not differ significantly at ($p<0.05$)

Eight (8) fungi were isolated in Fadama soil out of which *A. niger* has the highest percentage frequency (24.28%) followed by *A. flavus* (23.33), however *Mucor* sp. has the least percentage frequency (4.47%). Out of eight (8) fungi isolated from hydromophic soil, *A. flavus* has the highest percentage frequency of (23.70%) followed by *A. niger* (20.50%) however, *Fusarium* sp. has the least percentage frequency (4.40%).

Table 4: Frequency of Fungi Isolates from Rice field Soil

	Frequency of fungi in plots		
Isolated Fungi	Fadama	Hydromorphic	Uncultivated
A. flavus	23.33^{d}	23.70^{d}	25.07^{d}
A. niger	24.28^{d}	20.50^{c}	22.49^{c}
A. fumigatus	9.08^{b}	6.44^{a}	8.39^{b}
Fusarium sp.	5.68^{a}	4.40^{a}	8.37^{b}
Neurospora sp.	8.77^{b}	13.41^{b}	8.03^{b}
Penicillium sp.	16.36^{c}	19.71^{c}	19.47^{c}
R. stolonifer	8.06^{b}	6.23^{a}	4.09^{a}
Mucor sp.	4.47^{a}	5.61^{a}	4.11^{a}

Mean in column followed by the same superscript are not differ significantly at ($P<0.05$)

From the physical appearance, the colour of the soil sample from Fadama and uncultivated sites were grayish, while that of hydromorphic was brown (Table5). All soil texture of the farm sites were fine sand except in uncultivated sample that was granular. Moisture content of the Fadama and hydromorphic site ranged between (8.80±0.01 – 8.00±0.01) which was significantly different ($p<0.05$) from the uncultivated (3.00±0.01). Electrical conductivity of the soil samples for Fadama, hydromorphic and uncultivated field were (200,240, and 90µs/cm) respectively which are significantly different ($p<0.05$) (table5).

Table 5: The physicochemical properties of NCRI rice field soil

Sample	Colour	Texture	Moisture content	Conductivity
Fadama	Gray	fine sand	8.00 ± 0.01^{b}	200^{b}
Hydromorphic	Brown	fine sand	8.00 ± 0.01^{b}	240^{c}
Uncultivated	Gray	Granular	$3.00+0.01^{a}$	90^{a}

Mean in column followed by the same superscript are not differ significantly at ($P<0.05$).

The results on mineral content of the three soils revealed that their pH were not significantly different ($p<0.05$). The same results was obtained for organic carbon, phosphorus, exchange acidity, sand and clay, however, the percentage Nitrogen content were significantly different ($p<0.05$) with Fadama soil having the highest percentage of (0.43%). The hydromorphic soil and uncultivated soil were not significantly different. The calcium, potassium and sodium were significantly different ($p<0.05$) (table 6).

Table 6: Mineral content of the three types of soil from NCRI

SAMPLS	PH	Organic carbon %	Nitrogen %	Available Phosphorus PPM	CaCmolkg^{-1}	MgCmolkg^{-1}	KCmolkg^{-1}	NaCmolkg^{-1}	Exchangeable Acidity %	SAND %
Fadama	6.03^{a}	2.81^{a}	0.43^{b}	21.78^{a}	1.88^{b}	1.46^{a}	0.22^{a}	1.7^{b}	1.4^{a}	50.4^{a} 15.75^{b} 4.1^{a}
Hydromorphic		4.6^{a}	4.07^{a}	0.39^{a}	22.87^{a}	1.82^{b}	2.28^{b}	0.24^{a}	1.78^{b}	1.18^{a} 54.2^{a} 14.78^{b} 4.3^{a}
Uncultivated	5.15^{a}	3.47^{a}	0.32^{a}	22.33^{a}	0.51^{a}	1.27^{a}	0.18^{a}	0.52^{a}	1.58^{a}	52.5^{a} 12.76^{a} 5.07^{a}

Values along the column followed by the same superscript are not significantly different at ($P<0.05$)

DISCUSSION

The prevalence pattern of micro-organisms is related to the nature of the microbial resident in the particular type of soil. The high population density of micro-organisms in the studied rice cultivated field could be mainly due to the availability of nutrient content in the soil that determine the level of activity play by the microorganism in soil health, as earlier reported Akare (2003).

The dominant bacteria isolated; *E. coil, Bacillus cereus, Micrococcus luteus, Pseumodonas aureginosa, Staphylococcus aureus, Chromobacterium* sp. *Enterobacter aerogenes* and fungi; *Aspergillus flavus, A. niger A. fumigates, Fusarium* sp., *Neurospora* sp., *Penicillium* sp., *rhizopus stolonifer and Mucor* sp. was clear indication that they also play a significant role in nutrient retention used by rice plant.

Plant Growth Promoting Rhizobacteria (PGPR) is one of the numerous beneficial microorganisms i.e. free living bacteria that colonize rhizosphere soil and enhance plant growth by a wide variety of mechanisms (Malleswari and Bagyanarayana, 2013). Such PGPR strains use one or more direct or indirect mechanisms to improve the growth and health of plants. Direct mechanisms include fixation of atmospheric nitrogen, solubilization of phosphorous, synthesis of plant growth hormones such as indole acetic acid (IAA), gibberellins and cytokinn and production of siderophores, while indirect mechanisms involve biological control of pathogens and deleterious microbes through the production of antibiotics, hydrogen cyanide, lytic enzymes and catalase or through competition for nutrients and space can improve significantly plant growth and health as evidenced by increase in seedling emergence, vigour and yield (Kloepper and Beauhamp 1992 ; Glick 1995; Verma *et al.,* 2010; Silini-cherif *et al.,* 2012). However most of the bacteria isolated in this study were among the group of PGPR.

It is clear from the data that fungal population of Fadama which has the highest amount of *Aspergillus niger* (24.28%) was probably because of the farming activities carried out and the used of fertilizer. The beneficial effect of fertilizer in increasing fungal population was reported by many workers (Karmegan and Daniel, 2000; Marinara, 2000; ICRISAT and APRLP, 2003) and ascribe the higher nutrient supply as the reason for it.

The observation on the selected parameter such as: colour texture moisture, electrical conductivity, pH and mineral content such as organic carbon, Nitrogen, phosphorus, potassium, exchangeable Acidity, sodium greatly affected the diversity of these micro-organisms. Moisture content plays an important role in microbial activity in the soil. Increase hydromorphic while the uncultivated recorded lower content (3.00±0.01). The moisture content of the hydromorphic soil was slightly lower than the one from Fadama soil, this is because Fadama soil has much water content and the activities carried on Fadama soil were both during dry and raining season, while at hydromorphic soil, activities were done during the raining season. The uncultivated field soil was low compared to the other soil

samples due to less activity. Moisture content is chiefly responsible for the colonization of microorganisms (Marianara *et al.*, 2000).

Fadama soil colour was gray with fine texture with electrical conductivity was 200µs/cm. While hydromorphuc rice soil colour was black, the texture fine sand which is the same with the Fadama soil, but it has the highest electrical conductivity of 240µs/cm. Among all the three soil samples analyses, the uncultivated field soil was also gray colour but the texture was granular and the electrical conductivity was 90µs/cm which was also lower than that of the Fadama and hydromorphic soils samples. The Fadama soil and hyromorphic soil have the same soil texture, this is because their soil texture, that is fine sand hold a lot of water as earlier reported by Hole *et al.* (2005) who pointed out the important role of soil texture in the retention water and availability of nutrient in soil.

Soil pH is considered a master player in agriculture as it affects nutrient availability and other important chemical processes. In this study, the pH of sampling site range from 6.03 for Fadama soil, followed by uncultivated soil with 5.15 and the hydromorphic soil has the lowest pH of4.60 which has stronger acidity than the other soil. This seemingly wide range of pH values among sampling sites might be due to the differences in soil types and the fertilizers used by farmers. This, however is not apparently detrimental to the cultivation of rice since the crop is well adopted to different soil conditions including pH as reported by Lal (1998) who reported that pH less than 6.6 is best for growing rice and can produce higher yields than neutral and alkaline soil. This implies that fungi abundance increase slightly as soil pH decreases. Rousk and colleagues (2010) explained that fungi, unlike bacteria have wide pH tolerance for optimum growth and though are less affected by pH gradients.

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THE COMBINATORIAL GENERATION AND THE MATRIX ELIMINATION MODEL FOR THE LONG-RUN DIAGNOSTIC STABILITY OF AN AUTOREGRESSIVE DISTRIBUTED LAG (ARDL) MODEL

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ABSTRACT
This study examines the long – run stability of an Autoregressive Distributed Lag (ARDL) model with intercept and no constant trend, using quarterly data from CBN annual statistical bulletin that span from 1999Q1 to 2011Q2 and 2000Q1 to 2012Q4. The parameters of the long – run stability of ARDL model are not all statistically significant in their respective p – values and as such cannot be totally relied upon for statistical estimation. Then, we proposed a matrix elimination model which is motivated by the p – values of the respective parameters to systematically eliminate at a time an exogenous variable and its corresponding parameter whose p – value is less significant from the system. Using econometric package (EView), it was investigated that there is collaboration in the long – run stability between the ARDL model and the Binomial coefficient model for different order of n(K). The findings show that the system (model) now accommodates parameters whose p – values are all statistically significant at 5% level of significance. This now paves way for an extended understanding for decision and policymakers to formulate a mechanism to maintain long – run stability in Foreign Reserves.

KEYWORDS: *Binomial coefficients, Econometric View, Diagnostic Stability, Minimum information Criteria, Parsimonious Variables, Over-Parameterization*

Introduction
This paper considers an Autoregressive distributed lag model (ARDL) which is also known as the bound testing approach developed by Pesaran *et al*. (2001) to estimate long – run and short – run parameters in a single model. In this study, emphasis is on the long – run stability of foreign reserves in Nigeria and the binomial coefficients model to systematically examine the diagnostic stability for different order of n(k) of an ARDL model. Using econometric view package, we shall establish the long – run stability of an ARDL model on the sequence of models of the binomial coefficients. The result shall determine which family has its model least parsimonious. Then slight modification in the choice of exogenous variables as considered by Irefin and Yaaba (2013) with the inclusion of second lag in

reserves, third lag in Gross domestic product, trade openness and first lag in foreign debt shall be investigated. Accordingly Irefin and Yaaba (2013) were able to ascertain that income (GDP) is the major determinant of foreign reserves holding in Nigeria.

Ojo (2013) was able to analyze between the full and subset autoregressive polynomial distributed lag models without intercept, where both exogenous and dependent variables are stationary. He developed a scheme to eliminate irrelevant lags as a build – up for the subset model. He later discovered that the subset model perform better using information criteria (Akaike information criterion and Schwarz criterion) and residual variance.

Yi-Yi (2010) as cited in Ojo (2013) examined the inclusion and the consequence of lag variables in an autoregressive distributed lag model for dependent variable and for several exogenous variables. The level of outcome on policy variables are determined by time lags respectively.

Bankole and Shuaibu (2013) applied vector autoregressive model and ascertain that a drop in income generated from oil price has gross effect in reserves in the long – run with a minimal effect on reserves in the short – run. This again established the fact that gross domestic product is a major determinant in reserve holding in Nigeria.

Abdullateef and Waheed (2010) explored combination of ordinary least squares and vector error correction methods. It was observed that a variation in reserves has no influence on domestic investment and inflation rate, but it stimulates foreign direct investment and exchange rates. The finding stipulates the need for broader reserve management targeted in maximizing the advantage derived from oil exportation to enhance internal investment.

Atif *et al.* (2010) explored the influence of financial development and trade openness on gross domestic product exhausting autoregressive distributed lag approach formulated by Pesaran *et al*. (2001). The findings show that, trade openness and financial development induced economic growth.

The study carried by Shahbaz and Faridul (2011) adopted autoregressive distributed lag (ARDL) model to estimate the coefficients of the long run relationship and the error correction model. In their investigation, it was found that financial development reduces income differences and it is aggravated by financial instability.

Iwueze *et al.* (2013) discussed how levels and trend can influence foreign reserves in Nigeria. They explored data from annual CBN statistical bulletin 1999 – 2008 using ARIMA model. Their findings showed that there is need for data logarithmic transformation for variance stability and make the distribution normal. They claim that the autoregressive – integrated moving average for order (2, 1, 0) best disrobes the pattern in the transformed data.

Charles – Anyaogu (2012) analyzed data collected from CBN statistical bulletin that span from 1980 – 2009 using vector autoregressive model and Wald test, it was found that the exogenous variable (GDP) is significant in explaining foreign reserves. The outcome is different with difference in time lag in reserves and suggested that an enabling environment should be built for trade openness to increase GDP for Nigeria economy.

Ajayi and Oke (2012) investigated the burden external debt has on the Nigerian development and economy. They implemented ordinary least squares exploring data from CBN on variables such as national income, debt service payment, external reserves and interest rate among others. They found that high level of external debt on nation income and per capital income of the nation can lead to currency devaluation, retrenchment, continuous industrial strike and failure in educational system.

The Buffer stock model of Frenkel and Jovanovic (1981) defined reserve movements in continuous time period as a Weiner process given as:

$$dR(t) = -\mu dt + \sigma dW(t) \quad (1)$$

where: $R_t =$ reserves held in time t
$W_t =$ Standard Weiner process with zero mean and variance t
$\mu =$ Deterministic part of the instantaneous change in reserves
$\sigma =$ Standard deviation of the Weiner increment in reserves
$R^* =$ denotes the optimal stock of reserves
Then, $R(t)$ is characterized by

$$R(t) = R^* - \mu t + \sigma W(t) \quad (2)$$

Autoregressive Distributed Lag (ARDL) model by Pesaran *et al*. (2001) was developed to estimate Frenkel and Jovanovic's buffer stock model, but with a slight modification (Irefin and Yaaba (2013) The ARDL $(p, q_1, q_2, \dots, q_k)$ model following Pesaran *et al*. (2001) can be written as follows:

$$\emptyset(L,p)y_t = \sum_{i=1}^{k} \beta_i (L,q_i)x_{it} + \delta' w_t + u_t, i = location\ at\ time\ t;\ t = 1,2,\dots,n;\ i = 1,2,\dots,k \quad (3)$$

Where
$$\emptyset(L,p) = 1 - \emptyset_1 L - \emptyset_2 L^2 - \dots \emptyset_p L^p \quad (4)$$
and
$$\beta_i(L,q_i) = 1 - \beta_{i1}L - \beta_{i2}L^2 - \cdots - \beta_{iqi}L^{qi},\ \ i = 1,2,\dots,k \quad (5)$$

From equation (3), $y_t$is the dependent variable, x_{it} denotes the independent variables, L is the lag operator, and w_t is the $s \times 1$ vector of deterministic variables, including intercept terms, dummy variables, time trends and other exogenous variables as cited in Mosayeb *et al*. (2005).
According to Pesaran and Pesaran (2001), as cited in Wilson and Chaudhri (2004), the long-run coefficient can be estimated by:

$$\beta_i = \frac{\hat{\beta}_i(l,\hat{q}_l)}{\Omega(l,\hat{p})} = \frac{\hat{\beta}_{i0}+\hat{\beta}_{i1}+\cdots+\hat{\beta}_{qi}}{1-\hat{\emptyset}_1-\hat{\emptyset}_2-\cdots-\hat{\emptyset}_p} \qquad \forall\ i = 1,2,\dots,k \quad (6)$$

$$y_t - \hat{\theta}_0 - \hat{\theta}_1 x_{1t} - \hat{\theta}_2 x_{2t} - \cdots - \hat{\theta}_k x_{kt} = \varepsilon_t \qquad \forall\ t = 1,2,\dots,n \quad (7)$$

where $i = location\ at\ time\ t$.
(Pesaran and Pesaran (2001)).

In Equation (7) the constant term is equal to:

$\hat{\theta}_0 = \frac{\hat{\beta}_0}{1-\hat{\emptyset}_1-\hat{\emptyset}_2-\cdots-\hat{\emptyset}_p}$

The Error correction model of the selected ARDL model can be obtained by rewriting Equation (3) in terms of lagged levels, first difference and w_t as follows:

$\Delta y_t = -\emptyset(L,\hat{p})ECM_{t-1} + \sum_{i=1}^{k}\beta_{io}\,\Delta x_{1t} + \delta'\Delta w_t - \sum_{j=1}^{\hat{p}-1}\varphi^*\, y_{t-j} - \sum_{i=1}^{k}\sum_{j=1}^{\hat{q}_{i-1}}\beta_{ij}^{*}\,\Delta x_{i,t-j} + u_t$ (8)

where, the error correction term is defined by

$ECM_{t-1} = y_t - \sum_{i=1}^{k}\hat{\theta}_i\, x_{it} - \omega' w_t$ (9)

METHODOLOGY

Modified ARDL Model

Consider the function:

$R_t = \alpha G_t^{\alpha_1} T_t^{\alpha_2}\, M_t^{\alpha_3} E_t^{\alpha_4}\, S_t^{\alpha_5}\varepsilon_t$ (10)

where $R_t, G_t, T_t, M_t, E_t, S_t$ are the Foreign Reserve, Gross Domestic Product, Trade Openness, Monetary Policy Rate, Exchange Rate and Foreign Debt at time t respectively and $\alpha_1, \alpha_2, \alpha_3, \alpha_4, \alpha_5$ are parameters of the respective exogenous variables

ASSUMPTIONS OF ARDL MODEL:

i. The errors ε_t are serially independent with $\sigma^2, \varepsilon_t \sim\ iid\ (0, \sigma^2)$.

ii. The errors are uncorrelated with $\Delta_{t+h}, \forall\ h\ \in Z$.

iii. $\beta_0 - \Upsilon\alpha_0 = \rho_0, \Upsilon = \vartheta_1, -\Upsilon\alpha_1 = \vartheta_2, -\Upsilon\alpha_2 = \vartheta_3, -\Upsilon\alpha_3 = \vartheta_4, -\Upsilon\alpha_4 = \vartheta_5\ and - \Upsilon\alpha_5 = \vartheta_6$

iv. Maximum lag length (p, n, m, o, q, v) = (2, 3, 0, 0, 0, 1) respectively.

v. $-\beta_3 = a_0' = a_2' - a_1' = -a_3' = -\theta_0' = -\alpha_0' = -\lambda_0' = \mu_0' = -\mu_1' = \vartheta_3 - \theta_0' = \vartheta_4 - \alpha_0' = \vartheta_5 - \lambda_0' = 0.$

vi. $a = b = 1, i = n(K)$

vii. The modified ARDL model must stability in its parameters

viii. $n + 1 = n(k)$

ix. $\frac{n(k)!}{(n(k)-r)!r!} = 0, \forall\ n(k) < r$

x. $\frac{n(k)!}{(n(k)-r)!r!} = 0, \forall\ n(k)r < 0$

xi. $j = r - 1$

The logarithmic linear specification of Equation (10) which is applied in the multivariate cointegration technique is as follows:

$LogR_t = \alpha_0 + \alpha_1 LogG_t + \alpha_2 LogT_t + \alpha_3 LogM_t + \alpha_4 LogE_t + \alpha_5 LogS_t + \varepsilon_t, Log\alpha = \alpha_0$ (11)

$r_t = \alpha_0 + \alpha_1 g_t + \alpha_2 t_t + \alpha_3 m_t + \alpha_4 e_t + \alpha_5 s_t + \varepsilon_t$ (12)

Equation (12) represents the simple linear functional formulation of Equation (11).
where $r_t = LogR_t,\ g_t = LogG_t,\ t_t = LogT_t,\ m_t = LogM_t,\ e_t = LogE_t,\ s_t = LogS_t$ and ε_t is the residual term assumed to be normally distributed.

Consider the simple case for compact ARDL as:
$B(L,p)r_t = \beta_0 + a'(L,n)g_t + \varepsilon_t$ (13)
Equation (13) can be expressed as;
$r_t = \beta_0 + \beta_1 r_{t-1} + \beta_2 r_{t-2} + \ldots + \beta_p r_{t-p} + a_0 g_t + a_1 g_{t-1} + a_2 g_{t-2} + \cdots + a_n g_{t-n} + \varepsilon_t$ (14)

$r_t = \beta_0 + \sum_{i=1}^{p} \beta_i r_{t-i} + \sum_{i=0}^{n} a_i' g_{t-i} + \varepsilon_t$ (15)

Where L is the lag operator, $B(L,p)$ is the lag polynomial and $a'(L,n)$ is the vector polynomial defined as follows:
$B(L,p) = 1 - \sum_{i=1}^{p} \beta_i L^i = 1 - \beta_1 L^1 - \beta_2 L^2 - \ldots - \beta_p L^p$ (16)

$a'(L,n) = \sum_{i=0}^{n} a_i L^i = a_0 + a_1 L^1 + a_2 L^2 + \cdots + a_n L^n$ (17)

By substituting Equations (16) and (17) in Equation (13) we have Equation (18)
$\left(1 - \sum_{i=1}^{p} \beta_i L^i\right) r_t = \beta_0 + (a_0' + a_1' L^1 + a_2' L^2 + \cdots + a_n' L^n) g_t + \varepsilon_t$ (18)

$\left(1 - \beta_1 L^1 - \beta_2 L^2 - \ldots - \beta_p L^p\right) r_t = \beta_0 + a'_0 g_t + a'_1 L^1 g_t + a'_2 L^2 g_t + \cdots + a'_n L^n g_t + \varepsilon_t$ (19)

$r_t - \beta_1 L^1 r_t - \beta_2 L^2 r_t - \ldots - \beta_p L^p r_t = \beta_0 + a'_0 g_t + a'_1 L^1 g_t + a'_2 L^2 g_t + \cdots + a'_n L^n g_t + \varepsilon_t$ (20)

$r_t - \beta_1 r_{t-1} - \beta_2 r_{t-2} - \ldots - \beta_p r_{t-p} = \beta_0 + a'_0 g_t + a'_1 g_{t-1} + a'_2 g_{t-2} + \cdots + a'_n g_{t-n} + \varepsilon_t$ (21)

$r_t = \beta_0 + \beta_1 r_{t-1} + \beta_2 r_{t-2} + \ldots + \beta_p r_{t-p} + a'_0 g_t + a'_1 g_{t-1} + a'_2 g_{t-2} + \ldots + a'_n g_{t-n} + \varepsilon_t$ (22)

$r_t = \beta_0 + \sum_{i=1}^{p} \beta_i r_{t-i} + \sum_{i=0}^{n} a_i' g_{t-i} + \varepsilon_t$ (15)

Where the coefficients of $\beta_i\ (\beta_1, \beta_2, \beta_3, \ldots, \beta_p)$ are parameters of the autoregressive components and p is the lag length of the autoregressive component. While $a_i'(a_0', a_1', a_2', \ldots, a_n')$, $\theta_i'(\theta_0', \theta_1', \theta_2', \ldots, \theta_m')$, $\alpha_i'(\alpha_0', \alpha_1', \alpha_2', \ldots, \alpha_o')$, $\lambda_i'(\lambda_0', \lambda_1', \lambda_2', \ldots, \lambda_q')$ and $\mu_i'(\mu_0', \mu_1', \mu_2', \ldots, \mu_v')$ are the parameters of the polynomial distributed lag component whereas n, m, o, q and v are the lag length of the polynomial distributed lag component. Equation (15) can be extended to more exogenous variable. Thus, the ARDL compact (p, n, m, o, q, and v) model is given by:

$B(L,p)r_t = \beta_0 + a'(L,n)g_t + \theta'(L,m)t_t + \alpha'(L,o)m_t + \lambda'(L,q)e_t + \mu'(L,v)s_t + \varepsilon_t$ (16)

T

r + ε_t (17)

For we which to show that;
$\sum_{i=1}^{p} \beta_i r_{t-i} = -B(1)r_{t-1} + \sum_{i=1}^{p} \beta_i \Delta r_{t-i} + r_{t-1}$ (18a)

$\sum_{i=0}^{n} a'_i\, g_{t-i} = B(1)\beta' g_t + \sum_{i=1}^{n} a'_i\, \Delta g_{t-i}$ (18b)
$\sum_{i=0}^{m} \theta'_i\, t_{t-i} = B(1)\rho' t_t + \sum_{i=1}^{m} \theta'_i\, \Delta t_{t-i}$ (18c)
$\sum_{i=0}^{o} \alpha'_i\, m_{t-i} = B(1)\delta' m_t + \sum_{i=1}^{o} \alpha'_i\, \Delta m_{t-i}$ (18d)
$\sum_{i=0}^{q} \lambda'_i\, e_{t-i} = B(1)\varphi' d_t + \sum_{i=1}^{q} \lambda'_i\, \Delta e_{t-i}$ (18e)
$\sum_{i=0}^{v} \mu'_i\, s_{t-i} = B(1)\omega' s_t + \sum_{i=1}^{v} \mu'_i\, \Delta s_{t-i}$ (18f)

The purpose of Equation (18a) to (18f) is to formulate the ARDL unrestricted model that enable us estimate the long-run and short-run parameters respectively.

From Equation (16), we have

$B(L) = 1 - \sum_{i=1}^{p} \beta_i\, L^i = 1 - \beta_1 L^1 - \beta_2 L^2 - \ldots - \beta_p L^p$

Multiplying Equation (16) by $-r_{t-1}$ yields,

$-B(L)r_{t-1} = -\left(1 - \sum_{i=1}^{p} \beta_i\, L^i\right) r_{t-1}$

$= -(1 - \beta_1 L^1 - \beta_2 L^2 - \ldots - \beta_p L^p) r_{t-1}$ (19)

$-B(L)r_{t-1} = -r_{t-1} + \beta_1 L^1 r_{t-1} + \beta_2 L^2 r_{t-1} + \ldots + \beta_p L^p r_{t-1}$ (20)

where $L^p r_{t-1} = r_{t-p-1}$ (21)

$-B(L)r_{t-1} = -r_{t-1} + \beta_1 r_{t-2} + \beta_2 r_{t-3} + \ldots + \beta_p r_{t-p-1}$ (22)

Set $L = 1$

$-B(1)r_{t-1} = -r_{t-1} + \beta_1 r_{t-2} + \beta_2 r_{t-3} + \ldots + \beta_p r_{t-p-1}$ (23)

where $\sum_{i=1}^{p} \beta_i\, \Delta r_{t-i} = \beta_1 \Delta r_{t-1} + \beta_2 \Delta r_{t-2} + \beta_3 \Delta r_{t-3} + \ldots + \beta_p \Delta r_{t-p}$ (24)

where $\Delta = 1 - L$ from Δr_t and Δ is the first difference of the variables.

$\sum_{i=1}^{p} \beta_i\, \Delta r_{t-i} = \beta_1 (r_{t-1} - r_{t-2}) + \beta_2 (r_{t-2} - r_{t-3}) + \ldots + \beta_p (r_{t-p} - r_{t-p-1})$ (25)

$= \beta_1 r_{t-1} + \beta_2 r_{t-2} + \cdots + \beta_p r_{t-p} - \beta_1 r_{t-2} - \beta_2 r_{t-3} - \ldots - \beta_p r_{t-p-1}$ (26)

Summing Equation (23) and (26), we have

$-B(1)r_{t-1} + \sum_{i=1}^{p} \beta_i\, \Delta r_{t-i} = -r_{t-1} + \beta_1 r_{t-2} + \beta_2 r_{t-3} + \ldots + \beta_p r_{t-p-1} + \beta_1 r_{t-1} + \beta_2 r_{t-2} + \cdots + \beta_p r_{t-p} - \beta_1 r_{t-2} - \beta_2 r_{t-3} - \cdots - \beta_p r_{t-p-1}$ (27)

$-B(1)r_{t-1} + \sum_{i=1}^{p} \beta_i\, \Delta r_{t-i} = -r_{t-1} + \beta_1 r_{t-1} + \beta_2 r_{t-2} + \ldots + \beta_p r_{t-p}$ (28)

Renovating Equation (28) gives back Equation (18a).

Thus,

$-B(1)r_{t-1} + \sum_{i=1}^{p} \beta_i\, \Delta r_{t-i} + r_{t-1} = \sum_{i=1}^{p} \beta_i\, r_{t-i}$

To obtain Equation (18b):

$\sum_{i=0}^{n} a'_i\, g_{t-i} = B(1)\beta' g_t + \sum_{i=1}^{n} a_i\, '\Delta g_{t-i}$,

We consider,

$\beta = \frac{a(L)}{B(L)} \Rightarrow B(L)\beta = a(L)$ (28a)

$B(L)\beta' \Delta g_t = a'(L) \Delta g_t$ (28b)

$$= a'(L)(g_t - g_{t-1})$$
$$= a'(L)(g_t - Lg_t) \quad (28c)$$

if we assume $L = 1$ *in Equation* $(28c)$ *then,*

$$B(1)\beta'\Delta g_t = a'(1)(g_t - g_t) = 0 \quad (28d)$$

But,

$$B(L)\beta' g_{t-1} = a'(L)\, g_{t-1} \quad (28e)$$

$$B(L)\beta' g_{t-1} = \sum_{i=0}^{n} a_i' L^i g_{t-1} \quad (28f)$$

$$B(L)\beta' L g_t = \sum_{i=0}^{n} a_i' L^i g_{t-1} \quad (28g)$$

$$B(L)\beta' L g_t = \sum_{i=0}^{n} a_i' g_{t-i-1} \quad (28h)$$

$if\ L = 1$ to the left hand side of Equation (28h), gives

$$B(1)\beta' g_t = a'_0 g_{t-1} + a'_1 g_{t-2} + a'_2 g_{t-3} + \ldots + a'_n g_{t-n-1} \quad (29)$$

Thus $\sum_{i=0}^{n} a_i' \Delta g_{t-i} = a'_0 \Delta g_t + a'_1 \Delta g_{t-1} + \ldots + a'_n \Delta g_{t-n}$ (30)

$$= a'_0(g_t - g_{t-1}) + a'_1(g_{t-1} - g_{t-2}) + \ldots + a'_n(g_{t-n} - g_{t-n-1}) \quad (31)$$

Thus,

$$\sum_{i=0}^{n} a_i' \Delta g_{t-i} = a'_0 g_t - a'_0 g_{t-1} + a'_1 g_{t-1} - a'_1 g_{t-2} + \ldots + a'_n g_{t-n} - a'_n g_{t-n-1} \quad (32)$$

Summing Equation (29) and (32), gives Equation (18b);

$$\sum_{i=0}^{n} a_i' g_{t-i} = B(1)\beta' g_t + \sum_{i=0}^{n} a_i' \Delta g_{t-i}$$

$$\sum_{i=0}^{n} a_i' g_{t-i} = a'_0 g_{t-1} + a'_1 g_{t-2} + a'_2 g_{t-3} + \ldots + a'_n g_{t-n-1} + a'_0 g_t - a'_0 g_{t-1} + a'_1 g_{t-1} - a'_1 g_{t-2} + \ldots + a'_n g_{t-n} - a'_n g_{t-n-1}$$

$$\sum_{i=0}^{n} a_i' g_{t-i} = a'_0 g_{t-0} + a'_1 g_{t-1} + + a'_2 g_{t-2} + \cdots + a'_n g_{t-n}$$

Clearly, from Equation (15) substituting Equation (18a) and Equation (18b) in Equation (15) yield;

$$r_t = \beta_0 - B(1) r_{t-1} + \sum_{i=1}^{p} \beta_i \Delta r_{t-i} + r_{t-1} + B(1)\beta' g_t + \sum_{i=0}^{n} a_i' \Delta g_{t-i} + \varepsilon_t \quad (33)$$

$$r_t - r_{t-1} = \beta_0 - B(1) r_{t-1} + \sum_{i=1}^{p} \beta_i \Delta r_{t-i} + B(1)\beta'(g_{t-1} + \Delta g_t) + \sum_{i=0}^{n} a_i' \Delta g_{t-i} + \varepsilon_t \quad (34)$$

Where $\Delta g_t = g_t - g_{t-1} \Rightarrow g_t = g_{t-1} + \Delta g_t$ (35)

$$r_t - r_{t-1} = \beta_0 - B(1) r_{t-1} + B(1)\beta' g_{t-1} + \sum_{i=1}^{p} \beta_i \Delta r_{t-i} + B(1)\beta' \Delta g_t + \sum_{i=0}^{n} a_i' \Delta g_{t-i} + \varepsilon_t \quad (36)$$

and $B(1)\beta'\Delta g_t = a(1)(g_t - g_t) = 0$, from Equation (28d)

Where $\Delta r_t = r_t - r_{t-1}$ from first difference forward operator

Then,

$$\Delta r_t = \beta_0 - B(1) r_{t-1} + B(1)\beta' g_{t-1} + \sum_{i=1}^{p} \beta_i \Delta r_{t-i} + a(1)(g_t - g_t) + \sum_{i=0}^{n} a_i' \Delta g_{t-i} + \varepsilon_t \quad (37)$$

$$\Delta r_t = \beta_0 - B(1) r_{t-1} + B(1)\beta' g_{t-1} + \sum_{i=1}^{p} \beta_i \Delta r_{t-i} + 0 + \sum_{i=0}^{n} a_i' \Delta g_{t-i} + \varepsilon_t \quad (38)$$

$setting - B(1) = \Upsilon_1\ and\ B(1)\beta' = \Upsilon_2,\ so\ that\ Equation\ (38)\ becomes$

$$\Delta r_t = \beta_0 + \sum_{i=1}^{p} \beta_i \Delta r_{t-i} + \sum_{i=0}^{n} a_i' \Delta g_{t-i} + \Upsilon_1 r_{t-1} + \Upsilon_2 g_{t-1} + \varepsilon_t \quad (39)$$

Equation (39) is the ARDL (p, n) unrestricted model (UM) that examines the long-run and short-run relationships between the variables.
Equation (39) can be extended to ARDL (p, n, m, o, q, v) as follows:

$$\Delta r_t = \beta_0 + \sum_{i=1}^{p} \beta_i \Delta r_{t-i} + \sum_{i=0}^{n} a_i' \Delta g_{t-i} + \sum_{i=0}^{m} \theta_i' \Delta t_{t-i} + \sum_{i=0}^{o} \alpha_i' \Delta m_{t-i} + \sum_{i=0}^{q} \lambda_i' \Delta e_{t-i} + \sum_{i=0}^{v} \mu_i' \Delta s_{t-i} + \Upsilon_1 r_{t-1} + \Upsilon_2 g_{t-1} + \Upsilon_3 t_{t-1} + \Upsilon_4 m_{t-1} + \Upsilon_5 e_{t-1} + \Upsilon_6 s_{t-1} + \varepsilon_t \quad (40)$$

Where $-B(1) = \Upsilon_1$, $B(1)\beta' = \Upsilon_2$, $B(1)\rho' = \Upsilon_3$, $B(1)\delta' = \Upsilon_4, B(1)\varphi' = \Upsilon_5, B(1)\omega' = \Upsilon_6$, equation (40) is a consequence of Equation (18a) to Equation (18f).
$\beta_0 is\ the\ constant\ term,\ where\ \Upsilon_1, \Upsilon_2, \Upsilon_3, \Upsilon_4, \Upsilon_5\ and\ \Upsilon_6$ are the Long- run parameters. $\beta, a, \theta, \alpha, \lambda, and\ \mu$ are the short- run parameters of the ARDL UM respectively.

Therefore, the general unrestricted error correction model (UECM) of Equation (40) is as follows:

$$\Delta r_t = \beta_0 + \sum_{i=1}^{p} \beta_i \Delta r_{t-i} + \sum_{i=0}^{n} a_i' \Delta g_{t-i} + \sum_{i=0}^{m} \theta_i' \Delta t_{t-i} + \sum_{i=0}^{o} \alpha_i' \Delta m_{t-i} + \sum_{i=0}^{q} \lambda_i' \Delta e_{t-i} + \sum_{i=0}^{v} \mu_i' \Delta s_{t-i} + \Upsilon Z_{t-1} + \xi_t \quad (41)$$

Υ is the error correction parameter, Z_{t-1} is the residuals that are obtained from the estimated cointegration model and ξ_t is the disturbance term assumed to be uncorrelated with zero means.

Equation (41) is the error correction model of ARDL that gives adjustment back to the long – run stability. Υ is the error correction parameter, Z_{t-1} is the residuals that are obtained from the estimated cointegration model. The lagged error correction term Z_{t-1} derived from the Error Correction Model is an important element in the dynamics of cointegration system as it allows for adjustment back to the long - run equilibrium relationship given a deviation in the last quarter (Irefin and Yaaba (2013), Shahbaz and Faridul (2011)). and ξ_t is the disturbance term assumed to be uncorrelated with zero means.
From Equation (17), if we let $\varepsilon_t = z_t$, we have:

$$r_t = \alpha_0 + \alpha_1 g_t + \alpha_2 t_t + \alpha_3 m_t + \alpha_4 e_t + \alpha_5 s_t + z_t \quad (42)$$

From Equation (4212), Z_t is the error correction term in the Ordinary Least-Squares residuals series from the Long-run cointegration regression.

If we lag Equation (42) by one, that is $Lr_t = r_{t-1}$ and L is the lag operator, then we have

$$r_{t-1} = \alpha_0 + \alpha_1 g_{t-1} + \alpha_2 t_{t-1} + \alpha_3 m_{t-1} + \alpha_4 e_{t-1} + \alpha_5 s_{t-1} + z_{t-1} \quad (43)$$

$$z_{t-1} = r_{t-1} - \alpha_0 - \alpha_1 g_{t-1} - \alpha_2 t_{t-1} - \alpha_3 m_{t-1} - \alpha_4 e_{t-1} - \alpha_5 s_{t-1} \quad (44)$$

Thus, substituting Equation (44) into Equation (18) we have,

$$\Delta r_t = \beta_0 + \sum_{i=1}^{p} \beta_i \Delta r_{t-i} + \sum_{i=0}^{n} a_i' \Delta g_{t-i} + \sum_{i=0}^{m} \theta_i' \Delta t_{t-i} + \sum_{i=0}^{o} \alpha_i' \Delta m_{t-i} + \sum_{i=0}^{q} \lambda_i' \Delta e_{t-i} + \sum_{i=0}^{v} \mu_i' \Delta s_{t-i} + \Upsilon(r_{t-1} - \alpha_0 - \alpha_1 g_{t-1} - \alpha_2 t_{t-1} - \alpha_3 m_{t-1} - \alpha_4 e_{t-1} - \alpha_5 s_{t-1}) + \xi_t \quad (45)$$

$$\Delta r_t = \beta_0 + \sum_{i=1}^{p} \beta_i \Delta r_{t-i} + \sum_{i=0}^{n} a_i' \Delta g_{t-i} + \sum_{i=0}^{m} \theta_i' \Delta t_{t-i} + \sum_{i=0}^{o} \alpha_i' \Delta m_{t-i} + \sum_{i=0}^{q} \lambda_i' \Delta e_{t-i} + \sum_{i=0}^{v} \mu_i' \Delta s_{t-i} + \Upsilon r_{t-1} - \Upsilon \alpha_0 - \Upsilon \alpha_1 g_{t-1} - \Upsilon \alpha_2 t_{t-1} - \Upsilon \alpha_3 m_{t-1} - \Upsilon \alpha_4 e_{t-1} - \Upsilon \alpha_5 s_{t-1} + \xi_t \quad (46)$$

From assumption (iii) we have,

$$\beta_0 - \Upsilon\alpha_0 = \rho_0, \Upsilon = \vartheta_1, -\Upsilon\alpha_1 = \vartheta_2, -\Upsilon\alpha_2 = \vartheta_3, -\Upsilon\alpha_3 = \vartheta_4, -\Upsilon\alpha_4 = \vartheta_5\ and\ -\Upsilon\alpha_5 = \vartheta_6.$$

Thus,

$$\Delta r_t = \rho_0 + \sum_{i=1}^{p} \beta_i \Delta r_{t-i} + \sum_{i=0}^{n} \alpha_i' \Delta g_{t-i} + \sum_{i=0}^{m} \theta_i' \Delta t_{t-i} + \sum_{i=0}^{o} \alpha_i' \Delta m_{t-i} + \sum_{i=0}^{q} \lambda_i' \Delta e_{t-i} + \sum_{i=0}^{v} \mu_i' \Delta s_{t-i} + \vartheta_1 r_{t-1} + \vartheta_2 g_{t-1} + \vartheta_3 t_{t-1} + \vartheta_4 m_{t-1} + \vartheta_5 e_{t-1} + \vartheta_6 s_{t-1} + \xi_t \quad (47)$$

Equation (47) now accommodates both the long – run parameters ($\vartheta_1, \vartheta_2, \ldots, \vartheta_6$) and the short – run parameters ($\beta_i, \alpha_i', \theta_i', \alpha_i', \lambda_i', \mu_i'$) respectively. The maximum lag length of Equation (47) can be selected using information criteria. Therefore, several information criteria have shown great reliability in the selection of a statistical model. In time series analysis there are different information criteria for their respective model and all criteria are likelihood based with two major parts or components. The first part tackles the goodness of fit of the model to the data, whereas the other component penalizes more heavily complicated models (Tsay 2014). The goodness of fit of a model is often measured by the maximum likelihood. The selection of the penalty is relatively subjective, that is different penalties for different information criterion.

The Akaike information criterion (1974 and 1976), Schwarz information criterion (1978) and the Hanan-Quinn (1979) information criterion for selecting the most parsimonious model. Over-parameterization is taking care of by this process (Campos *et al*. (2005)) as cited in (Irefin and Yaaba, 2013). Raykov and Marcoulides (1999) stated that by the principle of parsimony, it is expedient to estimate the model that includes minimum lag the present absence of residual autocorrelation. The information criteria are;

Akaike (AIC): $c_T(k) = -\frac{2ln(L_T(k))}{T} + 2\frac{k}{T}$,

Schwarz (SIC): $c_T(k) = -\frac{2ln(L_T(k))}{T} + k\frac{l_n(T)}{T}$,

Hanan-Quinn (HQ): $c_T(k) = -\frac{2ln(L_T(k))}{T} + 2k\frac{l_n(l_n(T))}{T}$

respectively. These criteria take the general form

$$c_T(k) = -\frac{2ln(L_T(k))}{T} + k\frac{\Omega(T)}{T},$$

Where $\Omega(T) = 2$ in the Akaike case, $\Omega(T) = l_n(T)$ in the Schwarz case and $\Omega(T) = 2l_n(l_n(T))$ in the Hanan-Quinn case, the likelihood function $L_T(k) = -\frac{T}{2}(1 + \log(2\pi) + \log\left(\frac{\hat{e}'\hat{e}}{T}\right))$ and $\hat{e}'\hat{e} = e^2 = \sum_{t=1}^{T}(lR - X_i'b)$, where X_i' is a vector of exogenous variables and b is a vector of coefficients such that;

$$X_i' = (x_1\ x_2\ x_3 \ldots x_k), \quad b = \begin{pmatrix} b_1 \\ b_2 \\ b_3 \\ \vdots \\ b_k \end{pmatrix}$$

From assumption (iv) above, Equation (47) becomes;

$$\Delta r_t = \rho_0 + \sum_{i=1}^{2} \beta_i \Delta r_{t-i} + \sum_{i=0}^{3} \alpha_i' \Delta g_{t-i} + \sum_{i=0}^{0} \theta_i' \Delta t_{t-i} + \sum_{i=0}^{0} \alpha_i' \Delta m_{t-i} + \sum_{i=0}^{0} \lambda_i' \Delta e_{t-i} + \sum_{i=0}^{1} \mu_i' \Delta s_{t-i} + \vartheta_1 r_{t-1} + \vartheta_2 g_{t-1} + \vartheta_3 t_{t-1} + \vartheta_4 m_{t-1} + \vartheta_5 e_{t-1} + \vartheta_6 s_{t-1} + \xi_t \quad (48)$$

$$\Delta r_t = \rho_0 + \beta_1 \Delta r_{t-1} + \beta_2 \Delta r_{t-2} + \alpha_0' \Delta g_t + \alpha_1' \Delta g_{t-1} + \alpha_2' \Delta g_{t-2} + \alpha_3' \Delta g_{t-3} + \theta_0' \Delta t_t + \alpha_0' \Delta m_t + \lambda_0' \Delta e_t + \mu_0' \Delta s_t + \mu_1' \Delta s_{t-1} + \vartheta_1 r_{t-1} + \vartheta_2 g_{t-1} + \vartheta_3 t_{t-1} + \vartheta_4 m_{t-1} + \vartheta_5 e_{t-1} + \vartheta_6 s_{t-1} + \xi_t \quad (49)$$

where $\Delta r_t = r_t - r_{t-1}$

$r_t = \rho_0 + r_{t-1} + \beta_1(r_{t-1} - r_{t-2}) + \beta_2(r_{t-2} - r_{t-3}) + a_0'(g_t - g_{t-1}) + a_1'(g_{t-1} - g_{t-2}) + a_2'(g_{t-2} - g_{t-3}) + a_3'(g_{t-3} - g_{t-4}) + \theta_0'(t_t - t_{t-1}) + \alpha_0'(m_t - m_{t-1}) + \lambda_0'(e_t - e_{t-1}) + \mu_0'(s_t - s_{t-1}) + \mu_1'(s_{t-1} - s_{t-2}) + \vartheta_1 r_{t-1} + \vartheta_2 g_{t-1} + \vartheta_3 t_{t-1} + \vartheta_4 m_{t-1} + \vartheta_5 e_{t-1} + \vartheta_6 s_{t-1} + \xi_t$ (50)

Also from assumption (v) Equation (50) becomes;

$r_t = \rho_0 + (1 + \beta_1 + \vartheta_1)r_{t-1} + (\beta_2 - \beta_1)r_{t-2} + (a_1' - a_0' + \vartheta_2)g_{t-1} + (a_3' - a_2')g_{t-3} + \theta_0' t_t + \alpha_0' m_t + \lambda_0' e_t + (\vartheta_6 + \mu_1' - \mu_0')s_{t-1} + \xi_t$ (51)

Where $\rho_0 = C, (1 + \beta_1 + \vartheta_1) = C(1), (\beta_2 - \beta_1) = C(2), (a_1' - a_0' + \vartheta_2) = C(3), (a_3' - a_2') = C(4), \theta_0' = C(5), \alpha_0' = C(6), \lambda_0' = C(7), (\vartheta_6 + \mu_1' - \mu_0') = C(8)$

$LogR_t = C + C(1)LogR_{t-1} + C(2)LogR_{t-2} + C(3)LogG_{t-1} + C(4)LogG_{t-3} + C(5)LogT_t + C(6)LogM_t + C(7)LogE_t + C(8)LogS_{t-1} + \xi_t$ (52)

Equation (52) is the modified ARDL model to be estimated using least squares (LS). Then,

$C = 1, C(1) = 2, C(2) = 3, C(3) = 4, C(4) = 5, C(5) = 6, C(6) = 7, C(7) = 8 \; and \; C(8) = 9$

Thus,

$$K = \{1, 2, 3, 4, 5, 6, 7, 8, 9\} \; n(K) = 9$$

The elements of the set K are the parameters of the long – run stability for Equation (52) and the number of elements in the set K represents the order of Equation (52). Therefore, $n(K)$ now paved way in applying the binomial coefficient model to determine the total number of model each order can accommodate and their respective stability for different families of r.

The Binomial Coefficients Model

The binomial theorem is given by;

$$(a + b)^i = \sum_{r=0}^{i} \binom{i}{r} a^{i-r} b^r$$

From the above theorem, assumption (vi) $a = b = 1, i = n(K), n(K)$ is the number of elements in set K and it takes positive numerical values. Thus,

$$(1 + 1)^{n(K)} = \sum_{r=0}^{n(K)} \binom{n(K)}{r}$$

Then,

$$2^{n(K)} = C_0^{n(K)} + \sum_{r=1}^{n(K)} \binom{n(K)}{r}$$

$$2^{n(K)} - C_0^{n(K)} = \sum_{r=1}^{n(k)} \binom{n(K)}{r}$$

Where $C_0^{n(K)} = 1$, implies that

$$2^{n(K)} - 1 = \sum_{r=1}^{n(K)} \binom{n(K)}{r}$$

T_{nrm} (Total number of regression model) $= 2^{n(K)} - 1$

$$= \sum_{r=1}^{n(K)} \binom{n(K)}{r}$$

$$T_{nrm} \text{ (Total number of regression model)} \quad = \sum_{r=1}^{n(K)} \binom{n(K)}{r}, r = 1(1)n(K) \qquad (53)$$

Using one of the properties of combinatorial coefficients;

$$\binom{n}{j} + \binom{n}{j+1} = \binom{n+1}{j+1} \qquad (*)$$

From equation (*)

$$\binom{n}{j} = \binom{n+1}{j+1} - \binom{n}{j+1} \qquad (**)$$

Using assumptions (viii) and (ix) in equation **

$$\binom{n(k)-1}{r-1} = \binom{n(k)}{r} - \binom{n(k)-1}{r} \quad (***)$$

Equation (***) gives the number of models with fixed intercept. From equation (**) decompose $\binom{n}{j}$ such that,

$$\binom{n}{j} = \binom{n-1}{j} - \binom{n-1}{j-1} \quad (****)$$

Substitute equation (****) into equation (**)
Thus,

$$\binom{n-1}{j-1} = \binom{n+1}{j+1} - \binom{n}{j+1} - \binom{n-1}{j} \quad (*****)$$

Using assumptions (viii) and (xi) to obtain the stability rows for different order of $n(k)$ and their respective families of r.

$$\binom{n(k)-2}{r-2} = \binom{n(k)}{r} - \binom{n(k)-1}{r} - \binom{n(k)-2}{r-1} \quad (******)$$

Equation (53) gives the total number of regression model for different order of $n(K)$ for the long – run parameters. For a fixed $C = 1$ in Equation (53), it reduces T_{nrm} for which the stability of the models is obtained (see Appendix 3). In the estimation of Equation (52), if some of its parameters are statistically insignificant in their p – values, then we introduce the matrix elimination model.

Matrix Elimination Model

The modified ARDL model of Equation (52) is the least parsimonious model as suggested by the information criteria, which must have long run stability but with some parameters that are statistically insignificant at a specified level of percentage in their p-values (see Appendix 2). Thereafter, the matrix elimination model starts by eliminating at a time the least significant variable form the model whose p-value is greater than a specified level of significance. When this is done, the reduced ARDL model must also be stable. The process of matrix elimination is repeated for several times until no p-value in their respective parameters are greater than a specified significant level. Then the process of the matrix elimination is terminated for which all the parameters in their p-value are less than a specified level of significance. The model for

which all the p-values are less than a specified significance level, perhaps at 5% is statistically significant for parameter estimation. In the other hand, the process of matrix elimination is terminated when the model for which the variable and its parameter is eliminated becomes unstable, and then the preceding model is adopted as the choice model irrespective of whether there are parameters that are statistically insignificant.

From Equation (52)

$$LogR_t = C(1)LogR_{t-1} + C(2)LogR_{t-2} + C(3)LogG_{t-1} + C(4)LogG_{t-3} + C(5)LogT_t + C(6)LogM_t + C(7)LogE_t + C(8)LogS_{t-1} + C$$

is transformed to a matrix $M_{n\times m}$ and ɣ (also see Appendix 2) such that;

$$LogR_t = (LogR_{t-1}\ LogR_{t-2}\ LogG_{t-1}\ LogG_{t-3}\ LogT_t\ LogM_t\ LogE_t\ LogS_{t-1}\ 1)(C(1)\ C(2)\ C(3)\ C(4)\ C(5)\ \dots\ C)^T \quad (54)$$

$$LogR_t = M_{n\times m}ɣ \quad (55)$$

Thus,

$$M_{m\times n}^T LR_{n\times 1} = M_{m\times n}^T M_{n\times m}ɣ \quad (56)$$

for which $C(1) = ɣ_1, C(2) = ɣ_2, C(3) = ɣ_3, \dots, C = ɣ_m$ and $LogR_t = LR_{n\times 1}$

Therefore, $M_{(n\times m)}$ must be stable in all its parameters, $ɣ_j, j = 1(1)m$

Where ɣ is the unknown parameter, j=1(1) m, i=1(1) n.

Then the product,

$M_{m\times n}^T M_{n\times m} = Z_{m\times m}$ and $M_{m\times n}^T LR_{n\times 1} = H_{m\times 1}$,

$$Z_{(m\times m)}ɣ = H_{m\times 1}$$

Then,

$$ɣ = Z_{m\times m}^{-1} H_{m\times 1}$$

$$ɣ = ɣ_{m\times 1} \quad (57)$$

$$ɣ = \begin{bmatrix} ɣ_1 \\ ɣ_2 \\ ɣ_3 \\ ɣ_4 \\ ɣ_5 \\ ɣ_6 \\ . \\ . \\ . \\ ɣ_m \end{bmatrix}$$

If there exist any $ɣ_j$, $say\ ɣ_{m-1}$ in ɣ whose p - value > 0.05 , then there is need to carry out matrix elimination on the exogenous variable and its parameter value at the same time from the matrix $M_{(n\times m)}$ and its transpose. The reason for this matrix elimination is to obtain parameters that are stable and statistically significant at 5%, hence the result gives estimates that are statistically significant for parameter estimation. Thus, the new system becomes;

$$\dot{M}_{(m-1)\times n}^T, \dot{M}_{n\times(m-1)}, LR_{(n\times 1)}\ and\ ɣ̇$$

Therefore, $\dot{M}_{n\times(m-1)}$ must be stable in all its parameters, $ɣ_j\ j = 1(1)m-1$.

If m_{ij} is not statistically significant at 5% in $M_{(n\times m)}$, then the variable should be eliminated from the matrix $M_{(n\times m)}$ and $M_{m\times n}^{T}$. Where m_{ji}, m_{ij} had been removed from the matrix $M_{(n\times m)}$ and $M_{m\times n}^{T}$ respectively. Similarly, γ_{m-3} from γ , then the first elimination process becomes;

$$\dot{M}_{(\mathrm{m}-1)\times \mathrm{n}}^{\mathrm{T}}\dot{M}_{n\times(m-1)}\dot{\gamma} = \dot{M}_{(m-1)\times n}^{T}LR_{(n\times 1)}$$

Where,

$$\dot{M}_{(m-1)\times \mathrm{n}}^{\mathrm{T}}\dot{M}_{n\times(m-1)} = \dot{Z}_{(m-1)\times(m-1)}$$

and

$$\dot{M}_{(m-1)\times n}^{T}LR_{(n\times 1)} = \dot{H}_{(m-1)\times 1}$$

Thus,

$$\dot{Z}_{(m-1)\times(m-1)}\dot{\gamma} = \dot{H}_{(m-1)\times 1}$$

Then to obtain $\dot{\gamma}$, the matrix becomes

$$\dot{\gamma} = \dot{Z}_{(m-1)\times(m-1)}^{-1}\dot{H}_{(m-1)\times 1}$$

$$\dot{\gamma} = \gamma_{(m-1)\times 1} \qquad (58)$$

If there exist any $\dot{\gamma}_j,\ say\ \dot{\gamma}_{m-2}$ whose p - value > 0.05 in all its parameter values $\dot{\gamma}_j\ j = 1(1)m-1$, then there is need to carry out matrix elimination on the exogenous variable and its parameter value at the same time from the matrix $\dot{M}_{n\times(m-1)}$ and its transpose. The reason for this matrix elimination is to obtain parameters that are stable and statistically significant at 5%, hence the result gives estimates that are statistically significant for parameter estimation. Thus, the new system becomes;

$$\ddot{M}_{(m-2)\times n}^{T}, \ddot{M}_{n\times(m-2)}, LR_{(n\times 1)}\ and\ \ddot{\gamma}$$

Therefore, $\ddot{M}_{n\times(m-2)}$ must be stable in all its parameters, where s_{ji}, s_{ij} had been removed from the matrix $\dot{M}_{n\times(m-1)}$ and $\dot{M}_{(m-1)\times \mathrm{n}}^{\mathrm{T}}$ respectively. Similarly, $\dot{\gamma}_{m-2}$ from $\dot{\gamma}$.

Where the second elimination process becomes;

$$\ddot{M}_{(m-2)\times n}^{T}\ddot{M}_{n\times(m-2)}\ddot{\gamma} = \ddot{M}_{(m-2)\times n}^{T}\,LR_{(n\times 1)}$$

Where

and

$$\ddot{M}_{(m-2)\times n}^{T}LR_{(n\times 1)} = \ddot{H}_{(m-2)\times 1}$$

Thus,

$$\ddot{Z}_{(m-2)\times(m-2)}\ddot{\gamma} = \ddot{H}_{(m-2)\times 1}$$

$$\ddot{\gamma} = \ddot{Z}_{(m-2)\times(m-2)}{}^{-1}\ddot{H}_{(m-2)\times 1}$$

$$\ddot{\gamma} = \gamma_{(m-2)\times 1} \qquad (59)$$

Therefore, $\ddot{M}_{n\times(m-2)}$ must be stable in all its parameters, $\ddot{\gamma}_j$, $j = 1(1)m-2$.
If there exist any $\ddot{\gamma}_j$, $say\ \ddot{\gamma}_{m-3}$ whose p – value > 0.05 , then there is need to carry out matrix elimination on the exogenous variable and its parameter value at the same time from the matrix $\ddot{M}_{n\times(m-2)}$ and its transpose. The reason for this matrix elimination is to obtain parameters that are stable and statistically significant at 5%, hence the result gives estimates that are statistically significant for parameter estimation. Thus, the new system becomes;

$$\ddot{M}^{T}_{(m-3)\times n}, \ddot{M}_{n\times(m-3)}, LR_{(n\times 1)}\ and\ \ddot{\gamma}$$

$$\ddot{M}^{T}_{(m-3)\times n}\ \ddot{M}_{n\times(m-3)}\ddot{\gamma} = \ddot{M}^{T}_{(m-3)\times n}\ LR_{(n\times 1)}$$

Where,

$$\ddot{M}^{T}_{(m-3)\times n}\ \ddot{M}_{n\times(m-3)} = \ddot{Z}_{(m-3)\times(m-3)}$$

Similarly,

$$\ddot{M}^{T}_{(m-3)\times n}\ LR_{(n\times 1)} = \ddot{H}_{(m-3)\times 1}$$

Thus,

$$\ddot{Z}_{(m-3)\times(m-3)}\ddot{\gamma} = \ddot{H}_{(m-3)\times 1}$$

$$\ddot{\gamma} = \ddot{\gamma}_{(m-3)\times 1} \qquad (60)$$

$$\ddot{\gamma}^{T} = [\ddot{\gamma}_1 \quad \ddot{\gamma}_2 \quad \ddot{\gamma}_3 \quad \ddot{\gamma}_4 \quad \ddot{\gamma}_5 \quad . \quad . \quad . \quad \ddot{\gamma}_{m-3}]$$

DATA/ANALYSIS OF THE METHOD

The Modified ARDL Model

We analyzed the modified ARDL model of Equation (52) with the aid of econometric view (EView) package applying least squares to estimate the long – run parameters of order nine using quarterly data from CBN annual statistical bulletin that span from 1999Q1 to 2011Q2 and 2000Q1 to 2012Q4 (see Table 1 in Appendix 1). It was observed that the exogenous variables such as second lag of reserve, monetary policy rate, exchange rate are inverse related to reserves and others are positive related. The estimated parameters shows that the major determinant to foreign reserves are first and second lag of GDP and Trade Openness. In the estimated parameters for period (2000Q1- 2012Q4) only monetary policy rate is statistically insignificant and for period (1999Q1 – 2011Q2) monetary policy rate, exchange rate and foreign debt are statistically insignificant since they have p – values greater than 5% level of significance (see Table 1and 3 in Appendix 3). The adjustment back to the long – run stability is about 19.1% and 23% disequilibrium is corrected on a quarterly basis with variation in reserves (also see Table 2 and 4 in Appendix 3). The estimation of parameters in both periods shows long – run

stability in their cumulative sum of recursive residuals (CUSUM) and cumulative sum of squares of recursive residuals (CUSUMSQ) at 5% respectively (figures 1, 2, 3 and 4 in Appendix 3).

The Binomial Coefficients Model

In ARDL model of any order say $n(K)$, the long – run stability follows a sequence of pattern of the binomial coefficients model. That is the stability for order $n(K)$ follows a pattern around $n(K-2)$of the binomial coefficients model. Similarly, the stability for order $n(K-1)$ follows a pattern around $n(K-3)$ of the binomial coefficient model and the stability for order $n(K-2)$ follows a pattern around $n(K-4)$ of the binomial coefficients model and so on up to the stability for order $n(K-6)$ which also follows a pattern around $n(K-8)$. We observed that there is a synergy between the stability obtained from the binomial coefficients model and the econometric view package (see Table 5 and 6 in Appendix 3).

The Matrix Elimination Model for Sample (2000Q1-2012Q4)

The modified ARDL model of Equation (57) for order $n(K)=9$ has its estimated parameters and respective p – values as;

$$\text{ɣ} = \begin{bmatrix} 1.180918 \\ -0.344818 \\ 0.658327 \\ 0.657772 \\ 0.053448 \\ -0.144597 \\ -0.781727 \\ 0.114542 \\ -11.03724 \end{bmatrix} \quad \text{With respective} \quad p-values = \begin{bmatrix} 0.0000 \\ 0.0142 \\ 0.0001 \\ 0.0002 \\ 0.0004 \\ 0.0654 \\ 0.0038 \\ 0.0140 \\ 0.0000 \end{bmatrix}$$

LR = 1.18091777719 LR (-1) - 0.344817570867 LR (-2) + 0.658326678092 LG (-1) + 0.657771795343 LG (-3) + 0.0534476224896 LT - 0.144596977585 LM - 0.781727112739 LE + 0.11454201159 LS (-1) -11.0372378768

For other subsequent elimination process for equation (58), (59) and (60) gives;

$$\dot{\text{ɣ}} = \begin{bmatrix} 1.238195 \\ -0.380701 \\ 0.553822 \\ 0.551459 \\ 0.046226 \\ -0.597976 \\ 0.064158 \\ -9.504126 \end{bmatrix} \quad \text{With respective} \quad p-values = \begin{bmatrix} 0.0000 \\ 0.0081 \\ 0.0006 \\ 0.0008 \\ 0.0016 \\ 0.0179 \\ 0.0897 \\ 0.0002 \end{bmatrix}$$

LR = 1.23819509391 LR (-1) - 0.38070101803 LR (-2) + 0.5538219435 LG (-1) + 0.55145886501 LG (-3) + 0.0462256788374 LT - 0.59797561966 LE + 0.0641575429724 LS (-1) - 9.50412628637

$$\ddot{\gamma} = \begin{bmatrix} 1.331784 \\ -0.515163 \\ 0.463629 \\ 0.458765 \\ 0.046608 \\ -0.381848 \\ -7.385031 \end{bmatrix} \quad \text{With respective} \quad p-values = \begin{bmatrix} 0.0000 \\ 0.0001 \\ 0.0023 \\ 0.0030 \\ 0.0018 \\ 0.0804 \\ 0.0008 \end{bmatrix}$$

LR = 1.33178401462 LR (-1) - 0.515162841344 LR (-2) + 0.463628536411 LG (-1) + 0.458765210769 LG (-3) + 0.0466082670768LT - 0.381848461658LE -7.38503058454

$$\dddot{\gamma} = \begin{bmatrix} 1.395724 \\ -0.533517 \\ 0.300764 \\ 0.291693 \\ 0.040314 \\ -5.764844 \end{bmatrix}, \quad \text{With respective} \quad p-values = \begin{bmatrix} 0.0000 \\ 0.0000 \\ 0.0109 \\ 0.0148 \\ 0.0058 \\ 0.0039 \end{bmatrix}.$$

LR = 1.39572363349 LR (-1) - 0.533517479032 LR (-2) + 0.300764344044 LG (-1) + 0.291692664665 LG (-3) + 0.0403138779589 LT - 5.76484381234

Matrix Elimination Model for Sample (1999Q1-2011Q2)

Parameters for the full matrix and the respective p-values of Equation (57)

$$\gamma = \begin{bmatrix} 1.337251 \\ -0.502723 \\ 0.519842 \\ 0.523464 \\ 0.056753 \\ -0.153850 \\ -0.366501 \\ 0.077155 \\ -9.456340 \end{bmatrix} \quad \text{With respective } p-values = \begin{bmatrix} 0.0000 \\ 0.0006 \\ 0.0070 \\ 0.0104 \\ 0.0012 \\ 0.1568 \\ 0.1248 \\ 0.1229 \\ 0.0038 \end{bmatrix}$$

LR = 1.33725141305 LR (-1) - 0.502723321647 LR (-2) + 0.51984245871 LG (-1) + 0.523464485408 LG (-3) + 0.0567532948224 LT - 0.153849882343 LM - 0.366501008793 LE + 0.0771553543495 LS (-1) - 9.45633965002

For other subsequent elimination process for Equations (58), (59) and (60) gives;

$$\dot{\gamma} = \begin{bmatrix} 1.395996 \\ -0.559112 \\ 0.502722 \\ 0.505226 \\ 0.048285 \\ -0.282676 \\ 0.035195 \\ -9.420892 \end{bmatrix} \quad \text{with respective} \quad p-values = \begin{bmatrix} 0.0000 \\ 0.0001 \\ 0.0096 \\ 0.0140 \\ 0.0030 \\ 0.2247 \\ 0.3829 \\ 0.0043 \end{bmatrix}$$

LR = 1.39599636451 LR (-1) - 0.559112362247 LR (-2) + 0.502722010068 LG (-1) + 0.505226486255 LG (-3) + 0.048285044094 LT - 0.282676270411 LE + 0.0351953696723 LS (-1) - 9.420892416

$$\ddot{\gamma} = \begin{bmatrix} 1.428055 \\ -0.607328 \\ 0.439320 \\ 0.435513 \\ 0.047953 \\ -0.175127 \\ -7.876563 \end{bmatrix} \text{ with respective } p-values = \begin{bmatrix} 0.0000 \\ 0.0000 \\ 0.0133 \\ 0.0199 \\ 0.0031 \\ 0.3712 \\ 0.0038 \end{bmatrix}$$

LR = 1.42805536806 LR (-1) - 0.607327982857 LR (-2) + 0.439320321973 LG (-1) + 0.435513441743 LG (-3) + 0.047953118948 LT - 0.175127088498 LE - 7.87656294372

$$\ddot{\gamma} = \begin{bmatrix} 1.425515 \\ -0.575695 \\ 0.337152 \\ 0.327480 \\ 0.044101 \\ -6.512297 \end{bmatrix} \text{, with respective } \quad p-values = \begin{bmatrix} 0.0000 \\ 0.0000 \\ 0.0109 \\ 0.0189 \\ 0.0043 \\ 0.0031 \end{bmatrix}$$

LR = 1.42551453898LR (-1) - 0.575695249874 LR (-2) + 0.337151836674 LG (-1) + 0.327479811785 LG (-3) + 0.0441008704776 LT - 6.51229686678

In the above matrix elimination model, all parameters from $\ddot{\gamma}$ are statistically significant at their respective p – values. Hence, can be used for statistical estimation.

INTERPRETATION OF RESULTS

The Binomial Coefficients Model and ARDL Model

The stability of an autoregressive distributed lag model with intercept and no constant trend twirl along or twisted on the sequence of pattern of the binomial coefficients model for different order of n(k). The whole process gives an insight of how each family present their respective parsimonious model taking **r** family at a time. Then, a selection for the general parsimonious model is carried out with a clearer description form econometric view package. The binomial coefficients model cannot really tell the least parsimonious model and as such we introduce econometric view which explains the process by exploring information criteria (AIC, SIC and HQ). Over-parameterization is taking care of by this process Campos *et al*. (2005). The findings show that the full matrix has few of its parameters that are statistically insignificant at 5%. The result estimating Foreign Reserves from the estimated parameters for data 2000Q1-2012Q4 and 1999Q1-2011Q2 are stable and a positive indication that the model is reliable for statistical estimations and it performs better to the estimated parameters obtained by Irefin and Yaaba (2013). Υ is the error correction parameter, Z_{t-1} is the residual that is obtained from the estimated cointegration model and ξ_t is the disturbance term assumed to be uncorrelated with zero means.

The results for short - run of Reserves on exogenous variables within the error correction model are examined by using Equation (41). The short-run alteration process is examined from the error correction model. If the coefficient of Z_{t-1} lies between 0 and -1, the correction to Reserves in the period t is a fraction of the error in period $t-1$.As a result, the Z_{t-1} causes Reserves to converge monotonically to its long-run equilibrium path in reply to changes in the exogenous variables. If the coefficient Z_{t-1} is positive or less than -2,

this will cause the Reserves to diverge. If the value of the coefficient is between -1 and -2, Error Correction Model (ECM) will produce damped oscillations in the Reserves around its equilibrium path.

The Matrix Elimination Model

In the full matrix from our result, it was clear that few parameters were insignificant at 5% level of significance and the result obtained by Irefin and Yaaba (2013). Hence the need for matrix elimination on our model that is motivated by the p – values of the respective parameters. The systematic elimination now sprang a model whose p – values are statistically significant at 5%. Therefore, we say freely that the model now has its true determinants for statistical estimation.

CONCLUSION AND POLICY IMPLICATIONS

The result indicated by Irefin and Yaaba (2013) for R – squared explained over 98% movement in reserve and whereas ours explained over 99% for reserves (see Table 1 and 3 in Appendix 3). It was observed from our findings that gross domestic product (GDP) with first and third lags respectively exhibit a positive relationship with foreign reserves coinciding with the results from (Irefin and Yaaba (2013), Bankole and Shuaibu (2013), Mosayeb *et al* (2005)) and as such considered as the major determinant of foreign reserves. The distance second is trade openness with positive relationship and significant as reported by Atif *et al.* (2010). Again, it was found that monetary policy rates (MPR), Exchange rate (EXR) are inverse related to reserve, which again confirms Irefin and Yaaba (2013). By this, Ben – Bassat and Gottlied (1992) believes MPR could have been positive related and it shows from our findings that any percentage increase in MPR and EXR can drain foreign reserve in Nigeria (Table 1 and 3 in Appendix 3). The error correction parameters (-0.19126 and -0.22712) for the short – run dynamics are indicators towards long – run stability. Comparing the result of Irefin and Yaaba (2013) and our findings, it was obvious that about 19.1% and 23% disequilibrium is corrected as against 14% on a quarterly basis with variation in reserves. The implication of this is that, it will take disequilibrium to be corrected to equilibrium in five quarters and one month and four quarters, one month and two weeks respectively as against seven quarters and one month as obtained in Irefin and Yaaba (2013). Therefore, convergence towards equilibrium path in our findings is faster when compared with the result from Irefin and Yaaba (2013).

RECOMMENDATIONS

We therefore recommend that decision and policy makers formulate a mechanism to maintain long – run stability in Foreign Reserves for Nigeria. In this study intercept was fixed to the binomial coefficients model and further studies should embrace both intercept and constant trend fixed to the binomial coefficients model and investigation should be carried out to determine the difference in stability between the binomial coefficient model and the stability obtained from autoregressive distributed lag model.

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Appendix 1

TABLE 1: Data from CBN Annual Bulletin (2013)

Quarter	R	G	IM	M	T	E	S
1999Q1	5507.1	98099.48	167230	18	54.92043	86.32	28589.96
1999Q2	4772.3	98394.12	216085.5	18	74.19829	93.25	28406.38
1999Q3	5032.1	98546.73	231512.1	18	82.79302	94.88	28222.79
1999Q4	5424.6	98066.84	245697.7	18	90.22541	96.32	28039.21
2000Q1	6682.8	103201.2	160731.3	13.5	79.42832	99.88	28097.83
2000Q2	7272.4	103182.9	173001.3	13.5	84.18086	101.12	28156.45
2000Q3	8118.1	103234.4	177015.1	13.5	74.00978	103.53	28215.06
2000Q4	9386.1	102713.5	182873.3	13.5	68.93036	103.9	28273.68
2001Q1	10787.5	108099.8	311394.2	14.3	75.85664	110.62	28292.01
2001Q2	10166.7	108093.2	348097.9	14.3	76.97549	113.25	28310.34
2001Q3	10563.9	108083.7	364182.3	14.3	73.9196	111.71	28328.67
2001Q4	10267.1	107506.5	323791.8	14.3	60.18938	112.19	28347
2002Q1	9546.1	112633	376098.8	19	46.14942	114.76	29008.22
2002Q2	8674.7	113328.2	237339.6	19	39.70769	117.06	29669.44
2002Q3	7424	113096.1	325514.1	19	54.00043	125.31	30330.65
2002Q4	7681.1	112728.4	328984	19	59.65409	126.76	30991.87
2003Q1	8226.16	124036.8	543697.5	15.75	64.60292	127.18	31473.11
2003Q2	7673.09	123928.7	637173.7	15.75	64.1783	127.62	31954.34
2003Q3	7170.46	123782.6	587857.7	15.75	64.74057	128.08	32435.58
2003Q4	7467.78	123259	592867.6	15.75	69.97847	134.54	32916.81
2004Q1	9684.49	114617.6	542251	15	58.83204	135.23	33673.77
2004Q2	11441.36	123702.9	542199.2	15	63.4855	133.09	34430.74
2004Q3	13222.9	142373.6	548465.9	15	64.96385	132.82	35187.7
2004Q4	16955.02	146881.9	561051	15	69.51445	132.86	35944.66
2005Q1	21807.98	120048.9	606281.3	13	64.53038	132.85	32077.99
2005Q2	24367.12	128755.5	620972.6	13	63.33277	132.85	28211.32
2005Q3	28638.24	153933.6	631451.6	13	62.4192	132.3	24344.64
2005Q4	28279.06	159193.4	637718.2	13	60.55284	130.59	20477.97
2006Q1	36201.54	128579.8	639120.7	12.3	66.7117	129.53	16244.6
2006Q2	36479	135438.6	612365	12.3	53.46805	128.46	12011.23
2006Q3	40457.86	162498.8	839306.6	12.3	58.49019	128.33	7777.86
2006Q4	42298.11	169304.4	719713.3	12.3	46.90873	128.29	3544.49
2007Q1	42633.86	135774.9	1116317	8.8	58.42221	128.23	3287.73
2007Q2	42626.2	142763.5	1072817	8.8	55.67136	127.65	3348.22
2007Q3	47930.22	173067.5	1336873	8.8	57.94592	126.58	3397.48

2007Q4	51333.15	182618.2	813760.5	8.8	46.01118	120.87	3654.21
2008Q1	59756.51	142071.4	824101.6	9.8	60.34545	118.04	3670.748
2008Q2	59157.15	150862.2	857801.1	9.8	62.17349	117.84	3687.285
2008Q3	62081.86	183678.8	838843.8	9.8	52.98382	117.75	3703.823
2008Q4	53000.36	195590.1	778350.1	9.8	38.72028	120.65	3720.36
2009Q1	47081.9	149191.5	957725.1	7.4	36.53433	146.88	3719.8
2009Q2	43462.74	162101.2	994515.1	7.4	37.58924	147.76	3719.24
2009Q3	43343.33	197084.3	1310612	7.4	52.8128	150.92	3863.93
2009Q4	42382.49	210600.4	1785016	7.4	70.66926	149.96	3947.3
2010Q1	40667.03	160117	1453138	6.1	0.794283	149.8285	4306.18
2010Q2	37468.44	174734	1332006	6.1	0.841809	150.1915	4269.71
2010Q3	34589.01	212771.7	1668885	6.1	0.740098	151.0332	4534.19
2010Q4	32339.25	228709.5	1469228	6.1	0.689304	150.4799	4578.77
2011Q1	33221.8	171265.9	1672986	9.2	0.758566	152.5074	5227.05
2011Q2	31890.91	187833.1	2476227	9.2	0.769755	154.5029	5398.04
2011Q3	31740.23	228454.8	2276061	9.2	0.739196	155.2636	5633.71
2011Q4	32639.78	246447.1	2948707	9.2	0.62	158.2074	5666.58
2012Q1	35197.44	182119.4	2253505	12	0.58	157.5875	5993.54
2012Q2	35412.5	199831.6	2962151	12	0.56	157.4383	6035.66
2012Q3	40640.4	243263.1	2879259	12	0.52	157.3429	6296.17
2012Q4	43830.42	263678.9	3810044	12	0.53	157.324	6527.07

Appendix 2

$$M_{n\times m}=\begin{bmatrix} 0 & 0 & 0 & 0 & t_{115} & m_{116} & e_{117} & 0 & 1 \\ r_{121} & 0 & g_{123} & 0 & t_{125} & m_{126} & e_{127} & s_{12,m-1} & 1 \\ r_{131} & r_{232} & g_{133} & 0 & t_{135} & m_{136} & e_{137} & s_{13,m-1} & 1 \\ r_{141} & r_{242} & g_{143} & g_{244} & t_{145} & m_{146} & e_{147} & s_{14,m-1} & 1 \\ r_{151} & r_{252} & g_{153} & g_{254} & t_{155} & m_{156} & e_{157} & s_{15,m-1} & 1 \\ r_{161} & r_{262} & g_{163} & g_{264} & t_{165} & m_{166} & e_{167} & s_{16,m-1} & 1 \\ . & . & . & . & . & . & . & . & . \\ . & . & . & . & . & . & . & . & . \\ . & . & . & . & . & . & . & . & . \\ r_{1n-1,1} & r_{2n-1,2} & g_{1n-1,3} & g_{2n-1,4} & t_{1n-1,5} & m_{1n-1,6} & e_{1n-1,7} & s_{1n-1,m-1} & 1 \\ r_{1n,1} & r_{2n,2} & g_{1n,3} & g_{2n,4} & t_{1n,5} & m_{1n,6} & e_{1n,7} & s_{1n,m-1} & 1 \end{bmatrix}$$

$$M_{m\times n}^{T}=\begin{bmatrix} 0 & r_{112} & r_{113} & r_{114} & r_{115} & r_{116} & \dots & r_{11n-1} & r_{11n} \\ 0 & 0 & r_{223} & r_{224} & r_{225} & r_{226} & \dots & r_{22n-1} & r_{22n} \\ 0 & g_{132} & g_{133} & g_{134} & g_{135} & g_{136} & \dots & g_{13n-1} & g_{13n} \\ 0 & 0 & 0 & g_{244} & g_{245} & g_{246} & \dots & g_{24n-1} & g_{14n} \\ t_{151} & t_{152} & t_{153} & t_{154} & t_{155} & t_{156} & \dots & t_{15n-1} & t_{15n} \\ m_{161} & m_{162} & m_{163} & m_{164} & m_{165} & m_{166} & \dots & m_{16n-1} & m_{16n} \\ e_{171} & e_{172} & e_{173} & e_{174} & e_{175} & e_{176} & \dots & e_{17n-1} & e_{17n} \\ 0 & s_{1m-1,2} & s_{1m-1,3} & s_{1m-1,4} & s_{1m-1,5} & s_{1m-1,6} & \dots & s_{1m-1,n-1} & s_{1m-1,n} \\ 1 & 1 & 1 & 1 & 1 & 1 & \dots & 1 & 1 \end{bmatrix}$$

$$LR = \begin{bmatrix} r_1 \\ r_2 \\ r_3 \\ r_4 \\ r_5 \\ . \\ . \\ . \\ r_n \end{bmatrix}, \qquad \text{ɣ} = \begin{bmatrix} \text{ɣ}_1 \\ \text{ɣ}_2 \\ \text{ɣ}_3 \\ \text{ɣ}_4 \\ \text{ɣ}_5 \\ . \\ . \\ . \\ \text{ɣ}_m \end{bmatrix}$$

$$Z_{m\times m} = \begin{bmatrix} z_{11} & z_{12} & z_{13} & z_{14} & z_{15} & z_{16} & z_{1,m-2} & z_{1,m-1} & z_{1m} \\ z_{21} & z_{22} & z_{23} & z_{24} & z_{25} & z_{26} & z_{2,m-2} & z_{2,m-1} & z_{2m} \\ z_{31} & z_{32} & z_{33} & z_{34} & z_{35} & z_{36} & z_{3,m-2} & z_{3,m-1} & z_{3m} \\ z_{41} & z_{42} & z_{43} & z_{44} & z_{45} & z_{46} & z_{4,m-2} & z_{4,m-1} & z_{4m} \\ z_{51} & z_{52} & z_{53} & z_{54} & z_{55} & z_{56} & z_{5,m-2} & z_{5,m-1} & z_{5m} \\ z_{61} & z_{62} & z_{63} & z_{64} & z_{65} & z_{66} & z_{6,m-2} & z_{6,m-1} & z_{6m} \\ z_{m-2,1} & z_{m-2,2} & z_{m-2,3} & z_{m-2,4} & z_{m-2,5} & z_{m-2,6} & z_{m-2,m-2} & z_{m-2,m-1} & z_{m-2,m} \\ z_{m-1,1} & z_{m-1,2} & z_{m-1,3} & z_{m-1,4} & z_{m-1,5} & z_{m-1,6} & z_{m-1,m-2} & z_{m-1,m-1} & z_{m-1,m} \\ z_{m1} & z_{m2} & z_{m3} & z_{m4} & z_{m5} & z_{m6} & z_{m,m-2} & z_{m,m-1} & z_{mm} \end{bmatrix}$$

$$H_{(m\times 1)} = \begin{bmatrix} h_1 \\ h_2 \\ h_3 \\ h_4 \\ h_5 \\ h_6 \\ . \\ . \\ . \\ h_m \end{bmatrix}$$

$$\text{ɣ} = \begin{bmatrix} \text{ɣ}_1 \\ \text{ɣ}_2 \\ \text{ɣ}_3 \\ \text{ɣ}_4 \\ \text{ɣ}_5 \\ \text{ɣ}_6 \\ . \\ . \\ . \\ \text{ɣ}_m \end{bmatrix}$$

$$\dot{M}_{n\times(m-1)} = \begin{bmatrix} 0 & 0 & 0 & 0 & t_{115} & e_{116} & 0 & 1 \\ r_{121} & 0 & g_{123} & 0 & t_{125} & e_{126} & s_{12,m-1} & 1 \\ r_{131} & r_{232} & g_{133} & 0 & t_{135} & e_{136} & s_{13,m-1} & 1 \\ r_{141} & r_{242} & g_{143} & g_{244} & t_{145} & e_{146} & s_{14,m-1} & 1 \\ r_{151} & r_{252} & g_{153} & g_{254} & t_{155} & e_{156} & s_{15,m-1} & 1 \\ r_{161} & r_{262} & g_{163} & g_{264} & t_{165} & e_{166} & s_{16,m-1} & 1 \\ . & . & . & . & . & . & . & . \\ . & . & . & . & . & . & . & . \\ . & . & . & . & . & . & . & . \\ r_{1n-1,1} & r_{2n-1,2} & g_{1n-1,3} & g_{2n-1,4} & t_{1n-1,5} & e_{1n-1,6} & s_{1n-1,m-1} & 1 \\ r_{1n,1} & r_{2n,2} & g_{1n,3} & g_{2n,4} & t_{1n,5} & e_{1n,6} & s_{1n,m-1} & 1 \end{bmatrix}$$

$$\dot{M}^T_{(m-1)\times n} = \begin{bmatrix} 0 & r_{112} & r_{113} & r_{114} & r_{115} & r_{116} & . & . & . & r_{11n-1} & r_{11n} \\ 0 & 0 & r_{223} & r_{224} & r_{225} & r_{226} & . & . & . & r_{22n-1} & r_{22n} \\ 0 & g_{132} & g_{133} & g_{134} & g_{135} & g_{136} & . & . & . & g_{13n-1} & g_{13n} \\ 0 & 0 & 0 & g_{244} & g_{245} & g_{246} & . & . & . & g_{24n-1} & g_{14n} \\ t_{151} & t_{152} & t_{153} & t_{154} & t_{155} & t_{156} & . & . & . & t_{15n-1} & t_{15n} \\ e_{161} & e_{162} & e_{163} & e_{164} & e_{165} & e_{166} & . & . & . & e_{16n-1} & e_{16n} \\ 0 & s_{1m-1,2} & s_{1m-1,3} & s_{1m-1,4} & s_{1m-1,5} & s_{1m-1,6} & . & . & . & s_{1m-1,n-1} & s_{1m-1,n} \\ 1 & 1 & 1 & 1 & 1 & 1 & . & . & . & 1 & 1 \end{bmatrix}$$

$$LR_{(n\times 1)} = \begin{bmatrix} r_1 \\ r_2 \\ r_3 \\ r_4 \\ r_5 \\ . \\ . \\ . \\ r_n \end{bmatrix},$$

$$\dot{Y} = \begin{bmatrix} \dot{Y}_1 \\ \dot{Y}_2 \\ \dot{Y}_3 \\ \dot{Y}_4 \\ \dot{Y}_5 \\ . \\ . \\ . \\ \dot{Y}_{m-1} \end{bmatrix}$$

$$\dot{Z}_{(m-1)\times(m-1)} = \begin{bmatrix} z_{11} & z_{12} & z_{13} & z_{14} & z_{15} & z_{1,m-1} \\ z_{21} & z_{22} & z_{23} & z_{24} & z_{25} & z_{2,m-1} \\ z_{31} & z_{32} & z_{33} & z_{34} & z_{35} & z_{3,m-1} \\ z_{41} & z_{42} & z_{43} & z_{44} & z_{45} & z_{4,m-1} \\ z_{51} & z_{52} & z_{53} & z_{54} & z_{55} & z_{5,m-1} \\ z_{m-3,1} & z_{m-3,2} & z_{m-3,3} & z_{m-3,4} & z_{m-3,5} & z_{m-3,m-1} \\ z_{m-2,1} & z_{m-2,2} & z_{m-2,3} & z_{m-2,4} & z_{m-2,5} & z_{m-2,m-1} \\ z_{m-1,1} & z_{m-1,2} & z_{m-1,3} & z_{m-1,4} & z_{m-1,5} & z_{m-1,m-1} \end{bmatrix}$$

$$\dot{H}_{(m-1)\times 1} = \begin{bmatrix} h_1 \\ h_2 \\ h_3 \\ h_4 \\ h_5 \\ . \\ . \\ . \\ h_{m-1} \end{bmatrix}$$

$$\dot{Y} = \begin{bmatrix} \dot{Y}_1 \\ \dot{Y}_2 \\ \dot{Y}_3 \\ \dot{Y}_4 \\ \dot{Y}_5 \\ . \\ . \\ . \\ \dot{Y}_{m-1} \end{bmatrix}$$

$$\ddot{M}_{n\times(m-2)} = \begin{bmatrix} 0 & 0 & 0 & 0 & t_{115} & e_{116} & 1 \\ r_{121} & 0 & g_{123} & 0 & t_{125} & e_{126} & 1 \\ r_{131} & r_{232} & g_{133} & 0 & t_{135} & e_{136} & 1 \\ r_{141} & r_{242} & g_{143} & g_{244} & t_{145} & e_{146} & 1 \\ r_{151} & r_{252} & g_{153} & g_{254} & t_{155} & e_{156} & 1 \\ r_{161} & r_{262} & g_{163} & g_{264} & t_{165} & e_{166} & 1 \\ . & . & . & . & . & . & . \\ . & . & . & . & . & . & . \\ . & . & . & . & . & . & . \\ r_{1n-1,1} & r_{2n-1,2} & g_{1n-1,3} & g_{2n-1,4} & t_{1n-1,5} & e_{1n-1,6} & 1 \\ r_{1n,1} & r_{2n,2} & g_{1n,3} & g_{2n,4} & t_{1n,5} & e_{1n,6} & 1 \end{bmatrix}$$

$$\ddot{M}^{T}_{(m-2)\times n} = \begin{bmatrix} 0 & r_{112} & r_{113} & r_{114} & r_{115} & r_{116} & . & . & . & r_{11n-1} & r_{11n} \\ 0 & 0 & r_{223} & r_{224} & r_{225} & r_{226} & . & . & . & r_{22n-1} & r_{22n} \\ 0 & g_{132} & g_{133} & g_{134} & g_{135} & g_{136} & . & . & . & g_{13n-1} & g_{13n} \\ 0 & 0 & 0 & g_{244} & g_{245} & g_{246} & . & . & . & g_{24n-1} & g_{14n} \\ t_{151} & t_{152} & t_{153} & t_{154} & t_{155} & t_{156} & . & . & . & t_{15n-1} & t_{15n} \\ e_{161} & e_{162} & e_{163} & e_{164} & e_{165} & e_{166} & . & . & . & e_{16n-1} & e_{16n} \\ 1 & 1 & 1 & 1 & 1 & 1 & . & . & . & 1 & 1 \end{bmatrix}$$

$$LR_{(n\times 1)} = \begin{bmatrix} r_1 \\ r_2 \\ r_3 \\ r_4 \\ r_5 \\ . \\ . \\ . \\ r_n \end{bmatrix},$$

$$\ddot{Y} = \begin{bmatrix} \ddot{Y}_1 \\ \ddot{Y}_2 \\ \ddot{Y}_3 \\ \ddot{Y}_4 \\ \ddot{Y}_5 \\ . \\ . \\ . \\ \ddot{Y}_{m-2} \end{bmatrix}$$

$$\ddot{Z}_{(m-2)\times(m-2)} = \begin{bmatrix} z_{11} & z_{12} & z_{13} & z_{14} & z_{15} & z_{16} & . & . & . & z_{1,m-2} \\ z_{21} & z_{22} & z_{23} & z_{24} & z_{25} & z_{26} & . & . & . & z_{2,m-2} \\ z_{31} & z_{32} & z_{33} & z_{34} & z_{35} & z_{36} & . & . & . & z_{3,m-2} \\ z_{41} & z_{42} & z_{43} & z_{44} & z_{45} & z_{46} & . & . & . & z_{4,m-2} \\ z_{51} & z_{52} & z_{53} & z_{54} & z_{55} & z_{56} & . & . & . & z_{5,m-2} \\ z_{61} & z_{62} & z_{63} & z_{64} & z_{65} & z_{66} & . & . & . & z_{6,m-2} \\ z_{m-3,1} & z_{m-3,2} & z_{m-3,3} & z_{m-3,4} & z_{m-3,5} & z_{m-3,6} & . & . & . & z_{m-3,m-2} \\ z_{m-2,1} & z_{m-2,2} & z_{m-2,3} & z_{m-2,4} & z_{m-2,5} & z_{m-2,6} & . & . & . & z_{m-2,m-2} \end{bmatrix}$$

$$\ddot{H}_{(m-2)\times 1} = \begin{bmatrix} h_1 \\ h_2 \\ h_3 \\ h_4 \\ h_5 \\ h_6 \\ . \\ . \\ . \\ h_{m-2} \end{bmatrix}$$

$$\ddot{Y} = \begin{bmatrix} \ddot{Y}_1 \\ \ddot{Y}_2 \\ \ddot{Y}_3 \\ \ddot{Y}_4 \\ \ddot{Y}_5 \\ . \\ . \\ . \\ \ddot{Y}_{m-2} \end{bmatrix}$$

$$\ddot{M}_{n\times(m-3)} = \begin{bmatrix} 0 & 0 & 0 & 0 & t_{115} & 1 \\ r_{121} & 0 & g_{123} & 0 & t_{125} & 1 \\ r_{131} & r_{232} & g_{133} & 0 & t_{135} & 1 \\ r_{141} & r_{242} & g_{143} & g_{244} & t_{145} & 1 \\ r_{151} & r_{252} & g_{153} & g_{254} & t_{155} & 1 \\ r_{161} & r_{262} & g_{163} & g_{264} & t_{165} & 1 \\ . & . & . & . & . & . \\ . & . & . & . & . & . \\ . & . & . & . & . & . \\ r_{1n-1,1} & r_{2n-1,2} & g_{1n-1,3} & g_{2n-1,4} & t_{1n-1,5} & 1 \\ r_{1n,1} & r_{2n,2} & g_{1n,3} & g_{2n,4} & t_{1n,5} & 1 \end{bmatrix}$$

$$\ddot{M}^{T}_{(m-3)\times n} = \begin{bmatrix} 0 & r_{112} & r_{113} & r_{114} & r_{115} & r_{116} & . & . & . & r_{11n-1} & r_{11n} \\ 0 & 0 & r_{223} & r_{224} & r_{225} & r_{226} & . & . & . & r_{22n-1} & r_{22n} \\ 0 & g_{132} & g_{133} & g_{134} & g_{135} & g_{136} & . & . & . & g_{13n-1} & g_{13n} \\ 0 & 0 & 0 & g_{244} & g_{245} & g_{246} & . & . & . & g_{24n-1} & g_{14n} \\ t_{151} & t_{152} & t_{153} & t_{154} & t_{155} & t_{156} & . & . & . & t_{15n-1} & t_{15n} \\ 1 & 1 & 1 & 1 & 1 & 1 & . & . & . & 1 & 1 \end{bmatrix}$$

$$LR_{(n\times1)} = \begin{bmatrix} r_1 \\ r_2 \\ r_3 \\ r_4 \\ r_5 \\ . \\ . \\ . \\ r_n \end{bmatrix},$$

$$\ddot{Y} = \begin{bmatrix} \ddot{Y}_1 \\ \ddot{Y}_2 \\ \ddot{Y}_3 \\ \ddot{Y}_4 \\ \ddot{Y}_5 \\ . \\ . \\ . \\ \ddot{Y}_{m-3} \end{bmatrix}$$

$$\ddot{Z}_{(m-3)\times(m-3)} = \begin{bmatrix} z_{11} & z_{12} & z_{13} & z_{14} & z_{15} & z_{16} & . & . & . & z_{1,m-3} \\ z_{21} & z_{22} & z_{23} & z_{24} & z_{25} & z_{26} & . & . & . & z_{2,m-3} \\ z_{31} & z_{32} & z_{33} & z_{34} & z_{35} & z_{36} & . & . & . & z_{3,m-3} \\ z_{41} & z_{42} & z_{43} & z_{44} & z_{45} & z_{46} & . & . & . & z_{4,m-3} \\ z_{51} & z_{52} & z_{53} & z_{54} & z_{55} & z_{56} & . & . & . & z_{5,m-3} \\ z_{61} & z_{62} & z_{63} & z_{64} & z_{65} & z_{66} & . & . & . & z_{6,m-3} \\ z_{m-3,1} & z_{m-3,2} & z_{m-3,3} & z_{m-3,4} & z_{m-3,5} & z_{m-3,6} & . & . & . & z_{m-3,m-3} \end{bmatrix}$$

$$\ddot{H}_{(m-3)\times1} = \begin{bmatrix} h_1 \\ h_2 \\ h_3 \\ h_4 \\ h_5 \\ h_6 \\ . \\ . \\ . \\ h_{m-3} \end{bmatrix}$$

$$\ddot{Y} = \ddot{Y}_{(m-3)\times1}, \quad \ddot{Y}^{T} = [\ddot{Y}_1 \quad \ddot{Y}_2 \quad \ddot{Y}_3 \quad \ddot{Y}_4 \quad \ddot{Y}_5 \quad . \quad . \quad . \quad \ddot{Y}_{m-3}]$$

Appendix 3

The matrix $M_{n\times m}$ must be stable in its parameter values since we are considering the least parsimonious model.

Table 1: The least Parsimonious Model for Long – run Parameters

Dependent Variable: LR
Sample: 2000Q1 2012Q4
Included observations: 52

Variable	Coefficient	Std. Error	t-Statistic	Prob.
C	-11.03724	2.429650	-4.542728	0.0000
LR(-1)	1.180918	0.130865	9.023968	0.0000
LR(-2)	-0.344818	0.134846	-2.557121	0.0142
LG(-1)	0.658327	0.155491	4.233864	0.0001
LG(-3)	0.657772	0.158750	4.143449	0.0002
LT	0.053448	0.013895	3.846539	0.0004
LM	-0.144597	0.076465	-1.891015	0.0654
LE	-0.781727	0.255538	-3.059146	0.0038
LS(-1)	0.114542	0.044735	2.560432	0.0140

R-squared	0.990537	Mean dependent var	10.01357
Adjusted R-squared	0.988777	S.D. dependent var	0.749769
S.E. of regression	0.079431	Akaike info criterion	-2.071754
Sum squared resid	0.271297	Schwarz criterion	-1.734039
Log likelihood	62.86561	Hannan-Quinn criter.	-1.942282
F-statistic	562.6389	Durbin-Watson stat	1.964195
Prob(F-statistic)	0.000000		

Table 2: Error Correction Representation for the Selected ARDL Model (Short-run)

ARDL (3, 3, 0, 0, 1) selected based on Schwarz Bayesian Criterion

```
*****************************************************************************
 Dependent variable is dLR
 52 observations used for estimation from 2000Q1 to 2012Q4

*****************************************************************************
 Regressor              Coefficient       Standard Error         T-Ratio [Prob]
 dLR1                       .34197            .13630             2.5089[.016]
 dLR2                       .33445            .13373             2.5009[.016]
 dLG                       -.20034            .15538            -1.2894[.204]
 dLG1                      -.41951            .17266            -2.4296[.019]
 dLG2                      -.53121            .16392            -3.2406[.002]
 dLT                        .044387           .014414            3.0795[.004]
 dLM                       -.058236           .060824           -.95744[.344]
 dLE                       -.94057            .40824            -2.3039[.026]
 dINPT                     -6.6001            2.1323            -3.0953[.003]
 ecm(-1)                   -.19126            .053827           -3.5532[.001]
*****************************************************************************
R-Squared                     .58711   R-Bar-Squared                    .47357
 S.E. of Regression           .077199   F-stat.    F (9, 42)    6.3198[.000]
 Mean of Dependent Variable   .040180   S.D. of Dependent Variable      .10640
 Residual Sum of Squares       .23839   Equation Log-likelihood        66.2277
 Akaike Info. Criterion       54.2277   Schwarz Bayesian Criterion     42.5203
 DW-statistic                  2.0923
*****************************************************************************
```

Table 3: Revised version of Irefin's and Yaaba's model (including trade openness and external debt) with CUSUM/CUSUMSQ

Dependent Variable: LR
Date: 10/07/14 Time: 14:57
Sample: 1999Q1 2011Q2
Included observations: 50

Variable	Coefficient	Std. Error	t-Statistic	Prob.
C	-9.456340	3.079640	-3.070599	0.0038
LR(-1)	1.337251	0.125600	10.64692	0.0000
LR(-2)	-0.502723	0.135358	-3.714019	0.0006
LG(-1)	0.519842	0.183180	2.837879	0.0070
LG(-3)	0.523464	0.195021	2.684150	0.0104
LT	0.056753	0.016247	3.493193	0.0012
LM	-0.153850	0.106678	-1.442183	0.1568
LE	-0.366501	0.233858	-1.567195	0.1248
LS(-1)	0.077155	0.048987	1.575018	0.1229

R-squared	0.990445	Mean dependent var	9.838195
Adjusted R-squared	0.988581	S.D. dependent var	0.835348
S.E. of regression	0.089265	Akaike info criterion	-1.832869
Sum squared resid	0.326697	Schwarz criterion	-1.488705
Log likelihood	54.82172	Hannan-Quinn criter.	-1.701809
F-statistic	531.2647	Durbin-Watson stat	2.129299
Prob(F-statistic)	0.000000		

Table 4: Error Correction Representation for the Selected ARDL Model

```
        ARDL (3, 3, 0, 0, 1, 1) selected based on Schwarz Bayesian Criterion
*************************************************************************
 Dependent variable is dLR
 50 observations used for estimation from 1999Q1 to 2011Q2
*************************************************************************
 Regressor              Coefficient       Standard Error         T-Ratio [Prob]


dLR1                      .35836            .13626            2.6300[.012]
 dLR2                      .25833            .14442             1.7887[.081]
 dLG                      -.23281            .18181            -1.2805[.208]
 dLG1                     -.64165            .20948            -3.0631[.004]
 dLG2                     -.73639            .20934            -3.5177[.001]
 dLT                      .052277           .015401             3.3944[.002]
 dLM                      -.13927           .098342            -1.4162[.165]
 dLE                      -.65849            .26984            -2.4403[.019]
 dLS                      -.15022           .099817            -1.5049[.140]
 dINPT                    -8.8673            2.9692            -2.9864[.005]
 ecm(-1)                  -.22712           .068553            -3.3131[.002]
*************************************************************************
R-Squared                    .66769   R-Bar-Squared                   .54769
 S.E. of Regression          .079174   F-stat.    F (10, 39)    7.2333[.000]
 Mean of Dependent Variable  .030023   S.D. of Dependent Variable      .11772
 Residual Sum of Squares      .22567   Equation Log-likelihood        64.0710
 Akaike Info. Criterion      50.0710   Schwarz Bayesian Criterion     36.6868
 DW-statistic                 2.0878
*************************************************************************
```

Stability Test (CUSUM and CUSUMSQ) Sample 2000Q1 2012Q4

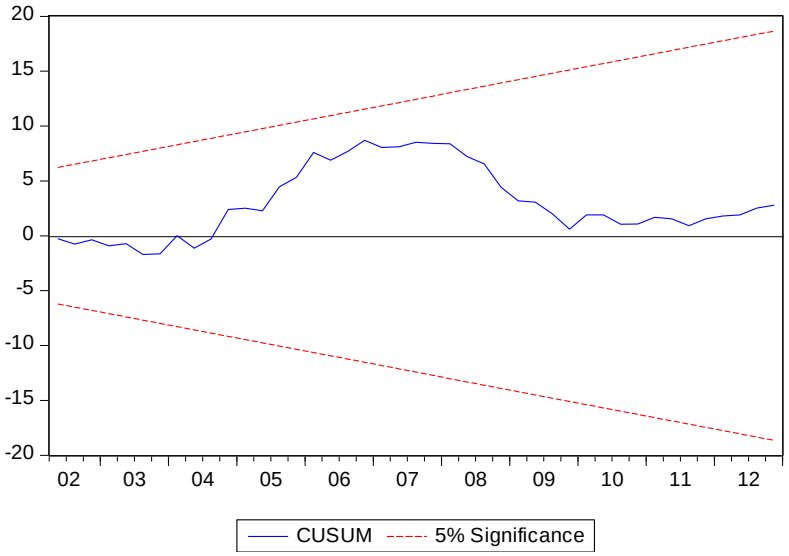

Figure 1: Stability of the Least Parsimonious Model with cumulative sum of recursive residuals at 5%

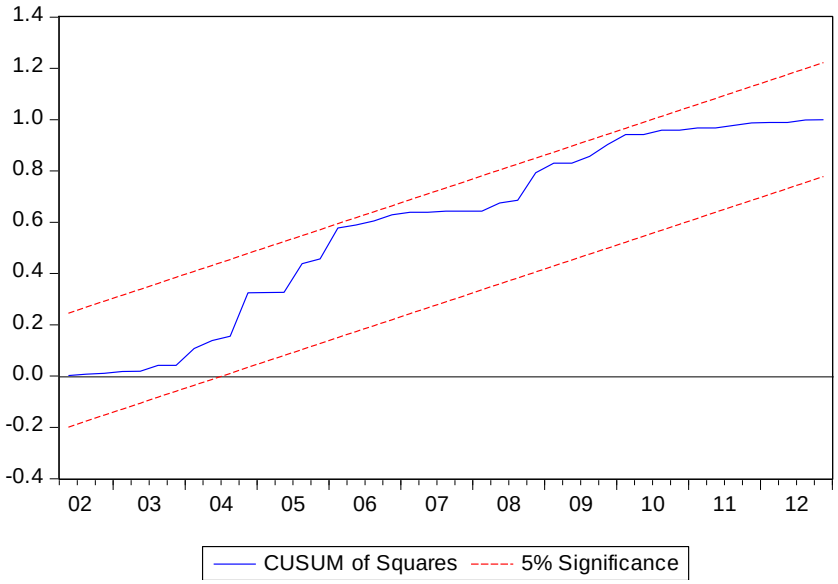

Figure 2: Stability of the Least Parsimonious Model with cumulative sum of squares of recursive residuals at 5%.

Stability Test (CUSUM and CUSUMSQ) Sample 1999Q1 2011Q2

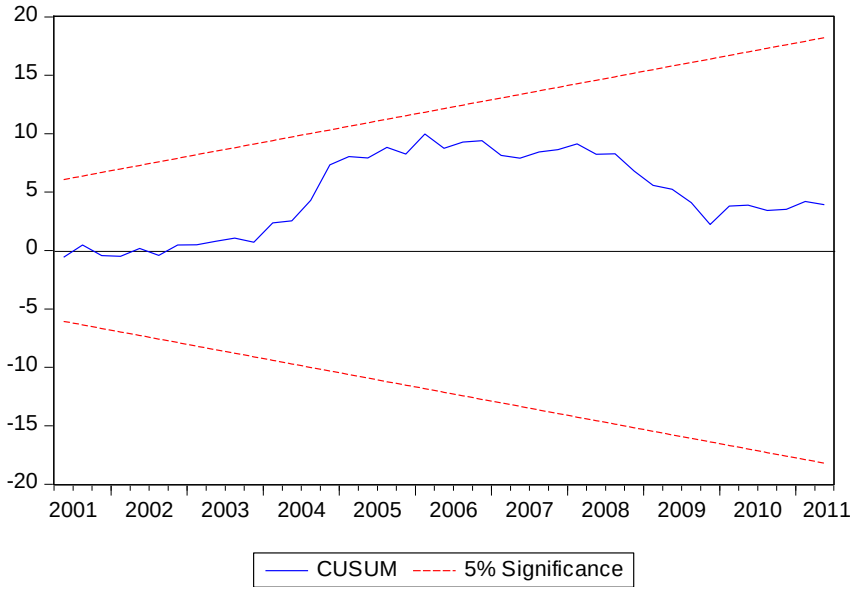

Figure 3: Stability of the Least Parsimonious Model by cumulative sum at 5%.

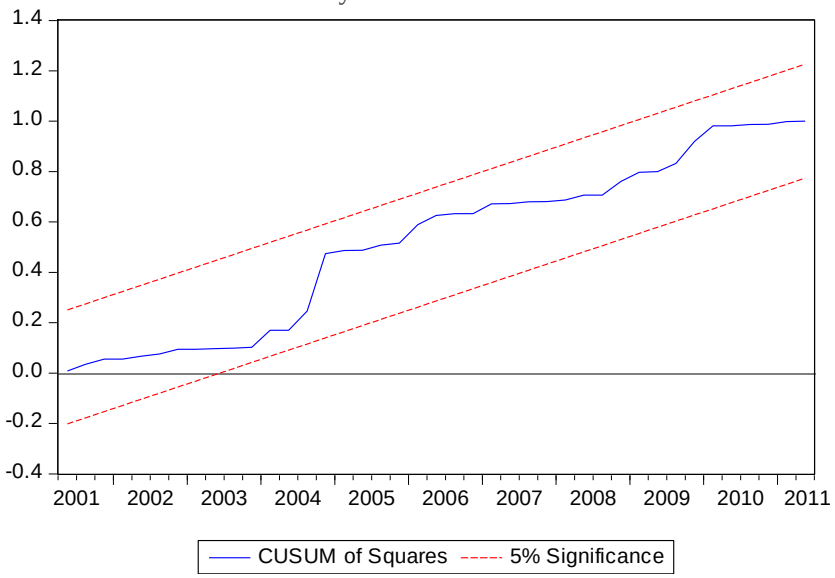

Figure 4: Stability of the Least Parsimonious Model by cumulative sum of squares of recursive residuals at 5%.

3.4 Analysis of the Binomial Model and Modified ARDL Model for different order of n(k)

Table 5: Data Period: 2000Q1-2012Q4

n(K) / r	3	4	5	6	7	8	9
1	NS	NS	NS	NS	NS	NS	NS
2	1, **1**	1,**1**	1,**1**	1,**1**	1,**1**	1,**1**	1, **1**
3	1, **NS**	2, **NS**	3,**2**	4,**4**	5,**5**	6,**6**	7, **7**
4	NA	1,**1**	3,**2**	6,**7**	10,**11**	15,**17**	21, **21**
5	NA	NA	1,**1**	4,**4**	10, **13**	20, **22**	35, **35**
6	NA	NA	NA	1,**1**	5,**5**	15,**16**	35, **37**
7	NA	NA	NA	NA	1,**1**	6,**7**	21, **24**
8	NA	NA	NA	NA	NA	1,**1**	7, **8**
9	NA	NA	NA	NA	NA	NA	1,**1**

Table 6: Data Period: 1999Q1-2011Q2

n(K) / r	3	4	5	6	7	8	9
1	NS	NS	NS	NS	NS	NS	NS
2	1, **1**	1,**1**	1,**1**	1,**1**	1,**1**	1,**1**	1, **1**
3	1, **NS**	2, **2**	3,**4**	4,**5**	5,**5**	6,**7**	7, **7**
4	NA	1,**1**	3,**4**	6,**9**	10,**12**	15,**18**	21, **20**
5	NA	NA	1,**1**	4,**5**	10, **9**	20, **20**	35, **26**
6	NA	NA	NA	1,**1**	5,**6**	15,**17**	35, **31**
7	NA	NA	NA	NA	1,**1**	6,**6**	21, **21**
8	NA	NA	NA	NA	NA	1,**1**	7, **7**
9	NA	NA	NA	NA	NA	NA	1,**1**

Where Bold face is $n(S_r(M))$ = Stability Results from EView, while faces not bolded is $n(S_r(B))$ = Stability from the respective families of the Binomial coefficients. NS means Not Stable and NA means Not Applicable.

Appendix 4

$$M_{n\times m}=\begin{bmatrix} 0 & 0 & 0 & 0 & 4.374855 & 2.60269 & 4.603969 & 0 & 1\\ 8.89184 & 0 & 11.54426 & 0 & 4.432968 & 2.60269 & 4.616308 & 10.24553 & 1\\ 9.00185 & 9.00185 & 11.54476 & 0 & 4.304197 & 2.60269 & 4.639861 & 10.24761 & 1\\ 9.14699 & 9.14699 & 11.5397 & 11.5397 & 4.233097 & 2.60269 & 4.643429 & 10.24969 & 1\\ 9.28614 & 9.28614 & 11.59081 & 11.59081 & 4.328845 & 2.66026 & 4.706101 & 10.25033 & 1\\ 9.22687 & 9.22687 & 11.59075 & 11.59075 & 4.343487 & 2.66026 & 4.729598 & 10.25098 & 1\\ . & . & . & . & . & . & . & . & .\\ . & . & . & . & . & . & . & . & .\\ . & . & . & . & . & . & . & . & .\\ 10.6125 & 10.6125 & 12.4019 & 12.4019 & -0.65393 & 2.484907 & 5.058428 & 8.747697 & 1\\ 10.6881 & 10.6881 & 12.48249 & 12.48249 & -0.63488 & 2.484907 & 5.058307 & 8.783713 & 1 \end{bmatrix}$$

$$M_{m\times n}^{T}=\begin{bmatrix} 0 & 8.89184 & 9.00185 & 9.14699 & 9.28614 & 9.22687 & . & . & . & 10.6125 & 10.6881\\ 0 & 0 & 9.00185 & 9.14699 & 9.28614 & 9.22687 & . & . & . & 10.6125 & 10.6881\\ 0 & 11.54426 & 11.54476 & 11.5397 & 11.59081 & 11.59075 & . & . & . & 12.4019 & 12.48249\\ 0 & 0 & 0 & 11.5397 & 11.59081 & 11.59075 & . & . & . & 12.4019 & 12.48249\\ 4.374855 & 4.432968 & 4.304197 & 4.233097 & 4.328845 & 4.343487 & . & . & . & -0.65393 & -0.63488\\ 2.60269 & 2.60269 & 2.60269 & 2.60269 & 2.66026 & 2.66026 & . & . & . & 2.484907 & 2.484907\\ 4.603969 & 4.616308 & 4.639861 & 4.643429 & 4.706101 & 4.729598 & . & . & . & 5.058428 & 5.058307\\ 0 & 10.24553 & 10.24761 & 10.24969 & 10.25033 & 10.25098 & . & . & . & 8.747697 & 8.783713\\ 1 & 1 & 1 & 1 & 1 & 1 & . & . & . & 1 & 1 \end{bmatrix}$$

$$LR^{T}=\begin{bmatrix} 8.80729 & 8.89184 & 9.00185 & 9.14699 & 9.28614 & 9.22687 & . & . & . & 10.6125 & 10.6881 \end{bmatrix}$$

$$\mathrm{y}=\begin{bmatrix} \mathrm{y}_1\\ \mathrm{y}_2\\ \mathrm{y}_3\\ \mathrm{y}_4\\ \mathrm{y}_5\\ .\\ .\\ .\\ \mathrm{y}_m \end{bmatrix}$$

Thus,

$$M_{m\times n}^{T}M_{n\times m}\mathrm{y}=M_{m\times n}^{T}\,LR$$

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PHYTOCHEMICAL ANALYSIS OF *Gongronema latifolium* ("Utazi") LEAF AND ITS BIOACTIVITY ON MICROBIAL ISOLATES FROM AFRICAN SORGHUM BEER (*Burukutu*)

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ABSTRACT
Forty-five (45) samples of prepared burukutu from Kaduna were microbiologically examined for microbial isolates using standard methods. Leaf extract of *Gongronema latifolium* was obtained using ethanol, hot and cold water as solvents. Phytochemical analysis of the plant was carried out using standard chemical methods. Antimicrobial properties of the extracts were evaluated using agar well diffusion method. The mean bacterial count of burukutu samples ranged from $1.2 \pm 0.10 \times 10^6$ to $8.3 \pm 0.30 \times 10^6$ cfu/ml in Kachia, $1.6 \pm 0.32 \times 10^6$ to $5.1 \pm 0.44 \times 10^6$ cfu/ml (Kajuru) and $1.4 \pm 0.21 \times 10^6$ to $7.2 \pm 0.40 \times 10^6$ cfu/ml in Kudan. The mean fungal count ranged from $1.47 \pm 0.31 \times 10^6$ to $3.87 \pm 0.35 \times 10^6$ cfu/ml in Kachia, $1.27 \pm 0.12 \times 10^6$ to $3.37 \pm 0.35 \times 10^6$ cfu/ml (Kajuru) and $1.50 \pm 0.27 \times 10^6$ to $4.00 \pm 0.30 \times 10^6$ cfu/ml in Kudan. Seven bacterial and four fungal species were identified. The phytochemistry of ethanol extracts of *G. latifolium* leaves showed phenols had the highest concentration of 15.74 ± 0.03 mg/100g and the least was anthraquinones (0.87 ± 0.02 mg/100g). The minimum inhibitory concentration (MIC) of the ethanol extract showed that *Staphylococcus aureus* and *Lactobacillus fermentum* were most susceptible to the leaf extract of *G. latifolium* with MIC of 6.25 mg/ml. The leaf extract of *G. latifolium* have the potential to preserve and extend the shelf-life of burukutu haven inhibited the microorganisms associated with it.

KEYWORDS: Burukutu, *Gongronema latifolium*, Phytochemical, Proximate

INTRODUCTION
Gongronema latifolium is a non-woody herbaceous plant from the family of Asclepiadaceae. It is widespread in the tropical and subtropical regions, especially in Africa and South America, with a moderate representation in Northern and South-Eastern Asia (Morebise *et al.*, 2002). In South-Eastern and South-Western Nigeria, *G. latifolium* is commonly called "Utazi" and "Arokeke" respectively (Agbo and Obi, 2006). *G. latifolium* is utilizable in many different ways to prepare delicacies in homes but primarily used as a spice and vegetable in traditional folk medicine. It can be consumed fresh, cooked or dried (Ugochukwu *et al.*, 2003). Reports by some authors show that it contains essential oils,

saponins and pregnanes (Schneider *et al.*, 1993). Morebise *et al.* (2002) showed that it has anti-inflammatory properties. In many situations it is part of herbal prescriptions or preparations administered by herbalists for treatment and or management of certain health challenges: pains, infertility, hypertension and ulcers (Schneider *et al.*, 1993).

Beer is an alcoholic beverage made from cereal grains, usually barley as well as corn, sorghum, millet, rice, wheat, and oats (Okafor, 2007). Beer is the world's oldest and most widely consumed alcoholic beverage and the third most popular drink overall after water and tea. Beer is made using a process called fermentation, in which yeast convert sugars in the grain to alcohol and carbon dioxide (Gutcho, 1976; Grossman, 1995: Bamforth, 2017). The tropical beers (African local beers) are known by different names in different parts of the world: *burukutu* and pito in Nigeria, maujek among the Nandi's in Kenya, mawe in Malawi, kaffir beer in South Africa, merisa in Sudan, bouza in Ethiopia and pombe in some parts of East Africa (Okafor, 2007; Ibrahim and Aondover, 2013).

Africa sorghum beer '*burukutu*' is a brownish-pink traditional alcoholic beverage consumed in the Northern Guinea savanna region of Nigeria, Republic of Benin and in Ghana (Okafor, 2007). The age long drink, serves as a source of alcohol for those who lack the financial means to patronize refined brew like beer and other foreign or imported drinks. It is produced mainly from the grains of guinea corn (*Sorghum vulgare* and *Sorghum bicolor*). The process of production of *burukutu* involves malting, mashing, fermentation and maturation (Ekundayo, 1969; Egemba and Etuk, 2007) . The production process of these indigenous drinks involves fermentation at its initial production stage which results in alcoholic drink. Its traditional processing method makes it susceptible to microbial contamination which results in the short shelf life of the product (Bamforth, 2017). This study was conducted to ascertain the antimicrobial potential of *G. latifolium* on microbial isolates from *burukutu* with a view to using it to extend its shelf life.

MATERIALS AND METHODS

Sample Collection

A total of forty-five (45) samples of *burukutu* used for this study were obtained from Kaduna metropolis. Fifteen (15) samples each of freshly prepared *burukutu* were obtained from the following locations; Kachia, Kajuru and Kudan. The samples were collected in 500 ml sterile plastic bottles and were transported to the laboratory in a cooler ice box containing ice packs for microbiological and physico-chemical analyses. Freshly harvested *G. latifolium* leaves were obtained from Uselu market, Benin City, Nigeria.

Isolation and enumeration of Microorganisms

Bacteria in *burukutu* were enumerated and isolated using nutrient agar and fungi with potatoe dextrose agar. The microbial isolates were identified phenotypically following standard microbiological procedures as described by Cheesbrough (2002), Lyn *et al.* (2014), Barnett and Hunter (1998).

Preparation of Ethanol Extract of Utazi Leaf

The "Utazi" leaves were collected by hand plucking from the plant and cleaned of debris by rinsing in running sterile distilled water. The leaves were then air-dried at ambient temperature (28 ± 2

°C) for 14 days to eliminate moisture (Atata *et al.,* 2003). The dried leaves were milled using a blender. Five grams (5g) of the milled leaves was soaked in 25 ml of each of the solvents (hot water, cold water and 99 % ethanol) separately. Each mixture was stirred occasionally and allowed to stay overnight. The samples was periodically shaken for at least 3 h a day to ensure complete extraction (Nenaah and Ahmed, 2011). The extract was filtered using Whatman filter paper No. 1 placed in a glass funnel and was concentrated by evaporation using water bath.

Antimicrobial Activity Bioassay

Standardization of the Bacterial Cell Suspension

Five colonies of each test organisms were inoculated into sterile test tube containing sterile nutrient broth and incubated at 35 °C for 24 h. The turbidity produced by these organisms were adjusted and used to match the McFarland turbidity (opacity) standard.

Agar Well Diffusion Method

Sterile nutrient agar was poured into sterile petri dishes aseptically and allowed to solidify. The surface of the sterile nutrient agar plate was streaked with pure culture of the standardized bacterial cell suspension. A cork borer (8 mm in diameter) was sterilized by flaming and used to create a ditch at the centre of each plate. The holes so created were then filled with the plant extract. The plates were allowed to stand for one hour for pre-diffusion of the extract and incubated at 28±2°C for 24 h. At the end of the incubation period, the diameter of the zone of inhibition was measured in millimeter (Ahmed and Aqil, 2007). This procedure was also carried out on the fungal isolates using potato dextrose agar as the medium.

Minimum Inhibitory Concentration (MIC) of the Extract

Test tubes were arranged on a test tube rack. The initial concentration of the plant extract (100 mg/ml) was diluted using double fold serial dilution by transferring 5 ml of the sterile plant extract (stock solution) into 5 ml of sterile nutrient broth to obtain 50 mg/ml concentration. The above process was repeated several times to obtain the different concentrations of the extract. Each concentration was inoculated with 0.l ml of each standardized bacterial cell suspension and incubated at 28±2°C for 24 h. Growth on the broth was indicated by turbidity or cloudiness of the broth and the lowest concentration of the extract which inhibited the growth of the test organisms, was taken as the minimum inhibitory concentration (MIC). Negative controls were set up as follows: nutrient broth only, nutrient broth and sterile plant extract, and finally positive control containing nutrient broth and test organism. This procedure was done for fungal isolates using potato dextrose broth.

Phytochemical Analysis

The qualitative and quantitative determination of phytochemical constituents of *G. latifolium* were carried out following standard techniques (Trease and Evans, 1996; AOAC, 2016).

RESULTS AND DISCUSSION

The total microbial count of "*Burukutu*" samples sold in Kaduna metropolis is shown in Table 1. The mean bacterial count ranged from 1.2 ± 0.10 × 10^6 cfu/ml to 8.3 ± 0.30 ×10^6 cfu/ml in Kachia, 1.6 ± 0.32 × 10^6 cfu/ml to 5.1 ±

0.44×10^6 cfu/ml in Kajuru and $1.4 \pm 0.21 \times 10^6$ cfu/ml to $7.2 \pm 0.40 \times 10^6$ cfu/ml in Kudan. The mean fungal count ranged from $1.47 \pm 0.31 \times 10^6$ cfu/ml to $3.87 \pm 0.35 \times 10^6$ cfu/ml in Kachia, $1.27 \pm 0.12 \times 10^6$ cfu/ml to $3.37 \pm 0.35 \times 10^6$ cfu/ml in Kajuru and $1.50 \pm 0.27 \times 10^6$ cfu/ml to $4.00 \pm 0.30 \times 10^6$ cfu/ml in Kudan. The presence, number and kind of microorganisms isolated in *Burukutu* could be as a result of processing and post-processing (fermentation) either from the handling or during mixing. The utensils used for storage may also be responsible for the microbial population and diversity in *burukutu*. Although *burukutu* is an acid fermented product, there is possibility of post processing contamination resulting from poor handling either at the processing site or from the market places during vending (Lyn *et al*., 2014).

Microorganisms isolated and their percentage occurrence frequencies are shown in Table 2. Bacteria isolated from the samples were *Bacillus subtilis, Escherichia coli, Staphylococcus aureus, Lactobacillus fermentum, Lactobacillus brevis, Pseudomonas aeruginosa* and *Lactobacillus plantarum* (Table 2). Fungi isolated from the samples were *Aspergillus* spp., *Candida* spp., *Penicillium* spp. and *Saccharomyces* spp. (Table 2). The lowest percentage occurrence of bacterial isolates was 26.7 % (*Lactobacillus brevis*) while the highest percentage occurrence was 73.3 % (*Staphylococcus aureus*). The lowest percentage occurrence of fungal isolates was 13.3 % (*Penicillium* spp.) while the highest percentage occurrence was 66.7 % (*Aspergillus niger*). The high mean microbial counts recorded shows exposure of the samples to heavy contamination by different genera of bacteria and fungi. The main sources of contamination may include humans, utensils, processing equipment and environment, handling and storage conditions (Eze *et al*., 2008). This observation is in agreement with the report of Kolawole *et al*. (2007), who documented information on the proximate and microbial analysis of *burukutu* and pito produced in Ilorin, Nigeria.

Table 3 shows the results of the susceptibility pattern of test organisms to the leaf extracts of *G. latifolium*. Ethanol extracts had the highest inhibitory effect while cold water extracts had no effect on all the isolates. *Bacillus subtilis, Escherichia coli, Staphylococcus aureus, Lactobacillus fermentum, Lactobacillus brevis, Lactobacillus plantarum, Aspergillus niger, Candida utilis* and *Saccharomyces cerevisceae,* were inhibited by both ethanol and hot water extracts. Results obtained showed that *G. latifolium* had no antimicrobial activity on *Pseudomonas aeruginosa* and *Penicillium* spp. The minimum inhibitory concentration (MIC) of the ethanol extract showed that *Staphylococcus aureus* and *Lactobacillus fermentum*, were most susceptible at 6.25 mg/ml followed by *Escherichia coli*, *Lactobacillus brevis and Lactobacillus plantarum* inhibited at 12.5 mg/ml. *Bacillus subtilis* and *Saccharomyces cerevisceae* were inhibited at 25 mg/ml, *Aspergillus niger and Candida utilis* were inhibited at 50 mg/ml, but *Pseudomonas aeruginosa* and *Penicillium* spp. were not susceptible at all concentrations used (Table 4).

Table 1: Total microbial count of "*burukutu*" samples sold in Kaduna

Sample Source (LGA)	Sample point	Mean bacterial count ± S.D ($\times 10^6$)	Mean fungi count ± S.D ($\times 10^6$)
Kachia	Dk	2.37 ± 0.15	1.47 ± 0.31
	Aw	1.2 ± 0.10	2.10 ± 0.46
	Bh	4.7 ± 0.20	1.70 ± 0.50
	Ak	7.5 ± 0.31	3.87 ± 0.35
	Sb	8.3 ± 0.30	2.20 ± 0.27
Kajuru	Rm	1.6 ± 0.32	1.27 ± 0.12
	Dt	3.5 ± 0.31	2.27 ± 0.45
	Fg	2.2 ± 0.25	1.47 ± 0.42
	Kt	3.7 ± 0.25	3.37 ± 0.35
	Yh	5.1 ± 0.44	2.77 ± 0.40
Kudan	Kd	1.4 ± 0.21	2.57 ± 0.35
	Lb	2.6 ± 0.30	1.50 ± 0.27
	Ma	3.7 ± 0.31	3.53 ± 0.32
	Dm	4.6 ± 0.40	2.67 ± 0.25
	Kn	7.2 ± 0.40	4.00 ± 0.30

Key: S.D = Standard deviation, LGA: Local Government Area
Dk = Doka, Aw = Awon, Bs = Bishini, Ak = Ankwa, Sb = Sabon, Rm = Rimau, Dt = Dokta, Fg = Fuga, Kt = Kutura, Yh = Yahaya, Kd = Kadama, Lb = Labalo, Ma = Ma-aji, Dm = Damaski, Kn = Kanawa

Table 2: Percentage occurrence of microbial isolates

Microbial isolates	No. of samples examined	Kachia Market	Kajuru Market	Kudan Market	Frequency (%)
			Occurrence		
Bacteria					
Staphylococcus aureus	5	4	3	4	11 (73.3)
Bacillus subtilis	5	3	0	2	5 (33.3)
E. coli	5	2	0	3	5 (33.3)
Lactobacillus brevis	5	0	0	4	4 (26.7)
Pseudomonas aeruginosa	5	3	4	0	7 (46.7)
Lactobacillus fermentum	5	3	3	3	9 (60)
Lactobacillus plantarum	5	4	0	3	7 (46.7)
Fungi					
Aspergillus niger	5	3	3	4	10 (66.7)
Candida utilis	5	4	0	3	7 (46.7)
Penicillium spp	5	0	2	0	2 (13.3)
Saccharomyces cerevisceae	5	0	3	2	5 (33.3)

Table 3: Susceptibility pattern of leaf extracts of *Gongronema latifolium* on test isolates at 100 mg/ml

S/N	Isolates	Diameter of Zone of Inhibition (mm)		
		Ethanol Extract	Hot Water Extract	Cold Water Extract
1	*Bacillus subtilis*	14	5	-
2	*Escherichia coli*	18	6	-
3	*Staphylococcus aureus*	28	12	-
4	*Lactobacillus fermentum*	26	10	-
5	*Lactobacillus brevis*	22	9	-
6	*Pseudomonas aeruginosa*	-	-	-
7	*Lactobacillus plantarum*	20	7	-
8	*Aspergillus niger*	10	-	-
9	*Candida utilis*	12	-	-
10	*Penicillium* spp.	-	-	-
11	*Saccharomyces cerevisceae*	14	5	-

Key - = No zone of inhibition,

Table 4: Minimum inhibitory concentration (MIC) of ethanolic extract of *G. latifolium* on microbial isolates

Test Organisms	Concentration (mg/ml)					MIC (mg/ml)
	3.125	6.25	12.5	25	50	
Bacillus subtilis	+	+	+	−	−	25
Escherichia coli	+	+	−	−	−	12.5
Staphylococcus aureus	+	−	−	−	−	6.25
Lactobacillus fermentum	+	−	−	−	−	6.25
Lactobacillus brevis	+	+	−	−	−	12.5
Pseudomonas aeruginosa	+	+	+	+	+	> 50
Lactobacillus plantarum	+	+	−	−	−	12.5
Aspergillus niger	+	+	+	+	−	50
Candida utilis	+	+	+	+	−	50
Penicillium spp.	+	+	+	+	+	> 50
Saccharomyces cerevisceae	+	+	+	−	−	25

Key: + = Growth, - = No growth, MIC = Minimum Inhibitory Concentration

Table 5: Quantitative Phytochemical analysis of ethanolic leaf extracts of *Gongronema latifolium* (Utazi leaf)

Parameter	Concentration (mg/100g)
Alkaloids	1.43 ± 0.03
Phenols	15.74 ± 0.03
Flavonoids	5.97 ± 0.02
Tannins	11.32 ± 0.02
Saponins	3.06 ± 0.03
Anthraquinones	0.87 ± 0.02
Cyanogenic glycosides	2.65 ± 0.01

In this study antimicrobial effect of *Gongronema latifolium* leaf extract was evaluated on strains of bacteria and fungi isolated from *burukutu*. The results of the antimicrobial properties of *G. latifolium* (Utazi leaf) revealed inhibitory activities of the ethanol extract against *Bacillus subtilis, Escherichia coli, Staphylococcus aureus, Lactobacillus fermentum, Lactobacillus brevis, Lactobacillus plantarum, Aspergillus niger, Candida utilis* and *Saccharomyces cerevisceae*. At 50 mg/ml, ethanol and hot water extract of *G. latifolium* were observed to possess varying degree of antimicrobial activities against isolated microorganisms. The zone of inhibition with ethanol extract ranged from 14 mm against *L. fermentum* to 28 mm against *Staphylococcus aureus*. The values ranged from 10-14 mm against fungal isolates. No antimicrobial activity was observed against *Pseudomonas aeruginosa* and *Penicillium* spp with the ethanol extract. In the aqueous extract, inhibition zone ranged from 5 - 12 mm against bacterial isolates and 5 - 7 mm against fungal isolates. No antimicrobial activity was observed against *P. aeruginosa* and *Penicillium* spp. It was observed that ethanol extract of *G. latifolium* was more active against microbial isolates than the hot water extract. Cold water extract had no antimicrobial activity against microbial isolates. This may be due to the fact that the extracting solvent (cold water) is less effective in extracting the phytochemicals present in the leaves of *G. latifolium* thus no activity was exhibited (Atata *et al*., 2003; Ahmed and Aqil, 2007).

A higher zone of inhibition was recorded for *Staphylococcus aureus* indicating the inhibitory effect on Gram-positive bacteria. This result is in agreement with the observation of Eleyinmi (2007) who reported inhibitory effect of methanol extract on *Bacillus subtilis* and *Staphylococcus aureus. Pseudomonas aeruginosa* and *Penicillium* spp were not inhibited by all the extracts of *G. latifolium* (Utazi leaf). The resistance exhibited by *P. aeruginosa* is in agreement with the report of Jarwertz *et al*. (1998) who showed that *P. aeruginosa* is highly resistant to many antimicrobial agents. Akortha *et al*. (2011) also showed the resistance of *P. aeruginosa* to antibiotics. Barry (1976) observed that the susceptibility of some test organisms varies with the concentration of the antimicrobial substance. Of all the extracts ethanol showed greater inhibitory activity. Cold water did not show any inhibitory property. A minimum inhibitory concentration of 6.25 mg/ml observed for *S. aureus* and *L. fermentum*; 12.5 mg/ml for *E. coli*, *L. brevis* and *L. plantarum* is a plus for the potential application of this plant extract for preserving and extending the shelf-life of sorghum beers.

The result of the phytochemistry of ethanol extracts of *G. latifolium* leaves showed it contains alkaloids, phenols, flavonoids, anthraquinones, tannins and saponins. Phenols had the highest concentration of 15.74 ± 0.03 mg/100g and the least which was anthraquinones (0.87 ± 0.02 mg/100g) as shown in Table 5. The phytochemical constituents of extracts of *G. latifolium* (Utazi leaf) revealed the presence of alkaloids, phenols, flavonoids, cyanides, tannins and saponins. These constituents are common in medicinal plants however, their concentration vary (Sofowora, 1993). In this study, results of the quantitative phytochemical analysis shows that phenols had the highest composition of

15.74 ± 0.03 mg/100g. Thus the antimicrobial activity of this plant leaf may be attributed to its phytochemical content (Egbung *et al*., 2011) Several plants possess phytochemicals like tannins, phenols and flavonoids and saponins which have been shown to exhibit antimicrobial activities against microorganisms (Funatogawa *et al*., 2004). The effective antimicrobial activity observed in this study is in concord with earlier reports (Banso and Adeyemo, 2007). This study has shown that *G. latifolium* extract has antimicrobial activity against the isolated organisms and hence may be used as a hop substitute in brewing industries to extend shelf life of the product by inhibiting several varieties of bacteria that can cause spoilage of the locally brewed alcoholic beverage.

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EXTENSION DELIVERY: THE BANE OF RUBBER PRODUCTION IN EDO SOUTH AGRO-ECOLOGICAL ZONE OF NIGERIA

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ABSTRACT
Rubber yield has been on the decline and below expectation due to the gap created by lack of extension services delivery. This study examined the awareness level of Agricultural Extension service delivery in rubber production in Edo South ecological zone of Nigeria. The main objective was to determine the respondents' awareness level of improved technologies and adoption rate. A sample size of 150 farmers was used from the list of rubber farmers obtained from Edo State Ministry of Agriculture and Rural Development and Tree Crop Unit of the Federal Ministry of Agriculture. Valid questionnaire used for the study were 137. A multi-stage sampling technique was adopted for the study. Data collected were analyzed using Frequency distribution, Percentage scores and Linear Regression. The result of the study indicated, that majority of the respondents (77.0%) were above 41 years of age. Greater proportion of the respondents (82%) had primary school education and could therefore be said to be literate. Most (66%) of the respondents had a household size above 9(nine). Respondent's contact with Agricultural extension agent was quiet low (18.2%). This means that as much as 76% of the respondents were practically without the necessary information and improve input that could facilitate productivity and improve income which was below N20,000 monthly for more than half of the respondents (55.3%). F value was statistically significant at 5% probability level and the t-value indicated that Ns were not significant. It was recommended that extension delivery should be given adequately priority to empower the farmers to bridge the deficit gap. Regular training should also be organized and more youth should be encouraged to go into rubber production.

KEYWORDS: *Extension, Awareness, Rubber farmers, Adoption, Edo South*

INTRODUCTION
Rubber (*Hevea brasiliensis*) is a dicotyledonous plant in the family Euphorbiacea (Agwu, 2006). It is cultivated in most part of Southern Nigeria as a result its suitable vegetative and climatic conditions conducive for its production. Rubber production in Nigeria dates back to 925 where thousands of hectares of rubber estates were established, but were predominantly owned by foreigners (the Europeans) and were located in the Southern part of the country. The yield of rubber has been

observed to be on the decline as a result of the gap created by lack of awareness of extension service delivery which is programmed to give rise to the effective dissemination of information on new and improved management practices. The consequence of the poor extension delivery culminated in the negative impact on production and led to reduce export which also affected the nation`s economy adversely (Agwu 2006 and Giroh *et al.*, 2007). Commenting on the relevance of Extension and the Extension worker, Oladele and Fawole (2007) described Agricultural Extension worker the "conduit pipe" through which information on research findings are made known to the farmers for implementation. This in turn improves their production capacity, income generation and living standard of farmers. Oladele and Fawole (2007) also identified the extension work as the most important public service because of the several roles it plays in Agriculture and rural development.

The relevance of rubber is indisputably high economically considering what it can be used to produce; which includes its latex being used for vital material in the automobile industry such as the manufacture of tyres, car bumpers, transmission belt, car mat, seats, adhesive, baby feeding bottle teat, condom, domestic and industrial gloves, balloons, balls, eraser among others (Abolagba and Giroh, 2006). It should be noted also that rubber tree produces seeds and wood, which are also of high economic value to the grower (farmer). Agwu (2006) highlighted that cake extracted from rubber seed after the extraction of oil can be used for the production of livestock feeds. The author also added that rubber seeds when processed could produce oil alkyd resins which is used for the production of paints, soap, skin cream and hair shampoo.

In enhancing the production of rubber, Cartwright *et al* (2002) further explained that the relevance of Extension when properly implemented would assist farmers in the rural communities to get relevant technical information that could help farmers to be more productive in their field of endeavour. Extension Service delivery also lead to the economic emancipation of the farmers, through the introduction of various programmes in Agriculture, forestry (where rubber belong), fishery, family and community development. This would empower them and create avenues through which their production output improves and boost their income generated and improved livelihood. Extension indeed has help individuals in various communities to succeed economically.

Omotayo (2005) and (Munyua 2000) highlighted that Agricultural Extension relies greatly on information sourced from the researcher and introduces synergy between the extension delivery stakeholders (ie. the farmer, extension agents, researcher and other actors) in the Agricultural Knowledge and Information System (AKIS) but when the farmers are deprived of the information that would have improves their knowledge and increase their performance level. The absence of adequate knowledge cause production to drops which also leads to the reduction of the expected income. The resultant effect introduction of dreaded poverty surge which is against the goals of extension delivery.

Ajokporise and Akpere (2010) in their study found that only 19.2% of the 120 respondents used for their study had

contact with government agencies specifically extension agent who were supposed to furnish the farmers with relevant technical information that could improve their production and impact the farmers positively. The authors strongly recommended that the government should provide effective extension services that could reach the farmer and also provide farm inputs such as planting materials as a way of encouraging farmers to plant rubber. Considering the low performance of Extension, Imarhiagbe *et al.* (2015) also recommended that extension services should be intensified to disseminate credible technical information, and improved technical assistance to rubber farmers by the extension practitioners on regular bases. They also recommended empowering the extension agents through improved skills (training).

Abolagba and Giroh (2006), complained that Rubber production has been on the decline over the years, from 155,000 metric tonnes in 1991 to 46,000 metric tonnes in 2004. This is brought about by several factors including over-aged trees and lack of adequate extension service delivery that could give the necessary information that would have improved production, etc. (Mgbeje, 2005). Complaining Further Igbenosa (2008) asserted that several improved technologies that could ameliorate the decline in rubber production has been generated, but there no evidence of improved adoption that could lead to increased production. Igbinosa, (2008) also asserted that in spite of the problems of the decline in natural rubber production, various research in rubber has been going on. However, there is no evidence currently to show if farmers in the study areas were aware of these new improved technologies generated by researchers, neither is there empirical support for their adoption or usage. This bring to light the fact that the farmers may not be aware of the new innovations hence no adoption. This study will attempt to provide empirical information relevant to making appropriate recommendations for solving the problems of information dissemination that could boost rubber production in the study area.

The main objective was to determine the respondents' awareness level of improved technologies and adoption rate.

1. Are there new innovations in rubber technologies that can improve rubber farming in the study area? If yes;
2. Did extension delivery in the study area improve the adoption of technology?
3. Are the new technologies made known to the farmers to enhance adoption (Are they aware)?

Objectives

i. Determine farmer's level of awareness of improved technologies in the study area
ii. Determine the level of extension contact the farmers had with the farmers.
iii. Ascertain whether the new technologies were adopted by the farmers

METHODOLOGY

Study Area

Edo state is made up of three (3) Senatorial zones namely: Edo North, Edo Central and Edo South. However, Oredo and Egor Local Government Areas which are part of Edo south Senatorial zone were not sampled because they are not agriculturally relevant and productive. This is as result of their fast growing

developments and urbanization tendencies. According to NPC, (2006) Edo State population stands at 3,233,366 person with a land mass spanning through of 19,819km^2. It has a population density of 163.14. The states lies between longitude 050 04' North and 060 43' East and latitude 050 44' and 070 34' North. It is bounded by Kogi State in the north, and Delta State by the south. At the west, it is bounded by Ondo State while Kogi and Anambra States on the eastern side. It consist of 18 Local Government Areas. It has two major vegetation belts namely: the Forest Belt of the south and central parts; while the Guinea Savannah belt sparse through the northern part of the state. The mean annual rainfall is between 127cm and 152cm in the northern part of the state, while the southern part records 252cm–254cm of rainfall. The average temperature ranges from a minimum of 24°C to about 33°C (FOS, 1994).

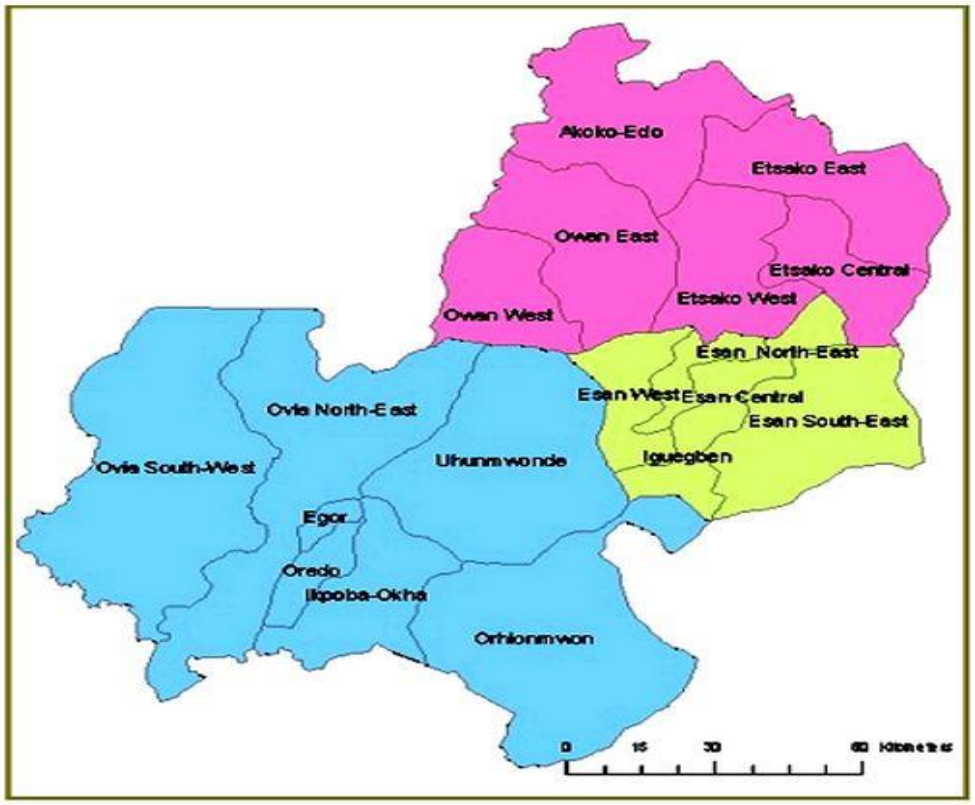

Fig. 1: Map of Edo State, Nigeria
Source: Ogbeide (2015)

Sample Collection

The focus of the study was on extension delivery in rubber production; it specifically concentrated on Extension being the bane of rubber production in Edo south agro-ecological zone of Nigeria. The study was carried out in three (3) local government areas of Edo South Senatorial Zone which include Ovia North-East, Ovia South-West and Uhunmwode. The rubber farmers used as the sample frame were 150 small scale farmers in the study area. The list of rubber farmers was obtained from Edo State Ministry of Agriculture and Rural Development and Tree Crops Unit of the

Federal Ministry of Agriculture. However, 137 of the farmer's questionnaire were found valid for analysis. A multi-stage sampling technique was deployed for the study as follows: One senatorial zone was purposively selected from the three senatorial zones of the state, because of the climatic condition and vegetation of these areas which favours the growth and establishment of rubber production. Three local government areas sampled were from farmers known to be highly involved in rubber production and are known small scale rubber farms in the area. Six (6) communities (i.e. two from each of the local government area producing rubber were further purposively sampled) from the list of the registered communities farmers provided by Tree Crop Unit of Edo State Ministry of Agriculture and Rural Development and Tree Crops Unit of the Federal Ministry of Agriculture. From the list provided by the above listed establishments; twenty-five (25) rubber farmers were randomly selected in each community, making a total of 150 farmers used for the study. The primary data were obtained through the use of well-structured questionnaire to elicit information from the (farmers) respondents in the study area. Data were collected with the assistance of Edo State Agricultural Development project (EDADP) and staff of the Rubber Research Institute of Nigeria (RRIN), Iyanomo-Benin (ie. the establishment`s extension workers) who were trained to administer the questionnaire. Data on socio-economic characteristics were elicited from the respondents include: Age, educational level, household size and the income including their level of awareness and adoption of improved rubber technologies and the constraints faced by the farmers in the adoption of these technologies. Oral interview was also used to obtain information that were not captured by the questionnaires and farmers who were not literate.

Measurement of variables: Contact with extension was measured by the number of times respondents were visited by Extension agents. Sources of information on improved rubber production practices; respondents were asked to indicate which of the following eight information sources were available to them and by ticking the one that was most appropriate; Ministry of Agriculture/ADP and RRIN organized Workshop/ Seminar which they participated in and acquired knowledge for their production.

Other sources of information were Trade fairs, Newspaper, Rubber Estates, Radio/TV, Friends and Cooperative societies. Adoption of Rubber Technology: This was measured by advising the respondents to tick either of the following options; aware, not aware, adopted and never adopted for each of the eleven (11) improved technology associated with rubber production in the study area. Adoption score were obtained by summing up the proportion of eleven technologies used.

Data Analysis

Factors that influenced the adoption of rubber technology were evaluated using percentages, multiple regression analysis. Four functional forms (Linear, Semi-log, Exponential and Cobb-Douglas) were tried using ordinary least square techniques (OLS). The estimated functions were evaluated in terms of the statistical significance of R^2 as indicated by F-value, the significance of the

coefficients as given by the t-value, the signs of the coefficient and the magnitude of standard errors.

RESULT AND DISCUSSION

Table 1 shows that most (39.5%) of the respondents were advanced adults of the age 60 years and above. Respondents within the age range of 31-40 years and 41-50 years both recorded equal number/ percentage of 19.7(%). Education Level: Table 1 also indicated few of the respondents (16.8%) having no formal education but majority (52.6%) had post primary education and only 5% had tertiary education. Summarily, most of rubber farmers (83%) had at least primary education, which shows appreciable literacy level of the respondents. It is expected that the level of adoption of innovations or technologies in rubber production would be appreciative. Onomolease *et al.* (2001) reported the educational level of farmers being positive and significant effect on farm productivity and adoption in Edo State, Nigeria. Table 1 also indicated that most (64.9%) of the respondents had a household size greater than 9 persons with only 44.5% of the respondents earning a monthly income that was more than N20,000. Large house size in the study area suggests availability of farm labour among farmers which could lead to increased productivity in rubber production.

Table 1: Demographic Characteristic of Respondents (N=137)

Characteristics	Category	Total
Age Group	Frequency	Percentage
21-30	5	3.6
31-40	27	19.7
41-50	24	17.5
51-60	27	19.7
>60	56	39.5
Sex		
Male	137	100
Female	0	0
Marital Status		
Married	135	98.5
Single	2	1.5
Education Status		
No Formal Education	23	16.8
Primary Education	35	25
Post Primary Education	72	52
Tertiary Education	7	5.1
House hold Size		
1-4	2	1.5
5-8	46	33.5
9-12	62	45.3
13-16	20	14.6
>16	7	5.1
Income		
<10,000	21	15.3
10,000 -20,000	55	40.2
>20,000	61	44.5

Table 2: Respondents Contact with Extension Agents

Variety	Frequency	Percentages
Response	Whether Visited or Not	
Yes	25	18.2
No	112	81.8
Frequency of Visit		
Never	112	81.8
Twice	25	18.2

Table 2 shows the distribution of respondents on the basis of extension agents' contact with rubber farmers. The result indicated that only 18.2 percent (%) of the respondents had contact with extension agents which invariably shows that extension delivery in the study area was very poor; this is in consonance with Agwu 2006 and Ajokporise and Akpere (2010) who asserted that low Extension contact was a great set back to the production of rubber; he found that only 24 % and 19.2% (respectively) of the farmers in the study area had contact with extension agent. Low or poor extension

contact is definitely a prerequisite for low rubber production in the study area.

Table 3 shows results on technology awareness and respondents adoption capacity. The table indicated that respondents in the study area lacked technological information from government agencies such as ADP/Ministry of Agriculture and Rubber Research Institute of Nigeria (RRIN); which are agency charged with the responsibilities of developing appropriate technology and disseminating same to the rubber farmers. The absence of extension agents discourages agricultural production (rubber inclusive); Mafimisebe and Mafemisebi (2008) highlighted that contact with extension workers is a prerequisite improved and high productivity. It aso increase their income because they are known to facilitate farmers' adoption of farm new innovations from research stations which leads to high productivity. The result of this study indicated that 17.5% of the respondents obtained information from other sources such as rubber estates (3.6%), cooperative societies and only 0.7% from Rubber Research Institute of Nigeria (RRIN) organized workshop/seminar. The result is a clear evidence of inadequate dissemination of technological information and adoption. The technologies adopted in the study area were weeding, (100%) fire trace (92.7) and pruning (49.6%). Igbinosa (2008) recommended that regular weeding of rubber plantations should be encouraged because good field hygiene creates airy and less humid environment which leads to the reduction of microbial attack on rubber latex thereby improving production the eventually leads to more income generation.

Table 3: Technologies awareness and Respondents' Adoption Capacity

Technology	Awareness Frequency	Percentage	Adopted Frequency	Percentage
Weeding	137	100	137	100
Fire trace	130	94.9	127	92.7
Pruning	72	52.6	68	49.6
Holing/dibbling	15	10.9	14	10.2
Intercropping	3	2.2	1	0.7
Improved clones of rubber {(NIG) 800 and 900 series}	-	-	-	-
Spacing (6.7m x 3.4m)	-	-	-	-
Thinning	-	-	-	-
Cover cropping	-	-	-	-

Table 4 explained the determinants of adoption of rubber technology in the study area. Based on these statistical, economic and econometric criteria, the linear form was selected as the best fit and result is presented in table 4. From the table, it was observed that age, total innovations that the farmers were aware of and their farm size carry the expected signs. Also, 83.8% variation in the regress and (adoption of rubber technology) was explained by the regressors. Similarly, the F value was statistically significant at 5% probability level indicating model fitness.

Table 4: Result of linear regression showing relationship between adoption and selected variables

Variable	Coefficient	Standard error	t. value
Constant			
X_1 = Farming experience	.330	.259	1.275Ns
X_2 = Household size	-.003	.005	-.667Ns
X_3 = Times visited by extension agents	-.040	.036	-1.108Ns
X_4 = Educational level	-.002	.040	-.040Ns
X_5 = Age	-.077	.043	-1.792Ns
X_6 = Income	.013	.058	.224Ns
X_7 = Total innovations aware of	-.011	.046	-.230Ns
X_8 = Farm size	.986	.040	24.400***
F value 90.389***			
R^2 .921			
R^2 .848			
R^2 adjusted .838			

*** (significant at 5% probability level)
Ns, not significant

CONCLUSION AND RECOMMENDATIONS

The study identified lack of awareness as the major reason why adoption of improved technologies in the study area were hindered. The most adopted technology were weeding, creation of fire trace and pruning which were regular agronomic practices in rubber production. The use of improved clones of rubber was not adopted in the study area due to lack of awareness. Contact with Agricultural Extension practitioners was not a regular phenomenon; thereby impeding adoption processes and reducing their expected improved yield and income. In view of the above findings, the study recommended that Agricultural Extension delivery activities should be vigorously encouraged, increased and improved. Collaborative effort should be made by all the extension delivery stakeholders in the area in order to increase extension contact which will improve the expected awareness. Regular training should be organized for the rubber farmers in order to improve their capacity for improved productivity, yield and income. Youth involvement in rubber production should be seriously encouraged order to sustain rubber production in the area.

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PROXIMATE COMPOSITION OF AGRO SHELL WASTES AND THEIR UTILISATION FOR BIOPROTEIN PRODUCTION WITH *Candida tropicalis* MYA- 3404

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ABSTRACT
An increasing interest for global food shortages for the world's expanding population necessitated the use of agro-waste as food sources or supplement for animal feeds. *Candida tropicalis* MYA- 3404 was isolated from pineapple fruit waste and identified by both phenotypic and molecular methods. Proximate analysis of three agro-wastes: melon, groundnut and walnut shell was carried out by standard chemical analytical methods. The protein content ranged between 3.73 to 12.14 % with groundnut shell having the highest. The shells contained 61.26 to 65.12 % carbohydrate. The moisture, fibre, ash and lipids were in the range 12.5 to 19.07 %, 6.45 to11.22 %, 1.43 to 6.50 % and 0.43 to 1.14 % respectively. Optimized production of bioprotein by *Candida tropicalis* MYA- 3404 was at pH 7.0, for 72h. Supplementation with nitrogen sources revealed highest biomass production with yeast extract. The optimization of a combination of carbon sources showed highest biomass production of 0.4640±0.053 mg/l for groundnut shell medium. Using submerged fermentation, maximum yield of cell biomass was produced from groundnut shell followed by melon shell as substrate for fermentation. *C. tropicalis* MYA 3404 can be used to produce bioprotein from agro-waste as a cheap source of protein in animal feed.

KEYWORDS: Yeast, Shells, Fermentation, Protein

INTRODUCTION
Dried cell biomass often referred to as bio-protein or Single cell protein (SCP) is a mixture of organic compounds such as proteins, lipids, carbohydrates, nucleic acids, and a variety of vitamins as well as inorganic compounds. The composition of single cell protein depends on the nature of substrate and also the microorganism employed for its production. Cell protein from yeast and fungi comprise 50-55 % and has high protein: carbohydrates ratio. It contains less methionine and cysteine and more of lysine. It possess a balance of amino acid, high B-complex vitamins hence more suitable as poultry feed (Mchoi and Park, 2003).

Before World War I the interest in SCP had already started (Ugalde and Castrillo, 2002). Using Baker's yeast Germany tried to supplement their protein supply in animal feed during World War

1. They were able to substitute as much as half of all the protein sources imported at that time with yeast (Ugalde and Castrillo, 2002). The yeast was cultivated on molasses as carbon source and ammonium salts were used as nitrogen source (Litchfield, 1983). After the end of the World War I the interest in yeast as fodder declined but arose again when World War II started. At this point yeast had been included into the army's diets, and after some time also into the diets of civilians.

However, the determination to produce more than 100 000 tonnes of yeast yearly were never reached (Ugalde and Castrillo, 2002). *Candida utilis* (Torula yeast) was of interest and it was cultivated on sulphite waste liquor from the pulp and paper industries and on wood sugar derived from acid hydrolysis of wood (Litchfield, 1983). Torula yeast production continued even after the World War II in the United States with regards to the utilization of natural sources for fodder (Ugalde and Castrillo, 2002).

The research into SCP production is growing extensively using agro-waste. An increasing interest for global food shortages for the world's expanding population necessitated the use of agro-waste as food sources or supplement for animal feeds. Microbial production is given due attention owing to microorganisms ability to utilize waste materials that cause pollution problems and/ or sanitary hazards.

In the production of microbial protein, several natural products are been tested. The utilization of agro-waste and industrial waste products for microbial cultivation appear to be a general trend in studies of this applied nature. Haider and EL-Hassy (2000) tested date extract supplemented with nitrogen source as a suitable substrate whereas, cashew and apple juice have also been used. Even though agricultural wastes are useful substrate for production of microbial protein, it is essential that they meet the following criteria; it should be nontoxic, abundant, totally regenerable, non-exotic, cheap and capable to initiate rapid growth of the organisms resulting in high quality cell biomass. Some agricultural and agro industrial waste products used for SCP and other metabolites production include; orange waste, mango waste, cotton salks, kinnow-mandarin waste, barley straw, corn cops, rice straw, corn straw, onion juice and sugar cane bagasse (Nigam *et al.*, 2000), cassava starch (Tipparat *et al.*, 1995), wheat straw (Abou Hamed, 1993), banana waste (Saquido *et al.*, 1981), capsicum powder (Zhao *et al.*, 2010) and coconut water (Smith and Bull, 1976). The presence of these waste abundantly in nature coupled with their cheap cost could be attributed to their usage as a sole carbon and nitrogen source for the production of SCP. Solid state fermentation (SSF) is currently being used for the production of protein-enriched feed (Rodriguez-Vazquez *et al*., 1992; Chaudhari *et al*., 1994). However employing industrial scale application of SSF for production of SCP would help in increased yields and improved conversion efficiency, which would reduce the overall cost of the final product. In the present study an attempt has been made to produce SCP with *Candida tropicalis* MYA- 3404 through submerged fermentation by using shells of walnut, groundnut and melon as energy sources.

MATERIALS AND METHODS

Source of culture and Agro-shell samples

The microbial culture used for fermentation was *Candida utilis* MYA-3404 isolated from soil sample rich in organic matter. Agro-shell wastes of groundnut and melon were obtained from small holder industries producing fried bottled groundnuts and melon seeds for commercial purpose. Walnut shell was obtained by aseptic mechanical cracking of the nuts in the laboratory. The shells were air-dried for 48 h and converted into powder with a household Lance grinding machine (model: LF80, Germany).

Determination of Proximate composition of Agro-shells

The kjeldahl technique was used for determination of protein content. The quantity of crude protein was calculated by multiplying the % nitrogen obtained by 6.25.The moisture content was determined by drying to a constant weight. Ash content was evaluated by dry ashing using a muffle furnace at 600^{o}C for 6h.The amount of lipid in the shells was determined by a using a soxhlet extractor petroleum ether as solvent. The residue obtained from ether extracted was further treated with 1.25 % sulphuric acid and 1.25 % of sodium hydroxide under heating for 30 min. The content was ashed in muffle furnace and reweighed to obtain the fibre content. Carbohydrate was obtained by difference of the other aforementioned components (A.O.A.C., 1995).

Isolation and identification of fungal isolate

One gram (1g) of the soil sample collected was mixed thoroughly with 9ml of sterile distilled water. A ten-fold serial dilution was then carried out. A measured 0.1ml was transferred into Sabouraud dextrose agar supplemented with 16 µg/ml of chloramphenicol. The plates were incubated at 28±2^{o}C for 72h. The discrete colonies on Sabouraud dextrose agar were selected, counted and examined macroscopically and microscopically using needle mount technique. They were identified based on the procedure outlined by Barnett and Hunter (1998).

Identification of Candida Isolates

Speciation was done by germ tube test, chlamydospore production on corn meal agar and sugar fermentation and assimilation tests using the Microexpress *Candida* identification kit (Tulip, India).

Direct Examination of the culture

Gram Stain: Smears were examined for the presence of gram positive budding yeast cells with pseudohyphae.

Potassium hydroxide (KOH) Mount: Smears were prepared for each sample by adding a loop full of colony to a drop of 10% KOH on a clean, grease- free slide and placing a cover slip over it. The preparations were slightly warmed to digest the materials and examined under the microscope with X10 and X40 objective for yeast cells, pseudohyphae.

Germ Tube Test: All presumptive *Candida* species were subjected to germ tube test by picking colonies from the purity plates. Small test tubes containing about 0.5 ml human serum were inoculated with colonies of test organisms in batches of three replicates per test. Each batch included a known positive and negative control. Inoculated tubes were incubated at 37˚C for 2 - 3 h. At the end of incubation, a drop of each serum was transferred to a clean slide, and a cover slip placed over it. These slides were examined microscopically under high power (×40) objective to detect the

presence of germ tubes, which are short hyphal initials.

Chlamydospore Formation: All Candida isolates were tested for the production of chlamydospores in corn meal agar (CMA) with 1% Tween 80. Subcultures on corn meal agar plates were made from SDA purity plate by furrowing the CMA plates (cut streak method), and incubating at room temperature (28˚C) for 2 - 5 days after which they were examined for the production of thick-walled chlamydospores in a lactophenol cotton blue mount of samples taken from the furrows of the corn meal agar plates.

Sugar Fermentation Test: All *Candida* isolates were subjected to carbohydrate fermentation and utilization test using Candida identification kit (Tulip Diagnostics Limited, India). Each kit contained sterile media for colorimetric identification using biochemical test and carbohydrate utilization tests based on the principle of pH change and substrate utilization designed to identify various metabolic properties of different *Candida* species that can be used to differentiate even closely related *Candida* species. The media were inoculated by adding 100 µl of the *Candida* suspension, incubated at 28˚C and read after 24 - 48 h incubation. The Candidal suspension was prepared by purifying on a Brain Heart Infusion Agar (BHIA) medium, after which a single discrete colony was picked up and streaked on to BHIA slant for enrichment and incubated at 28˚C (room temperature) for 24 - 48 h. The growth on the slant was washed with 2 - 3 ml of sterile saline and the turbidity of the suspension compared with Mcfarland standard number 5. The results were interpreted as per the standards given by the manufacturer in the result interpretation chart.

Molecular identification of Candida

DNA Extraction and PCR Amplification of the Fungal ITS gene

DNA extraction was carried out on the isolate using the Zymo ZR Fungi DNA miniprep Kit (Zymo Scientific, USA) according to manufacturer's instructions Polymerase chain reaction was carried out to amplify the Internal Transcribed Spacer ITS gene of the fungi using the primer pair ITS-1 (tccgtaggtgaacctgcgg) and ITS-4(tcctccgcttattgatatgcc) (Manter and Vivanco, 2007). The PCR reaction was carried out using the Solis Biodyne 5X HOT FIREPol Blend Master mix. PCR was performed in 25 µl of a reaction mixture, and the reaction concentration was brought down from 5X concentration to 1X concentration containing 1X Blend Master mix bufferBuffer (Solis Biodyne), 1.5mM $MgCl_2$, 200µM of each deoxynucleoside triphosphates (dNTP) (Solis Biodyne), 25pMol of each primer (BIOMERS, Germany), 2 unit of Hot FIREPol*DNA* polymerase (Solis Biodyne), proofreading enzyme, 2µl of the extracted DNA, and sterile distilled water was used to make up the reaction mixture. Thermal cycling was conducted in an Eppendorf Vapo protect thermal cycler (Nexus Series) at an initial denaturation temperature of 95°C for 15 minutes followed by 35 amplification cycles of 30 seconds at 95°C; 1 minute at 55°C and 1 minute 72°C. This was followed by a final extension step of 10 minutes at 72°C. The amplification product was separated on a 1.5% agarose gel and electrophoresis was carried out at 80V for 1 hour 30 minutes. During electrophoresis, DNA bands were visualized by ethidium bromide staining. 100bp DNA ladder was used as DNA molecular weight standard.

PCR amplification of the 18S rRNA gene and sequencing

The 18S rRNA amplified was sequenced with a BigDye Terminatory v3.1 cycle sequencing kit using an automated capillary sequencer (model 3100 Avant Genetic Analyzer, Applied Biosystems). Analyses of the gene sequences were carried out as described by Yakimov *et al*. (2006) in conjunction with the similarity rank from Ribosomal Database Project (RDP) Maidak *et al*. (1997). Finally, the FASTA nucleotide database query available through European Bioinformatics Institute were employed to determine the partial 18S rRNA sequences and the degree of similarity to other 18S rRNA gene was estimated.

Fermentation

A batch fermenter was used for the production of Single Cell Protein (SCP) from shell of melon, groundnut and walnut. The experiment set consisted of the fermenter, the air supply and the digital based data acquisition control system. The fermenter and all accessories were chemically treated with 2% potassium metabisulphate solution and then washed with hot water several times. The reactor was filled with one litre (1L) of mineral salt medium (MSM) containing 20g each of melon shell, walnut shell, and groundnut shell as a sole substrate and immediately inoculated with 40 ml of the inoculum. Cell suspensions were prepared from 24h old cultures in sterile media and aseptically introduced to the flask containing MSM. The pH of the medium was adjusted as desired before autoclaving. The pH was maintained with the addition of HCl or NaOH and determined using digital based pH measurement and control system. The reactor was operated at mixing speed of 300rpm. The samples were checked after 48 h for maximum yield of biomass.

Optimization of process parameters for bioprotein production

Inoculum Size: Different concentrations of 1.5 x 10^8 Mcfarland inocula sizes of 2, 3, 4, 5 and 6% v/v were used to analyze for the maximum production of single cell biomass of isolate at pH 6 for a period of 72h at 120 rpm

Fermentation period: The effect of fermentation period on bioprotein production was evaluated by harvesting samples at 24, 48, 72, 96 and 120h to check for the maximum yield of biomass at the different incubation period.

Medium pH: The initial pH values of 4, 5, 6, 7 and 8 of the growth media was checked before sterilization to enhance the maximum production of biomass of isolate.

Carbon source addition: Different carbon source such as glucose, sucrose, maltose, fructose and mixture of the four carbon source (glucose + sucrose + maltose + fructose) each at 0.5 % w/v were used to supplement the shell medium to check for the maximum production of biomass.

Nitrogen source supplementation: Organic nitrogen sources (urea, peptone and yeast extract powder) and inorganic (ammonium nitrate, ammonium sulphate,) nitrogen sources were supplemented to the growth media, each at 0.25% w/v to test for the maximum production of yeast biomass.

Isolation of yeast bioprotein from fermenter

A measured 10ml of the fermented media was centrifuged for 20min at 6000rpm. The supernatant obtained was subjected to ammonium sulphate precipitation. Ammonium sulphate [40%

(w/v)] was added to the cell-free supernatant and stirred for 4 h at 4^{o}C. The precipitate obtained was allowed to stand for 2 h and then collected by centrifugation at 15000 rpm for 20 min. The pellet obtained was dissolved in 2ml of 2mM glycine NaOH buffer, pH at 11 and protein was assayed by Lowry's method (Lowry *et al*., 1951). The blue colour developed by the phosphomolybdic phosphorus in the folinciocalteau reagent by the amino acid tyrosine and the tryptophan present in the protein plus the colour developed by the biuretic reaction of the protein with the alkaline cupric tartarate were measured by Lowry's method. Several dilutions of 0.3 mg/ml bovine serum albumin (BSA) was prepared in the same buffer having the unknown of 30 to 150 micrograms/ml (0.03 to 0.15 mg/ml). 1.0 ml was added to each dilution of standard, protein-containing unknown, or buffer (for the reference) to 0.90 ml reagent A (0.1 N of NaOH +0.1N of Na_2CO3) in different test tubes and mixed. The test tubes were incubated for 10 min in a 50 ^{o}C bath, and cooled to room temperature. A measured 0.1 ml reagent B (0.02M of $CuSO_4.5H_2O$+0.052M of Na_2Tartrate) was delivered to each test tube, mixed and incubated for 10min at room temperature. This was followed by rapidly adding 3 ml of reagent C (0.124 M of Na_2Tarterate.$2H_20$) to each test tube, mixed and incubated for 10 min in the 50 ^{o}C water bath and cooled to room temperature. Five mililitre (5ml) of samples was added to each test-tube and 5 ml of distilled water was added to the blank test tube. It was thoroughly mixed and allowed to stand for 10 minutes The 5 ml of the alkaline cupric tatrate reagent was added to each tube including the blank. It was well mixed and incubated at room temperature in the dark for 30 min to allow for blue colour development. The reading was taken at 660 nm in the colorimeter. Working standard of tyrosine and tryptophan of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 1mg/l were also prepared into the series of test tubes which were treated in the same way as the samples and run alongside with the samples.

RESULTS AND DISCUSSION

The phenotypic characteristics of the yeast isolate used in this experimentation is shown in Table 1. The Gram reaction showed it as positive blue cells and growth on corn meal tween 80 agar revealed the formation of pseudohyphae or chlamydospore. The organism fermented glucose and sucrose but was negative for others. In plate 1 is shown the result of the molecular characterisation with band at 600bp positive for *Candida tropicalis.* The base sequence identity of *Candida tropicalis* strain MYA-3404 is shown in Table 2 with Ascension number NW_0030200391 and 94 % homology.

Plate 1: Polymerase chain reaction results for fungal isolate analyzed with 1.5% agarose gel electrophoresis. Lane M is 100bp-1kb DNA ladder (molecular marker). Lane 1 is positive for *Candida tropicalis* with band at 600bp.

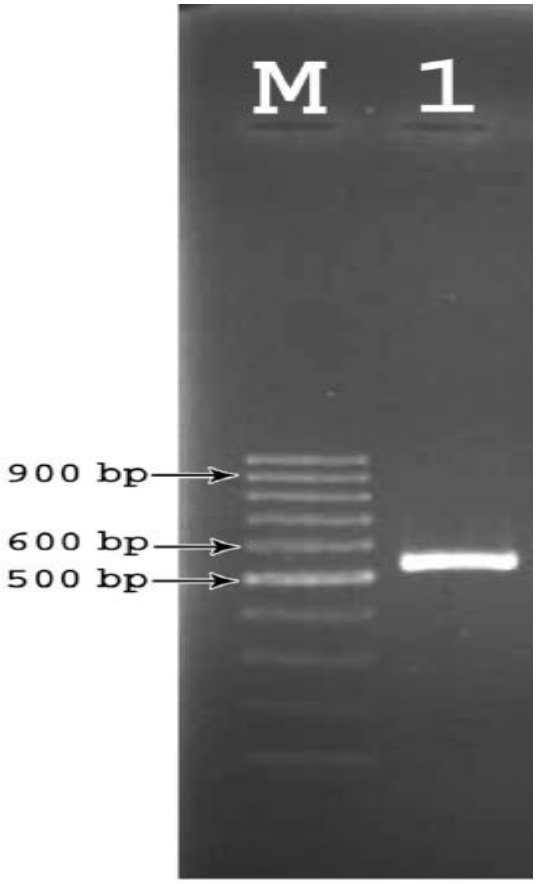

Table 2: Base sequence of *Candida tropicalis* strain MYA-3404

Sequence blast	Ascension no.	Sequence identity	Query coverage length	Score bits/percent homology
GCACCACATGTGTTTTTTATTGAACAAATTTCTTTGGTGGCGGGAGCAATCCTACCGCC AGAGGTTATAACTAAACCAAACTTTTTATTTACAGTCAAACTTGATTTATTATTACAAT AGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGCATCGATGAAGAACGCAGCGAA ATGCGATACGTAATATGAATTGCAGATATTCGTGAATCATCGAATCTTTGAACGCACA TTGCGCCCTTTGGTATTCCAAAGGGCATGCCTGTTTGAGCGTCATTTCTCCCTCAAACC CCCGGGTTTGGTGTTGAGCAATACGCTAGGTTTGTTTGAAAGAATTTAACGTGGAAAC TTATTTTAAGCGACTTAGGTTTATCCAAAACGCTTATTTTGCTAGTGGCCACCACAATT TATTTCATAACTTTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCA ATAAGCGGAGGAAAAG	NW_003020039.1	*Candida tropicalis* strain MYA-3404	745	460/487(94 %)

The proximate analysis of the three agro-waste with respect to their percentage occurrence are shown in Table 3. Groundnut shell had the highest crude protein content of (12.14±0.92 %) and walnut shell (3.73±0.31 %) the least. Moisture content analysis showed a range between 12.50±0.50 % to 19.07±1.52%. Walnut shell had the highest crude fibre content of 11.22±1.60 % % compared to groundnut shell which had the least of 6.45±0.80 %. The result of lipid content indicated that groundnut shell had the highest (1.14±0.01 %), followed by walnut shell (0.49±0.00 %) and melon shell had the least (0.43±0.00 %). Crude ash content of walnut shell was highest (6.50±0.21 %), while melon shell had the least (1.43±0.15 %). The three agro-waste shells had high carbohydrate content which ranged from 61.26±0.32 % to 65.12±1.14 % with melon shell having the least.

The effect of pH in this study revealed that maximum cell biomass yield was produced at pH 6. The cell yield of 0.1086 mg/l was highest with melon shell as the substrate for fermentation. Followed by 0.1054 mg/l cell yield obtained at pH 7 using melon shell containing medium. The least cell biomass (0.0188 mg/l) was produced with walnut shell as substrate for fermentation at pH 5.0. Although bioprotein production was on the increase from pH 5.0 for the agro shell media, a decrease occurred with progression from pH 7.0 to higher values (Table 4).

The higher the inoculum the greater the biomass yield at first. Among various inoculum sizes, 4 ml (v/v) inoculum size gave a maximum yield of cell biomass of 0.0956 mg/l with groundnut shell as substrate fermentation. This was followed by cell yield of (0.0874 mg/l) from melon shell containing medium. The least biomass obtained (0.0028 mg/l) was from walnut shell substrate inoculated with 2 ml (v/v) inoculum size of *C. tropicalis* MYA-3404 as shown in Table 5.

The result of the effect of incubation time showed that 72 h incubation period using groundnut shell as substrate yielded in maximum cell biomass of 0.1546 mg/l as shown in Table 6. The least cell biomass of 0.0050 mg/l was produced after 24 h with melon shell containing medium.

Supplementation of the three substrates with organic and inorganic nitrogen sources showed that the maximum cell biomass of 0.4840 mg/l was produced with yeast extract addition using walnut shell as substrate for fermentation. This was followed by 0.3410 mg/l cell biomass from groundnut shell as substrate supplemented with ammonium sulphate in Table 7. Peptone addition produced the least cell yield (0.0430 mg/l) from walnut shell containing medium.

The incorporation of all the carbon sources (glucose, maltose, sucrose and fructose) gave the maximum biomass (0.4640 mg/l) after fermentation of the medium containing groundnut shell as substrate for fermentation. Medium supplemented with glucose had the second highest biomass yield (0.3166 mg/l) after inoculating groundnut shell containing medium as shown in Table 8. The least cell yield of (0.0743mg/l) was obtained from walnut shell containing medium that was supplemented with maltose.

Table 3: Proximate analysis of agro-shell wastes

Parameter (%)	Walnut shell	Groundnut shell	Melon shell
Moisture	12.50±0.50^{b}	13.7±0.80^{b}	19.07±1.52^{a}
Protein	3.73±0.31^{c}	12.14±0.92^{a}	9.26±0.71^{b}
Fibre	11.22±1.60^{a}	6.45±0.80^{b}	7.57±0.40^{b}
Lipid	0.49±0.00^{b}	1.14±0.01^{a}	0.43±0.00^{b}
Ash	6.50±0.21^{a}	2.79±0.11^{b}	1.43±0.15^{c}
Carbohydrate	65.12±1.40^{a}	63.52±0.81b^{c}	61.26±0.32^{c}

Legend: Values are means of triplicates ± standard deviations (SD).Mean differences are presented across column and values with significant difference carry different alphabets a, b and c.

Table 4: Optimization of pH for the biosynthesis of single cell protein from agro-wastes

pH	Walnut shell	Groundnut shell	Melon shell	p-value
5	0.0188±0.007^{A}	0.0287±0.009^{C}	0.0382±0.010^{C}	0.000
6	0.0628±0.023^{B}	0.0888±0.041^{C}	0.1086±0.051^{D}	0.000
7	0.0568±0.014^{B}	0.0804±0.009^{CD}	0.1054±0.017^{E}	0.000
8	0.0496±0.043^{B}	0.0766±0.031^{C}	0.0927±0.019^{D}	0.000
9	0.0386±0.062^{BC}	0.0402±0.046^{C}	0.0468±0.058^{C}	0.008

Legend: Values are means of triplicates ± standard deviations (SD).Mean differences are presented as $^{A, B, C, D,}$ and ^{E} across column and values with significant difference carry different alphabets.

Table 5: Optimization of inoculum sizes for the biosynthesis of single cell protein from agro-wastes

Inoculum size ($1.5x10^{8}$) McFarland (ml)	Walnut shell	Groundnut shell	Melon shell	*p*-value
2	0.0028±0.005^{A}	0.0108±0.050^{B}	0.0042±0.002^{AB}	0.1884
3	0.0346±0.007^{B}	0.0658±0.005^{E}	0.0611±0.010^{D}	0.000
4	0.0508±0.043^{B}	0.0956±0.031^{D}	0.0874±0.048^{D}	0.000
5	0.0276±0.036^{AB}	0.0494±0.050^{C}	0.0485±0.052^{C}	0.001
6	0.0238±0.007^{A}	0.0432±0.013^{C}	0.0487±0.049^{C}	0.000

Legend: Values are means of triplicates ± standard deviations (SD).Mean differences are presented as $^{A, B, C, D,}$ and ^{E} across column and values with significant difference carry different alphabets.

Table 6: Optimization of incubation period for the biosynthesis of single cell protein from agro-wastes

Incubation period (hr)	Walnut shell	Groundnut shell	Melon shell	*p*-value
24	0.0112±0.039^{A}	0.0096±0.012^{A}	0.0050±0.006^{A}	0.635
48	0.0708±0.054^{A}	0.1320±0.020^{C}	0.1044±0.027^{B}	0.000
72	0.0936±0.036^{A}	0.1546±0.025^{D}	0.1283±0.045^{C}	0.000
96	0.0502±0.057^{A}	0.0877±0.054^{CD}	0.0682±0.009^{B}	0.001
120	0.0487±0.050^{A}	0.0751±0.0363^{BC}	0.0682±0.019^{AB}	0.000

Legend: Values are means of triplicates ± standard deviations (SD).Mean differences are presented as $^{A, B, C, D,}$ and ^{E} across column and values with significant difference carry different alphabets.

Table 7: Optimization of nitrogen sources for the biosynthesis of single cell protein from agro-wastes

Nitrogen Sources	Walnut shell	Groundnut shell	Melon shell	*p*-value
Yeast extract	0.1916±0.008^{A}	0.4840±0.022^{D}	0.2950±0.006^{B}	0.000
Peptone	0.0430±0.012^{A}	0.1173±0.010^{A}	0.0643±0.013^{A}	0.025
Urea	0.0866±0.006^{A}	0.2126±0.002^{C}	0.1360±0.004^{B}	0.000
Ammonium nitrate	0.0650±0.005^{A}	0.1433±0.004^{C}	0.0883±0.006^{B}	0.000
Ammonium sulphate	0.1586±0.007^{A}	0.3410±0.039^{C}	0.2666±0.028^{B}	0.000
Control	0.0629±0.023^{B}	0.0888±0.041^{C}	0.1086±0.051^{D}	0.000

Legend: Values are means of triplicates ± standard deviations (SD).Mean differences are presented as $^{A, B, C, D,}$ and ^{E} across column and values with significant difference carry different alphabets.

Table 8: Optimization of carbon sources for the biosynthesis of single cell protein from agro-wastes

Carbon source	Walnut shell(mg/l)	Groundnut shell(mg/l)	Melon shell(mg/l)	*p*-value
Glucose	0.2153±0.015^{A}	0.3166±0.029^{C}	0.2433±0.006^{AB}	0.000
Maltose	0.0743±0.021^{A}	0.1583±0.040^{B}	0.1673±0.055^{B}	0.006
Sucrose	0.1833±0.017^{AB}	0.2506±0.045^{C}	0.1890±0.005^{AB}	0.002
Fructose	0.1200±0.004^{B}	0.1860±0.005^{C}	0.1376±0.006^{B}	0.000
G+M+S+F	0.2926±0.011^{B}	0.4640±0.053^{D}	0.3803±0.041^{C}	0.000
Control	0.0629±0.023^{B}	0.0888±0.041^{C}	0.1086±0.051^{D}	0.000

Legend: Values are means of triplicates ± standard deviations (SD).Mean differences are presented as $^{A, B, C, D,}$ and ^{E} across column and values with significant difference carry different alphabets.

A host of raw materials have been selected as substrate for SCP biosynthesis (Nasseri *et al.,* 2011). The most widely used are carbohydrate sources which are natural microbial substrates which also comprise a renewable feedstock (Ugalde and Castrillo, 2002). Agro-shells of walnut, groundnut and melon are rich in carbohydrate (61.26±0.32 to 65.12±1.40 %) which can provide carbon source for growth and metabolism of *Candida tropicalis* MY-3404. Ezekiel *et al*. (2002) reported that a large scale production of SCP both for human and animal feed depends on cheap and available substrates with carbon source.

The acidity or alkalinity of a fermentation media is important to growth and biosynthesis of materials. A pH optimum of 6.0 was found for effective biomass formation in this study with the agro-shells. This is in conformity with the results obtained by Rajoka *et al*. (2005). Rosma and Ooi (2006) however reported a pH of 4.5 for *Candida utilis*. Halasz and Radomie, 1991 have noted that increase in bioprotein production increases with pH from 4 to 7 .On the whole Candida species are capable of good growth in media with pH between 3.0 to 6.2 (Adoki 2007).

Inoculum size is key to breakdown of suitable substrate for biomass production. An inoculum size of 4 ml (v/v) resulted in maximum cell yield (0.0956 mg/ml) in this experiment with groundnut shell. Ravinder *et al.* (2003) obtained the maximum growth yield with 3% (v/v) inoculum size working with deoiled rice

bran. A 10 % (v/v) inoculum size resulted in maximum biomass of *Candida utilis* on rice bran as reported by Rajoka *et al*. (2005). Yunus *et al*. (2015) also obtained a maximum biomass yield for *Candida utilis* and *Rhizopus oligosporus* grown on wheat bran. The differences in maximum yield with different inoculum sizes may be attributed to the diverse organisms, substrates and fermentation techniques employed.

The maximum production of yeast biomass (0.0936±0.036 to 0.1546±0.025 mg/ml) was found after 72 h of incubation for all agro shells used in this study with groundnut shell taking the lead in production. However no further increase was seen after 72 h. While this corroborates the report of Ravinder *et al.* (2003), it is discordant with Adoki (2007) who reported maximum biomass of *Candida* spp at 96 h as well as Yunus *et al*. (2015) who reported 48h for *Candida utilis* and *Rhizopus oligosporus.* Lubna *et al.*(2004) observed a maximum cell biomass of *Aspergillus niger* after 120 h of incubation.

In other to improve cell mass production of *Candida tropicalis* in this study, different nitrogen sources: organic and inorganic were added to the fermentation media containing agro shells. The results clearly demonstrates that nitrogen compounds influenced the biomass yield. It was observed that the maximum biomass yield of 0.4840 mg/ml was obtained with yeast extract using groundnut shell medium. The least was obtained for peptone. This result is in concordance with the report of Rajoka *et al. (*2004) and Zhao *et al*. (2010*)* who reported the production of single cell protein using waste capsicum powder produced during capsanthin extraction.

The supplementation with all carbon source resulted in maximum biomass yield of 0.4640 mg/ml during fermentation with groundnut shell medium. The least biomass yield (0.0743 mg/ml) was produced with maltose using walnut shell as substrate. The addition of glucose as a carbon source resulted in an appreciable biomass yield of 0.3166 mg/ml with groundnut shell. This is concordance with the report of Kurbanoulu who applied it on bioprotein production using horn hydrolysate. The use of groundnut shell supplemented with organic nitrogen source such as yeast extract and subsequent fermentation with *Candida tropicalis* MY-3404 can lead to effective bioprotein production using this organism while maintaining other parameters of pH, temperature and inoculum size. Thus instead of burning groundnut shell which can lead to environmental hazard, they can be effectively harnessed into single cell protein as animal feed.

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ANTIBIOTICS SUSCEPTIBILITY PROFILE OF BACTERIA ISOLATED FROM FISH PONDS IN BENIN CITY

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ABSTRACT

Fish farming is a recognized means of livelihood in Nigeria; however, the intensive use of antibiotics to prevent infection and increase fish yield calls for concern. This practice may lead to the growth of antibiotics resistant bacteria which contains resistant genes that can be transferred to human pathogens, causing treatment- resistant illness. This study investigated the antibiotics susceptibility profile of bacteria isolated from five fish ponds in Benin City using standard microbiological procedures. Isolates were identified using morphological characteristics and conventional biochemical tests, while antibiotic susceptibility test was determined by the Kirby-Bauer disc diffusion method. Results showed that total aerobic count of the ponds ranges from minimum 7.85 x 10^4 cfu/ml recorded in Ekewan fish pond to maximum 135.06 x 10^4 cfu/ml recorded in Sapele Road fish pond. Bacteria species isolated were *Staphylococcus aureus, Bacillus* spp *and Klebsiella* spp, *Pseudomonas* spp, *Proteus* spp and *E. coli.* Frequency of occurrence and percentage distribution of the isolates showed that *Bacillus* spp had the highest while *Klebsiella* spp had the lowest. Community fish pond recorded the highest number of isolates while Adesuwa fish pond recorded the lowest number of isolates. Antibiotics susceptibility patterns showed that majority of the isolates were resistant to perfloxacin (85.18%) and amoxicillin (74.81%). It is suggested that the prophylactic and indiscriminate use of antibiotics may be a predisposing factor in the development of antibiotics resistance among the bacteria isolates. This may pose a threat to human population by transferring these resistant genes to other bacteria of human clinical significance.

KEYWORDS: Bacteria isolates, Susceptibility, Antibiotics, Fish ponds, Frequency

INTRODUCTION

Fish has a nutrient profile superior to all terrestrial meats (Feldhusen *et al.*, 2000). It is a good source of sulphur and essential amino acids such as lysine, leucine, valine and arginine. Fish contains thiamine and a rich source of Omega-3 polysaturated fatty acids, fat soluble vitamins (A, D and E) and water soluble vitamins (B complex) and minerals (calcium, phosphorus, Iron, Iodine and Selenium). High content of

polyunsaturated (Omega –III) fatty acid is important in lowering blood cholesterol level and high blood pressure (Zárate *et al*., 2017)

As the population of humans increase, the demand for fish will also grow flanking it (FAO, 2007). Several efforts have been made both in developing and developed countries to meet this demand for fish. However, it has been forecasted that the demand for fish will grow beyond levels that can be sustained (Vignesha *et al*., 2011). To meet the much needed demand for animal proteins, make profits and create jobs, people engage in fish aquaculture. A fish pond is a type of aquaculture usually filled with fresh water, fairly shallow and is usually non-flowing. Tidal ponds, reservoirs, storage tanks, raceway and fish farm tanks are not included (Wilcox, 1985). Fish ponds have been referred to be self-contained ecosystems which are often teeming with rich vegetable and diverse organisms (Olukunle and Oyewumi, 2017). However, fish farmers face huge loss as a result of infections by pathogenic bacteria, among the common fish pathogens are *Staphylococcous* sp., *Aeromonas* sp., *Salmonella* sp., *Shigella* sp., *Enterococcus faecalis*, *E. coli*, *Yersinia* sp, *V. cholera and* other Vibrios (Schmidt *et al*., 2000). Others are *Pseudomonas* sp. and *Streptococcous* sp. Diseases caused by these pathogenic bacteria include white- skin, haemorrhagical septicaemia, furunculosis etc. (Ponnerassery *et al*., 2012).

For optimum production, fish farmers use antibiotics for both prophylactic and remedial purposes. According to Cabello *et al*. (2006), large amount of antibiotics is used in the fish industry to avoid infection in developing countries. According to Vignesha *et al*. (2011) most antibiotics used in fish aquaculture are non-biodegradable and remain in the aquaculture environment for long periods of time. This may lead to increase in population of bacteria that can survive in the presence of these antibiotics, thereby acquiring resistance which can passed to their progenies. Beyond increased resistance in the ecosystem, these resistant bacteria can be transferred to humans leading to the development of infectious diseases which are difficult to treat and thereby pose public health risk.

Monitoring antibiotics resistant bacteria in animals reared for the purpose of human consumption is paramount for the regulation of resistance in animals and man; this will help to detect trends and changes of resistance pattern (WHO, 2001). According to World Health Organization for Animal Health (OIE), regular surveillance of resistant microorganisms in aquatic is necessary (Smith *et al*., 2013). Hafsat *et al*. (2015), reported the presence of multidrug resistant bacteria in fresh fish in Maiduguri. Olukunle and Oyewumi (2017) reported that the physicochemical parameters of two fish ponds investigated in Akure where within the recommended range for fish production. According to Dunba *et al*. (2015), *Staphylococcus aureus*, *Salmonella paratyphi*, *Pseudomonas aeruginosa*, *Shigella* spp, *Enterococcus faecalis* and *Enterobacter aerogenes* were found in fish pond in Taraba State. The aim of this research is to isolate, identify as well determine the antibiotics

susceptibility profile of bacteria species isolated from fish ponds in Benin City.

MATERIALS AND METHOD

Study Area

This research was conducted in the metropolitan area of Benin City (Lat. 6º 17' to 7º 12′N and Long. 5° 15' to 5° 41'E), the capital of Edo State in southern Nigeria. The average precipitation rate and relative humidity are 79.2mm and 87% respectively. Benin City experiences all year round rainfall which peaks around July, with a mean annual rainfall of 2980 mm.

Sample Collection.

Water samples were aseptically collected from five fish ponds within Benin City namely; Ekewan fish pond, GRA fish pond, Sapele Road fish pond, Adesuwa fish pond and Community fish pond. These fish ponds contained fishes of various sizes and ages, most of them were active and they range from two months to ten months. Community fish pond was constructed with large plastic container while the others were constructed with concrete, the average fish stock and depth ranged from 2000-3500 and 6-7 feet respectively. Samples were collected with a sterile bottle at a depth of 14 cm two times a day; morning and evening. The water samples were transferred to the laboratory for analysis.

Sterilization

The work bench was sterilized by cleaning with a wet swab (cotton wool soaked with 75% alcohol). The solid and liquid media were sterilized by autoclaving at 121°C for 15minutes. Glass wares such as conical flask and test tubes were sterilized in oven at 160°C for one hour. The inoculating loop and inoculating needle were sterilized by holding the nichrome wire in flame until it glowed red. The glass wares were allowed to cool before being used while the media was left to cool to 45°C.

Sample Preparation

The samples were shaken properly and diluted serially to sixth dilution using saline peptone water as the diluent; this diluent is used for maximum recovery of bacteria from a sample. About9ml of peptone water was put into test tubes (15ml) and autoclaved at 121°C for 15minutes and allowed to cool. Using sterile pipette sterilized at 160°C for 1hour in the oven, 1ml of pond water from each bottle was diluted to the sixth dilution. About 1ml of aliquot of each dilution was inoculated on nutrient agar, MacConkey agar, and Eosin Methylene Blue (EMB). Nutrient agar was used to determine the total aerobic bacterial heterotrophic count, while MacConkey agar and Eosin Methylene Blue (EMB) were used to isolate coliforms. Culturing of the bacteria was carried out using the pour plate method for MacConkey agar and Nutrient agar and streak plate method for the EMB agar. This was done in triplicate and was then incubated for 24-48 hours at 37°C.

Isolation and Identification of Isolates

After incubation, the morphology of colonies which appeared on different agar plates were observed. Pure cultures of bacteria were obtained by aseptically streaking representative colonies of different morphological types onto freshly prepared nutrient agar plates. Discrete colonies, which developed on the plates were transferred into a nutrient agar slant and preserved in the refrigerator at 4ºC. Identification of isolates were done based on their motility, colonial morphology and

biochemical properties (Cheesbrough, 2006)

Identification of Isolates

Identification of isolates was done by cultural and microscopic analysis as well as conventional, physiological and biochemical test. Distinct colonies were picked from incubated plates, purified by sub culturing before being examined microscopically for Gram reaction cell morphology, pigmentation and biochemical analysis included.

Gram Staining Process

Smears of each colony that were picked were made on a grease free slide, after which the slides were flooded with crystal violet for 30 seconds. The stains were rapidly washed off under the tap, and then the water was tipped off. The slides were covered with iodine for 30seconds and washed off with clean water. The smears on the slides were decolourized rapidly using alcohol, which was immediately washed off again. The slides were placed on a rack for the smears to air dry. After drying, each slide was examined under the microscope.

Antibiotics Susceptibility Tests

Antibiotics susceptibility test were performed for the colonies by using the disc diffusion method as described by National Committee for Clinical Laboratory Standards (NCCLS, 1997), with antibiotics containing disc. Antibiotics used include Ofloxacin (30 µg), Chloramphenicol (30 µg), and Ciprofloxacin (10 µg), Amoxicillin (30µg), Augmentin (30µg), Gentamycin (10 µg), Perfloxacin (30 µg), Streptomycin (30 µg), and Erythromycin (19 µg). Respective colonies were streaked on nutrient agar, the disc were then transferred into the plate with a sterile forceps and incubated at 37^0C for 24 - 48 hours. The zone diameters for each antibiotic disc were translated in prefixed susceptibility (S) and resistant (R) categories by referring to the clinical laboratory standards (NCCLS, 1999). Resistance was recorded when there was no zone of inhibition around the respective disc and sensitivity was recorded when there was presence of inhibition.

RESULTS

Total Aerobic Bacteria Count

Table 1 shows the total aerobic bacteria count expressed in cfu x 10^4 obtained from the five fish ponds at different times of the day (morning and evening). In the morning, the mean total aerobic bacteria count ranged from minimum 8.43 x 10^4 cfu/ml recorded in Ekewan fish pond to maximum 115.17 x 10^4 cfu/ml recorded in Sapele road fish pond pond. In the evening, the mean total aerobic bacteria count ranged from minimum 7.25 x 10^4 cfu/ml recorded in Ekewan fish pond (GRA) to maximum 155.00 x 10^4 cfu/ml recorded in Sapele road fish pond. The average total aerobic bacteria count recorded in this analysis is 54 x 10^4 cfu/ml.

Table 1: Total Aerobic Bacteria Count

POND LOCATION	MEAN COUNT x 10^4 ±SD MORNING	EVENING	AVERAGE
Ekewan Fish Pond	9.68 ± 1.05	10.77 ± 2.4	10.23 ± 1.73
G.R.A Fish Pond	100.3 ± 2.08	106. 8 ± 6.33	103 ± 4.21
Sapele Road Fish Pond	115.17 ± 1.04	155.00 ± 7.82	135.06 ± 4.43
Adesuwa Fish Pond	8.43 ± 1.60	7.25 ± 0.92	7.85 ± 1.26
Community Fish Pond	14.77 ± 4.07	13.25 ± 3.44	14.01 ± 3.76
			54.14 ± 1.47

Bacteria Isolated from the Fish Ponds

Table 3 showed the bacteria species isolated, they belonged to six genera. *Staphylococcus aureus, Bacillus* sp., *Klebsiella* sp., *Pseudomonas* sp., *Proteus* sp., and *E. coli* were present in Ekewan fish pond, Sapele road fish pond and Community fish pond. However, G.R.A fish pond had the above bacteria species except *Klebsiella* sp. while Adesuwa fish pond had the above isolates except *E. coli and Klebsiella* sp.

Table 3: Bacteria Species Isolated from the Fish Ponds

POND LOCATIONS	BACTERIA ISOLATES
Ekewan Fish Pond	*Staphylococcus aureus, Bacillus* spp., *Klebsiella* spp., *Pseudomonas* spp., *Proteus* spp. *and E. coli.*
G.RA Fish Pond	*Staphylococcus aureus, Bacillus* spp., *Pseudomonas* spp., *Proteus* spp. *and E. coli.*
Sapele Road Fish Pond	*Staphylococcus aureus, Bacillus* spp ., *Pseudomonas* spp., *Proteus* spp., *Klebsiella* spp. *and E. coli.*
Adesuwa Fish Pond	*Staphylococcus aureus, Bacillus* spp., *Pseudomonas* spp, *Proteus* spp.,
Community Fish Pond	*Staphylococcus aureus, Bacillus* spp., *Pseudomonas* spp., *Proteus* spp., *Klebsiella* spp., *and E. coli.*

Frequency and Percentage Distribution of Bacteria

Table 4 showed the frequency of occurrence of bacteria isolated from the five fish ponds. A total of 135 isolates belonging to six genera were recorded namely; *Staphylococcus aureus* (36), *Bacillus* sp. (40), *E. coli* (13), *Proteus* sp. (24), *Pseudomonas* sp. (18), *Klebsiella* sp. (4). *Bacillus* sp. had the highest 40 (29.63%), while *Klebsiella* sp. had the lowest 4 (2.96%). Community fish pond recorded the highest number of isolates (40) while Adesuwa fish pond recorded the lowest number of isolates (18)

Table 4: Frequency and Percentage Distribution of Bacteria

Isolates	Frequency of occurrence (no/percentage)					Total
	Ekewan Fish pond	G.R.A Fish Pond	Sapele Road Fish Pond	Adesuwa Fish Pond	Community Fish Pond	
Staphylococcus aureus	6 (28. 60%)	7 (24.14%)	9 (33.30%)	4 (22.22%)	10 (25.00%)	36(26.67%)
Bacillus sp.	3 (14.30%)	11 (37.93%)	7 (25.90%)	5 (27.78%)	14 (35.00%)	40(29.63%)
E. coli	3 (14.30%)	5 (17.24%)	3 (11.10%)	0 (0%)	2 (5.00%)	13(9.63%)
Proteus sp.	5 (23.80%)	2 (6.90%)	4 (14.80%)	6 (25.00%)	7 (17.50%)	24(17.78%)
Pseudomonas sp.	3 (14.30%)	4 (12.50%)	2 (7.40%)	3 (12.50%)	6 (15.00%)	18(13.33%)
Klebsiella sp.	1 (4.76%)	0 (0%)	2 (7.40%)	0 (0%)	1 (2.50%)	4(2.96%)
Total	21	29	27	18	40	135

Pond location	Isolates	AM	OFX	PEF	CPX	AU	GEN	S	E	CH
ESEOSA FISH POND	6	5(83.33%)	5(83.33%)	3(50%)	5(83.33%)	6(100%)	6(100%)	4(66.7%)	4(66.7%)	3(50%)
Staphylococcus aureus										
Bacillus sp.	3	0(0%)	0(0%)	0(0%)	3(100%)	3(100%)	0(0%)	0(0%)	0(0%)	2(66.6%)
E. coli	3	0(0%)	1(33.33%)	1(33.33%)	3(100%)	0(0%)	1(33.3%)	0(0%)	0(0%)	2(66.6%)
Proteus sp.	5	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	5(100%)	0(0%)	0(0%)	3(60%)
Pseudomonas sp.	3	0(0%)	0(0%)	1(33.33%)	2(66.67%)	0(0%)	0(0%)	0(0%)	0(0%)	1(33.33%)
Klebsiella sp.	1	0(0%)	0(0%)	1(100%)	1(100%)	1(100%)	0(0%)	0(0%)	1(100%)	0(0%)
EMMA FISH POND										
Staphylococcus aureus	7	7(100%)	5(7.4%)	1(14.25%)	7(100%)	7(100%)	6(85.7%)	5(71.5%)	5(71.46%)	5(71.46%)
Bacillus sp.	11	0(0%)	5(45.45%)	0(0%)	6(59.54%)	10(90.9%)	0(0%)	11(100%)	0(0%)	1(100%)
E. coli	5	0(0%)	2(40%)	0(0%)	0(0%)	0(0%)	2(40%)	0(0%)	0(0%)	0(0%)
Proteus sp.	2	0(0%)	0(0%)	0(0%)	0(0%)	1(50%)	0(0%)	0(0%)	0(0%)	2(100%)
Pseudomonas sp.	4	0(0%)	0(0%)	0(0%)	2(50%)	2(50%)	1(75%)	0(0%)	2(50%)	1(25%)
Dr SAM FISH POND										
Staphylococcus aureus	9	8(88.90%)	6(60.66%)	1(11.11%)	3(33.3%)	0(0%)	5(55.6%)	3(33%)	1(11.11%)	5(55.55%)
Bacillus sp.	7	2(28.57%)	7(100%)	1(14.28%)	7(100%)	7(100%)	2(28.57%)	6(85.7%)	0(0%)	7(100%)
E. coli	3	0(0%)	1(33.33%)	1(33.33%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)
Proteus sp.	4	0(0%)	2(50%)	2(50%)	2(50%)	2(50%)	0(0%)	1(25%)	0(0%)	4(100%)
Pseudomonas sp.	2	0(0%)	1(50%)	1(50%)	1(50%)	0(0%)	0(0%)	1(50%)	0(0%)	2(100%)
Klebsiella sp.	2	1(50%)	0(0%)	0(0%)	2(100%)	2(100%)	0(0%)	0(0%)	0(0%)	0(0%)
OSULA FISH POND	4	0(0%)	2(50.0%)	4(100%)	4(100%)	4(100%)	4(100%)	4(100%)	2(50%)	2(50%)
Staphylococcus Aureus										
Bacillus sp.	5	1(20%)	0(0%)	0(0%)	3(60.0%)	5(100%)	0(0%)	0(0%)	0(0%)	5(100%)
Proteus sp.	6	0(0%)	3(50.0%)	0(0%)	3(50.0%)	0(0%)	6(100%)	0(0%)	0(0%)	3(50%)
Pseudomonas sp.	3	1(33.3%)	0(0%)	3(100%)	3(100%)	0(0%)	0(0%)	0(0%)	0(0%)	0(0%)
COMMUNITY FISH POND										

Staphylococcus aureus	10	6(60%)	5(50.0%)	0(0%)	6(60.0%)	10(100%)	5(50%)	5(50%)	10(100%)	10(100%)
Bacillus sp	14	6(42.86%)	10(78.5%)	0(0%)	10(71.4%)	8(57.1%)	0(0%)	5(38%)	4(28.6%)	0(0%)
E. coli	2	0(0%)	1(50.0%)	0(0%)	2(100.0%)	1(50%)	0(0%)	2(100%)	0(0%)	14(100%)
Proteus sp.	7	0(0%)	0(0%)	0(0%)	2(28.6%)	0(0%)	0(0%)	0(0%)	0(0%)	5(71.4%)
Pseudomonas sp.	6	0(0%)	6(100%)	0(0%)	2(33.3%)	0(0%)	1(16.6%)	0(0%)	3(50%)	5(83.3%)
Klebsiella sp.	1	1(100%)	1(100%)	0(0%)	1(100%)	0(0%)	1(100%)	0(0%)	0(0%)	1(100%)

Table 5: Antibiotics Susceptibility Patterns of Bacteria Isolated from Fish Ponds

AM Amoxicillin, PEF Perfloxacin, OFX Ofloxacin CPX Ciprofloxacin, AU Augmentin, GEN Gentamycin, S Streptomycin, E Erythromycin, CH Chloramphenicol

DISCUSSION

Intensive use of antibiotics in domestic and commercial fish farming has resulted in the growth of antibiotics resistant bacteria in the aquatic ecosystem. These resistant bacteria can be transferred to humans directly or their resistant genes can be transferred to human pathogens (Hafsat *et al*., 2015.)

The presence of *Staphylococcus aureus, Bacillus* spp, *Esherichia coli, Proteus* spp, *Pseudomonas* spp *and Klebsiella* spp in the fish ponds under study agrees with earlier findings by Danba *et al*., (2015) who reported that these organisms are found in fish ponds. *Staphylococcus aureus* has been linked to bacteria sepsis; osteomyelitis, bacteraemia and otitis, in humans, while *Pseudomonas* spp can cause inflammation (Udeze *et al*., 2012). *E. coli* can cause diseases of the gastrointestinal tract, Bacillus spp can cause food poisoning and deep seated soft tissue infections and systemic infections (Tena *et al*., 2007). *Klebsiella* sp can cause a wide range of community related diseases like *pneumonia* and more generalized infections (Botelho-Nevers *et al*., 2007). *Proteus* spp has been implicated in urinary tract infection and septicemias (Endimiani *et al*., 2005).

The high bacteria load recorded in the fish pond located in Sapele road could be due to the nature of operation of the fish pond as well as maintenance, the owner of this fish pond occasionally apply organic manure on the fish pond. This practice has been associated with buildup of organic matter and bacteria in the pond environment. Sharmila *et al*. (1996), reported that bacteria load in pond ecosystem is greatly influenced by the presence of organic matter. High bacteria load could also be due run-off from roads and surrounding soil which can carry animal wastes into the pond. Birds and dogs which are free roaming animals can also be responsible for a significant source of fecal contamination of ponds (Green *et al*., 2012).

Ekewan fish pond recorded low total bacteria count probably because of good pond management which includes using continuous flow of water. This may prevent the buildup of bacteria load hence the low total aerobic bacteria count. There was a variation in the frequency and percentage distribution of the bacteria isolates. *Bacillus species* and *Staphylococcus aureus* recorded the highest frequency of occurrence and percentage distribution of 40 (29.63%) and 36 (26.67%) respectively. The high frequency of *Staphylococcus aure*us in this study compares with the findings of Dalgaard *et al*. (2006). The high presence of *Staphylococcus aure*us could be from the fish handlers as humans harbour Staphylococcus aureus as a normal flora (Danba *et al*., 2015)

Antibiotics susceptibility test was done for all the 135 isolates, the isolates showed varied resistance patterns to all the antibiotics used, 85.18% of the 135 isolates showed resistance to amoxicillin while 74.18% showed resistance to perfloxacin, this is consistent with earlier findings of Sunder *et al*., (2006). However majority of the isolates were very sensitive to chloramphenicol and ciprofloxacin.

The organisms from different genera also showed variable range of resistance to the antibiotics used, majority of the *E. coli* isolates showed absolute resistance to amoxicillin and a very low resistance to chloramphenicol. This finding is in

agreement with Samuel *et al*. (2011) who reported minimum resistance of *E.coli* isolated from cat fish pond to chloramphenicol. This finding is also supported by reports of Overdevest *et al*. (2011) that antibiotic resistance among Enterobacteriaceae has increased drastically during the past decade.

All the *Pseudomonas Species and Proteus Species* isolated from cat fish pond showed a high resistance to majority of the antibiotics used, this may be associated with the excessive or indiscriminate use of antibiotics (Macpherson *et al*., 1991; Schmiat *et al*., (2000). The high resistance of *Pseudomonas* sp. to antibiotics used in this study is consistent with earlier report by (Al-Jebouri and Al-Meshhadani, 1985), who reported that *Pseudomonas* sp. are known to be highly resistant to antimicrobial drugs. High incident of multiple resistance patterns in bacteria isolates fish have also be reported by (Hafsat *et al*., 2015).

CONCLUSION

Poor pond management could be a major reason for bacteria buildup in the pond ecosystem; some of these bacteria are pathogenic. The indiscriminate use of antibiotics for both prophylactic and remedial purposes can lead to the presence of antibiotic resistant bacteria in the pond environment. Their presence can be a major threat to public health. These bacteria can transfer resistant gene to other bacteria of human clinical significance.

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MAXIMUM LIKELIHOOD PARAMETER BASED ESTIMATION FOR IN-DEPTH PROGNOSIS INVESTIGATION OF STOCHASTIC ELECTRIC FIELD STRENGTH DATA

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ABSTRACT
This work centers on parametised investigation of the field strength fading characteristics of microcellular wireless LTE channels operating in typical urban terrains. Due to the blockades between the transmitting NodeBs and the mobile station receiver, the fading characteristics of LTE channel are highly stochastic with respect to time or transmitting distance and possesses their own distinctive property. To robustly investigate the field strength fading characteristics, detail drive test measurement has been conducted in five different site locations in waterline area of Port Harcourt City Nigerian at 2.6GHz. From the measured field data, the parametised amplitude of the measured field data is estimated by using the Maximum Likelihood Estimation (MLE) based on different probability distribution functions. The results of the parametised MLE estimate for the acquired field data has been shown, analyzed and reported for each studied location. It is supposed that results of this research this work would serve as a first-hand information for effective communication system design and deployment of future cellular broadband mobile network in similar radio signal propagation terrains.

KEYWORDS: Fading, Channel, Field strength, Maximum likelihood estimate, Probability distribution function**,** Broadband networks

INTRODUCTION
In cellular broadband communication systems, the natural and man-made obstructions between the transmitting and mobile station antenna largely influence the received field strength of the propagated signal power, thus leading to fluctuating and degraded system quality of service. An accepted criterion of performance monitoring and optimization such communication systems is to have detail knowledge about stochastic field signal statistics the channels for re-planning of its networks. Also, according to Isabona *et al*. (2013) and Isabona and Konyeha, (2013), quantified and parametised understanding of the propagated field strength behaviour in the radio links is required to carry out in-depth prognosis analysis of the level of signal coverage fluctuations in the cellular broadband communication networks (Isabona and Obahiagbon, 2014).

In the past years, scholars have explored a number of approaches to study stochastic signal fading phenomenon. For

example, in Xiao-Li *et al*. (2018), transmit power estimation centered on Signal Strength of wireless network with cooperative receiver nodes is presented using Maximum Likelihood (ML) estimation technique. Their results reveals that numerical experiments validate the explored ML theoretical discoveries. In Nikola *et al*. (2005), the authors explored maximum likelihood estimation method combined with signal statistics is explored to determine the intensity modulated fiber optic links.

Abiodun and Ojo, (2019), worked on realistic predictive modelling of stochastic path attenuation losses in wireless channels over microcellular urban, suburban and rural terrains using probability distribution functions. The results of their study revealed the normal distribution was most suitable for the statistical predictive modelling signal path loss data. Similar predictive analysis and reports in (Krishnamoorthy, 2006; Salo *et al*., 2005; Fengyu *et al*., 2005).

This work centres on parametised investigation of the field strength fading characteristics of microcellular wireless LTE channels operating in typical urban terrains. To robustly investigate the field strength fading characteristics, detail drive test measurement is piloted in five different locations in waterline area of Port Harcourt City Nigerian at 2.6GHz. From the measured field data, the parametised amplitude of the measured field data is estimated by using the Maximum Likelihood Estimation (MLE) based on different probability distribution functions. The results of parametised MLE estimate of the acquired field data fading characteristics has been revealed, analyzed and reported for each studied location.

THEORETICAL FRAMEWORK

Maximum Likelihood Estimate and Statistical Probability Models

Maximum likelihood estimation (MLE) is a technique of estimating to obtain the parameters of a statistical model, given the observations (i.e. the observed data). This statistical model contains the unknown parameters. Those values of the parameters that maximize the probability of the observed data are referred to as the maximum likelihood estimates. The likelihood function or model is the probability density function (PDF) of the particular observations, and the MLE solution is the parameter that maximizes this joint PDF.

In communication theory, though there exist a number of PDF models, but the problem is in choosing the right one for effective prognosis analysis a particular datasets. In this work, the concentration is on Normal, lognormal, Nakagami, Rician, Weibull and Rayleigh PDFs.

(a) Normal Distribution Model

The normal distribution possess key two distribution parameters. The first parameter is tagged the mean (μ), and the second one is called the standard deviation (σ) or the variance (σ^2). The normal PDF and PDF can be determine using (Isabona and Konyeha, 2015; Krishnamoorthy, 2006):

$$f(x,\mu,\sigma)=\frac{1}{\sigma\sqrt{2\pi}}\exp\left[\frac{-(x-\mu)^2}{2\sigma^2}\right] \quad (1)$$

$$F(x,\mu,\sigma)=\frac{1}{2}\left[1+erf\left(\frac{(x-\mu)}{\sigma\sqrt{2}}\right)\right] \quad (2)$$

The maximum likelihood estimators for the normal distribution are the μ and σ; they can be obtained using the expression in (3) and (4):

$$\mu = \frac{1}{N}\sum_{i=1}^{N}(x_i) \quad (3)$$

$$\sigma = \frac{1}{N}\sum_{i=1}^{N}(x_i - \mu_i)^2 \quad (4)$$

where *x* and N indicate the measured sample and the measurement sample number.

(a) Lognormal Distribution Model

The lognormal distribution, also generally termed Galton or Gaussian distribution, is applicable the desired quantity of interest must be positive. The lognormal PDF and CDF can be defined by (Krishnamoorthy, 2006):

$$f(x,\mu,\sigma) = \frac{1}{x\omega\sqrt{2\pi}}\exp\left[\frac{-(\ln x-\mu)^2}{2\omega^2}\right] \quad (5)$$

$$F(x,\mu,\sigma) = \frac{1}{2}+\frac{1}{2}erf\left[\frac{-(\ln x-\mu)^2}{\sqrt{2}\omega}\right] \quad (6)$$

In (7), μ and ω maximum likelihood estimators and they represent the shape and scale distribution parameters for the lognormal. The mean and standard deviation for lognormal can be expressed as:

$$\mu = \exp\left(\mu+\frac{\omega^2}{2}\right) \quad (7)$$

$$\sigma = \exp(2\mu+\omega^2)\left[\exp(\omega^2)-1\right] \quad (8)$$

where *x* stands for the measured sample.

(b) Weibull Distribution Model

The weibull distribution, which generally employed for reliability analysis, make use of λ, and c as its shape and scale (Weibull slope) distribution parameters. The Weibull PDF and CDF can be defined by (Krishnamoorthy, 2006, Abiodun and Ojo, 2019):

$$f(x,\lambda,c) = \frac{c}{\lambda}\left(\frac{x}{\lambda}\right)^{c-1}\exp\left[-\left(\frac{x}{\lambda}\right)^{c}\right] \quad (9)$$

$$F(x,\lambda,c) = 1-\frac{c}{\lambda}\left(\frac{x}{\lambda}\right)^{c-1}\exp\left[-\left(\frac{x}{\lambda}\right)c\right] \quad (10)$$

The distribution parameters, μ and σ can be obtained the expressions in (11) and (12) respectively:

$$\mu = \lambda\Gamma\left(1+\frac{1}{c}\right) \quad (11)$$

$$\sigma = \sqrt{\lambda^2\Gamma\left(1+\frac{2}{c}\right)-\left[\Gamma\left(1+\frac{1}{c}\right)\right]^2} \quad (12)$$

where *x* stands for the measured sample.

(c) Rayleigh Distribution Model

The Rayleigh distribution is a continuous probability distribution and also a special (singular) case of the Weibull distribution. The Rayleigh PDF and CDF are given by (Krishnamoorthy, 2006, Abiodun and Ojo, 2019):

$$f(x,\sigma) = \frac{x}{\sigma}\exp\left[-\left(\frac{-x^2}{2\sigma^2}\right)\right] \quad (13)$$

$$F(x,\sigma) = 1-\exp\left[-\left(\frac{-x^2}{2\sigma^2}\right)\right] \quad (14)$$

The distribution parameters, μ and σ_m can be obtained the expressions in (15) and (16) respectively:

$$\mu = \sigma\sqrt{\frac{\pi}{2}} \quad (15)$$

$$\sigma_m = \sigma\sqrt{\frac{4-\pi}{2}} \quad (16)$$

where *x* stands for the measured sample.

(d) Nakagami Distribution Model

The Nakagami distribution, also termed Nakagami-*m* distribution, behave roughly and evenly near its mean value. The Nakagami PDF and CDF can expressed as (Krishnamoorthy, 2006):

$$f(x,m,a)=\frac{2m^m}{\Gamma(m)a^m}x^{2m-1}\exp\left[-\frac{m}{a}x^2\right] \quad (17)$$

$$F(x,m,\Omega)=\frac{Y\left(m,\frac{m}{a}x^2\right)}{\Gamma(m)} \quad (18)$$

In (20), μ and ω represent the shape and scale distribution parameters for the Nakagami. The mean and standard deviation for Nakagami can be expressed as:

$$\mu=\frac{\Gamma\left(m+\frac{1}{2}\right)}{\Gamma(m)}\left(\frac{a}{m}\right)^{1/2} \quad (19)$$

$$\sigma=\sqrt{a\left(1-\frac{1}{m}\left(\frac{\Gamma\left(m+\frac{1}{2}\right)}{\Gamma(m)}\right)^2\right)} \quad (20)$$

where *x* stands for the measured sample.

(e) Rician Distribution

In communication, the Rician distributions model are usually employed to study stronger line-of-sight fading channels. The Rician PDF and CDF can expressed as (Krishnamoorthy, 2006):

$$f(x,v,\sigma)=\frac{x}{\sigma^2}\exp\left[\left(\frac{-x^2+v^2}{2\sigma^2}\right)I_o\left(\frac{xv}{\sigma^2}\right)\right] \quad (21)$$

$$F(x,v,\sigma)=1-Q_1\left(\frac{v}{\sigma},\frac{x}{\sigma}\right) \quad (22)$$

In (24), μ and ω represent the shape and scale distribution parameters for the Rician. The mean and standard deviation for Rician can be expressed as:

$$\mu=\sigma\sqrt{\frac{\pi}{2}}L_{1/2}\left(\frac{-v^2}{2\sigma^2}\right) \quad (23)$$

$$\sigma=2\sigma^2+v^2-\frac{\pi\sigma^2}{2}L_{1/2}^2\left(\frac{-v^2}{2\sigma^2}\right) \quad (24)$$

where *x* stands for the measured sample. $I_o(z)$ and $Q_1(z)$ represent the modified Bessel function and Marcum Q function, respectively.

METHODOLOGY

Data Collection

The measurement campaign has been performed around five operational Long term Evolution (LTE) cellular networks base station (BS) sites in Waterline areas of Port Harcourt City, with concentration on built-up busy urban streets, and roads. It is a typical urban area with a flat topography and mixed commercial and residential building edifices. As revealed in table 1, the BS antenna heights range from 28m to 45m, elevated above the ground level to broadcast signals in three sectors configuration. With the aid of drive test equipment which include the Global Positioning System (GPS), HP Laptop, two Samsung Galaxy mobile Handsets (Model-SY 4) and network scanner, signal power measurements were conducted along different routes round the cell sites, in active mode. Specifically, drive tests around sites I, II and IV were performed via non-line of sight (NLOS) routes, while that of sight III was piloted through line of sight (LOS) route, such that there were no obstructions between the BS and user equipment terminal. A snap shot of data collection in route I is revealed in figure 3. All the test equipment were connected together with USB cables and housed in a Gulf car before the field

drive test measurement. Also, both the Samsung handsets and the HP laptop were both enhanced with Telephone Mobile Software (TEMS, 15.1 version), which enable us to access, acquire and extract signal power data, including serving BS information after measurement. A total of 1,502 signal power data points were extracted for further analysis using MapInfo and Microsoft Excel spreadsheet.

The measured signal power, which is called RSRP, is related to electric field strength, E_{FS} by (Isabona, *et al*, 2013):

$$E_{FS}(dB\mu/m) = RSRP(dBm) - G + A \quad (25)$$

$$A = 10\log\left(\frac{1.64\lambda}{4\pi}\right) + 145.8 \quad (26)$$

where, G is the antenna gain in dB, λ is the signal transmitting wavelength in m and RSRP indicates Reference Signal Receive Power.

Table 1: Measurement Campaign Parameters in LTE Network

	Site I	Site II	Site III	Site IV	Site V
Parameter			Value		
Operating Frequency (MHz)	2600	2600	2600	2600	2600
BS Antenna Height (m)	28	30	45	32	38
BS antenna gain (dBi)	17.5	17.5	17.5	17.5	17.5
Transmit power (dB)	43	43	43	43	43
Feeder Loss (dB)	3	3	3	3	3
Transmitter cable loss (dB)	0.5	0.5	0.5	0.5	0.5
Mobile antenna height (dB)	1.5	1.5	1.5	1.5	1.5

RESULTS AND DISCUSSION

Displayed in figures 1 to 5 are acquired stochastic electric field strength data via drive test over the period of measurement in each study terrain sites. Their stochastic fading distributions characteristics are shown in Figure 6 (a-f) using the six probability distribution models (i.e. Normal, lognormal, Nakagami, Rician, Weibull and Rayleigh distributions). Figure 7 (a-f) reveals the corresponding cumulative distribution functions (CDFs) model fittings connected to the pdfs depicted in Figure 6(a-f). A fluctuating fading pattern of measured field strength along the measurement locations can been seen in all the Figures of 1 to 5 and this is roughly constant across the study location sites. The scenario can be attributed to the uneven proliferation of natural and man-made obstructions between the transmitting and mobile station antenna.

More importantly, to ascertain how accurately each statistical distribution fits with the measured electric field strength data, the estimated Maximum Likelihood Parameter Estimation method were considered as benchmark**.** Also, to reveal how well the investigated distribution function models fit into the stochastic fading behaviour of the measured electric field strength data, MLE statistics were further explored and the resultant estimated values using MLE are revealed in table 2. From the table, the lognormal distribution model fitting display the best maximum log likelihood values of -312 and 415 in locations 1 and 2. It is an indication that lognormal distribution

model is a better fit to the measured electric field strength data in the two locations compared to other distributive models. As revealed in from table 2, while Nakagami model gave the optimal maximum log likelihood estimate in location 3, Normal and Rician models provided the best maximum log likelihood estimates of -200 and 210 in locations 4 and 5, respectively. Unlike the results in (Abiodun and Ojo, 2019), different distributive fitting results on the measured electric field strength data in the respective locations is revealed in this work; it simply shows that one particular distributive model cannot be generalized as a fitting model to the study locations. Such performances may be attributed to differences that exist in building height structures, roof tops, street/roads widths around the five study location sites. In general, the graphs in figure 2 (a-f) clearly shows that Rayleigh and Weibull distribution displayed the poorest fits to empirical electric field strength. This simply implies that there are no multiple paths of condensed scattered electric field signals reaching a receiver along the measurement routes in the study location sites

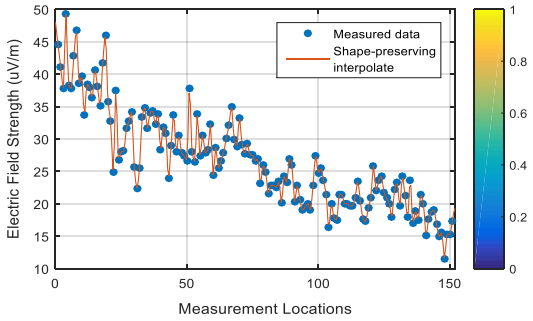

Fig. 1: Stochastic electric field strength data in Site location I

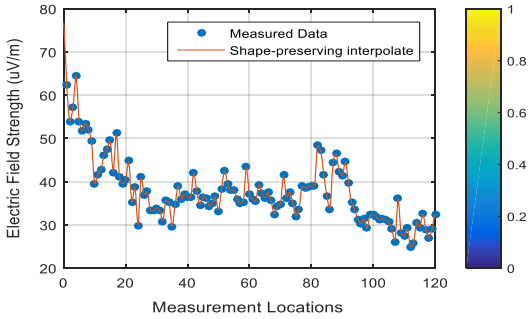

Fig. 2: Stochastic electric field strength data in Site location II

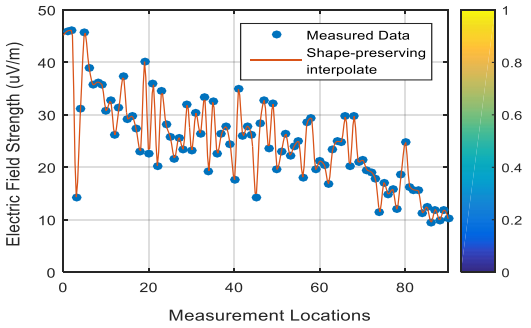

Fig. 3: Stochastic electric field strength data in Site location III

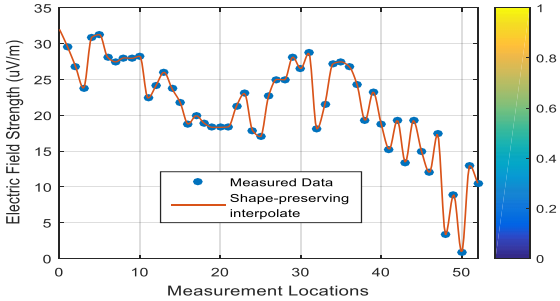

Fig. 4: Stochastic electric field strength data in Site location IV

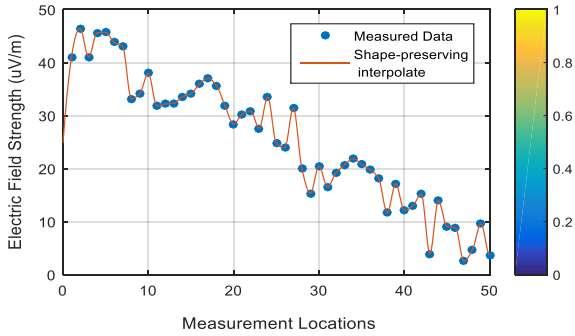

Fig. 5: Stochastic electric field strength data in Site location V

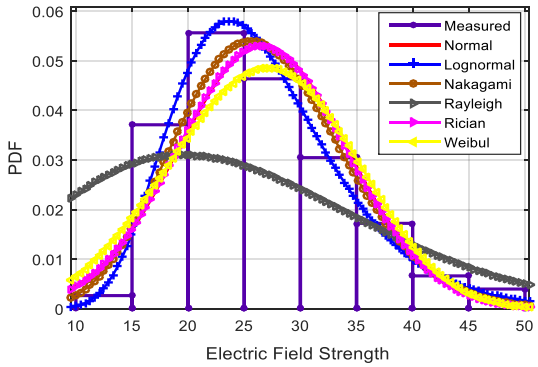

Fig. 6 (a): Stochastic electric field strength data with different PDFs fitted plots in Site location I

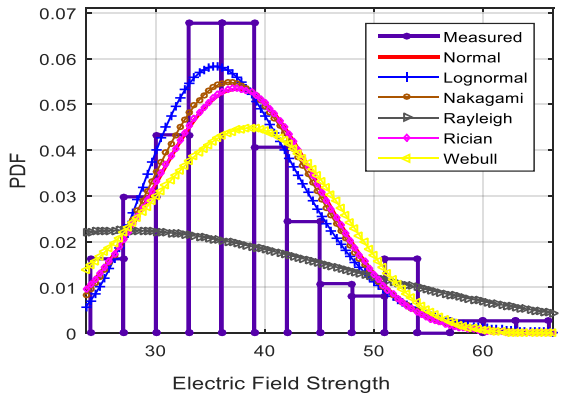

Fig. 6 (b): Stochastic electric field strength data with different PDFs fitted plots in Site location II

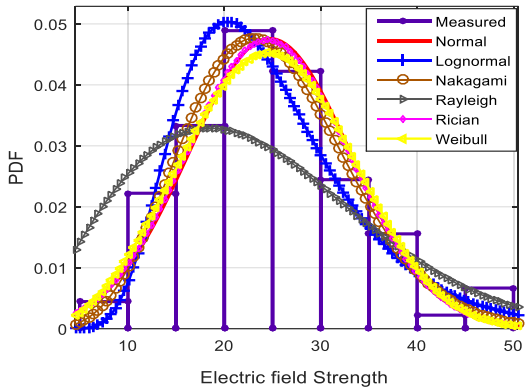

Fig. 6 (c): Stochastic electric field strength data with different PDFs fitted plots in Site location III

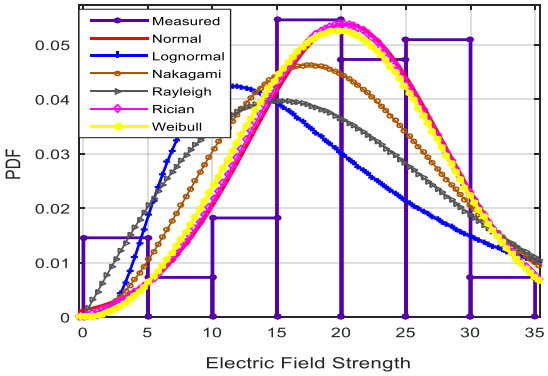

Fig. 6 (d): Stochastic electric field strength data with different PDFs fitted plots in Site location IV

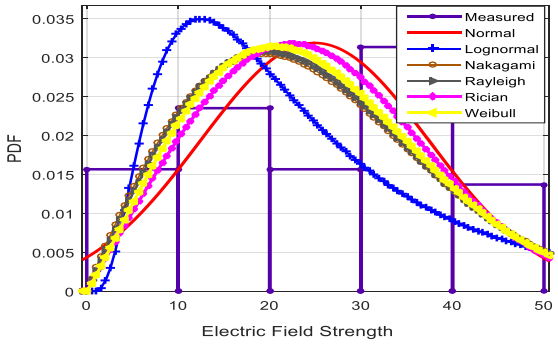

Fig. 6 (f): Stochastic electric field strength data with different PDFs fitted plots in Site location V

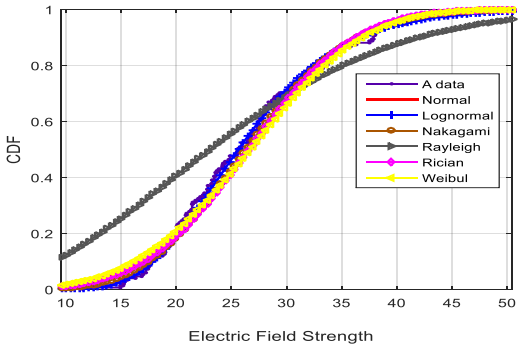

Fig. 7 (a): Stochastic electric field strength data with different CDFs fitted plots in Site location I

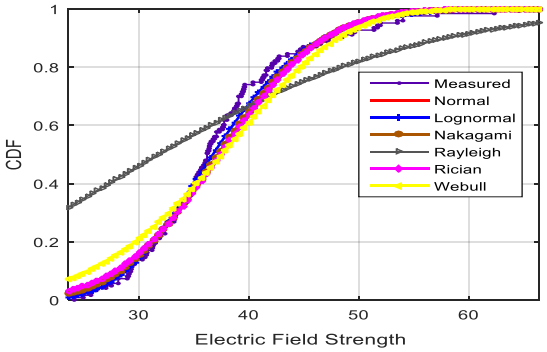

Fig. 7 (b): Stochastic electric field strength data with different CDFs fitted plots in Site location I

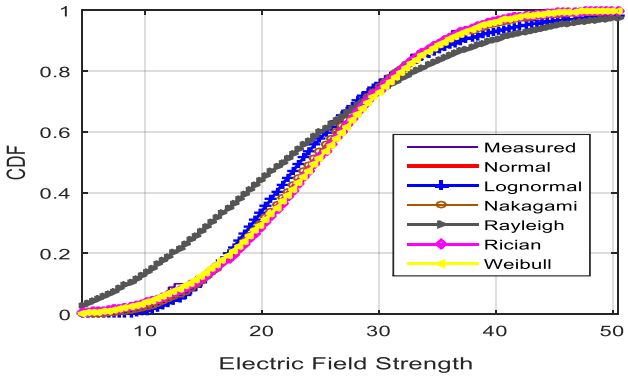

Fig. 7 (c): Stochastic electric field strength data with different CDFs fitted plots in Site location III

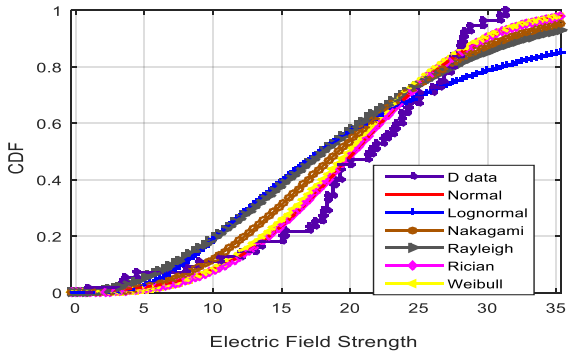

Fig. 7 (d): Stochastic electric field strength data with different CDFs fitted plots in Site location IV

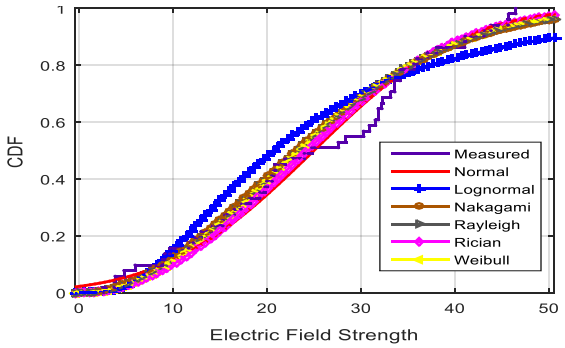

Fig. 7 (f): Stochastic electric field strength data with different CDFs fitted plots in Site location V

Table 2: Statistical Maximum Likelihood Estimation Parameters and Values

		Location				
Distribution function	MLE Parameters	1	2	3	4	5
Normal	Log likelihood Estimate	-518	-421	-318	-187	-200
	Mean	26.63	37.41	24.70	20.33	24.92
	Variance	56.57	55.97	70.19	55.14	156.40
Lognormal	Log likelihood Estimate	-510	-412	-319	-212	-208
	Mean	26.65	37.39	24.82	22.08	26.59
	Variance	57.56	51.22	87.51	259.61	463.95
Nakagami	Log likelihood Estimate	-514	-417	-316	-194	-200
	Mean	26.68	37.44	24.71	19.85	24.58
	Variance	53.90	52.67	69.00	73.08	170.20
Rayleigh	Log likelihood Estimate	-554	-490	-331	-196.50	-200
	Mean	24.52	33.80	23.10	19.16	24.66
	Variance	164.35	312.27	145.90	100.32	166.23
Rician	Log likelihood Estimate	-517	-421.39	-318	-188	-199
	Mean	26.64	37.41	24.70	20.35	25.11
	Variance	56.13	55.51	69.40	53.24	144.06
Weibull	Log likelihood Estimate	-520	-431	-317.80	-191.24	-200
	Mean	26.60	37.13	27.59	20.09	24.82
	Variance	62.97	77.13	71.39	52.71	156.80

CONCLUSION

Through the use of the detailed maximum likelihood estimator in correspondent with different stochastic distribution models, the statistical fading characteristic of measured electric field strength data acquired in five uneven microcellular urban terrains of deployed LTE broadband communication channels has been investigated and analyzed in this research work. From the results summary, the lognormal distribution model fitting display the maximum log likelihood values of -510 and -412 in locations 1 and 2. It is an indication that lognormal distribution model is better fit the measured electric field strength data in the two locations compared to other distributive models. Also from the results, while Nakagami model gave the maximum log likelihood estimate of -316 in location 3, Normal and Rician models provided the maximum log likelihood estimates of -194 and -199 in locations 4 and 5, respectively. These different distributive fitting results on the measured electric field strength data in the respective locations clearly reveals that one particular model cannot be generalized as a fitting function terrain. It is also mainly caused by differences that exist in building height structures, roof tops, street/roads widths in the five study locations

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