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Articles

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Efficacy of Polyphosphoric Acid in Reducing the Degree of Thermal Aging of Agbabu Natural Bitumen

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***Absract*:** Bitumen is a complex mixture of hydrocarbons which occurred naturally or obtained from crude oil distillation. The degradation of bitumen causes its rapid aging leading to deterioration of asphalt based pavement readily induced by cracking, rutting, potholes etc. Aging in bitumen is attributed to chemical oxidation of its molecules and exposure to environmental factors, such as heat, moisture and sunlight. This study therefore investigates the viability of using polyphosphoric acid (PPA) to reduce the oxidative degradation effect of heat (60^{o}C) on constituents of Agbabu natural bitumen (ANB) with a view to making it more durable when used for road pavement. Modification of ANB with PPA was carried out at 150-55^{o}C using melt blend technique. Thereafter, the PPA modified and neat (unmodified) ANB samples were thermally aged in a hot air oven at 60^{o}C. Fourier Transform Infrared (FTIR) Spectroscopy was used to evaluate the changes in the structure of ANB and aging indexes of neat and PPA modified samples of ANB were calculated. The neat and PPA modified ANB samples aged as a result of their exposure to temperature at 60^{o}C. However, the aging indexes of PPA modified samples of ANB were found to be lower compared with that of the neat sample of ANB. This implies that the rate of aging (60^{o}C) of the neat ANB was faster than that of PPA modified ANB samples. Thus, a good potential for reducing thermal aging in ANB was found in PPA.

***Keywords*:** Agbabu natural bitumen, Polyphosphoric acid, FTIR analysis, Aging, Oxidative degradation

1.0 Introduction

Flexible road pavement (FRP) is made up of bitumen, mineral aggregate, filler and additives. It is a known fact that FRP deteriorates with time as a result of usage and contact with some environmental factors, such as heat, moisture, sunlight etc. The chemical oxidation of bitumen molecules and interaction with some environmental

factors leads to aging of bitumen which manifests in pavement surface distresses such as cracks, rutting, potholes, ravelling etc.

Reduction of the rate of bitumen aging has been a challenge and the incorporation of materials such as polymers, wastes, PPA etc into the matrix of bitumen for this is well reported in the literatures (Iliya and Adam, 2011; Shulga *et al.*, 2012). The incorporation of materials such as polymers into bitumen is known as bitumen modification, and the objective of this study is to reduce the susceptibility of bitumen molecules to oxidation and thus increase aging resistance of the bitumen.

The application of PPA in modification of bitumen has been the usual practice as far back as 1939 (Burke *et al*., 1939). Daranga *et al., (*2009) used PPA to modify some binders and reported an increase in stiffness and reduction in oxidation process in the modified binders. Similarly, the study of Masson and Collins, (2009) showed that PPA modified asphalt when used in road pavement led to increase in pavement upper service temperatures and reduction of rutting. A mixture of PPA and SBR was used by Feng and Jianyin, (2010) to modify asphalt. An improvement in some physical properties and rheological properties of the modified asphalt was reported by these authors.

Bitumen aging has been identified as the major factor responsible for deplorable condition of our roads (Sa Araujo, 2013). Two aging mechanisms have been identified, which are reversible and irreversible mechanisms. The reversible mechanism (physical hardening) is revocable by application of heat and occurs as a result of molecular re-organisation in bitumen which is a function of time (Tabatabaee *et al.*, 2012). The irreversible mechanism occurs as a result of oxidation, dehydrogenation, polymerisation and condensation reactions and it is accompanied with loss of volatile components. It is either thermally induced by heating or light induced by exposing modified or unmodified bitumen sample to sunlight or ultra violet (UV) radiation (Mouillet *et al.*, 2008; Lins *et al.*, 2008).

Oxidative aging causes hardening of bitumen and occurs throughout the service life (Chen and Huang, 2000). It is induced by the chemical reaction of bitumen molecules with oxygen. Xiaohu *et al.*, (2008) carried out investigation on how different laboratory aging tests, viz: Rolling Thin Film Oven Test (RTFOT), Pressure aging Vessel (PAV) Test and Rotating Cylinder Aging Test (RCAT) under various conditions relate to field aging of bituminous binders. They concluded that the field aged bitumens showed a much higher level of sulphoxides but lower level of carbonyls compared to laboratory aging. The reason for this according to the authors was that higher temperature in laboratory aging results in higher amount of carbonyls while longer time in the field produces higher amount of sulphoxides. It was also concluded that aging kinetics and formation of sulphoxides and carbonyls were strongly temperature dependent. For the SBS modified binder, the polymer was found to inhibit the formation of sulphoxides on aging.

Most of the studies on application of PPA to modifying bitumen were based on artificial or petroleum bitumen. There is paucity of literatures on the use of PPA to modify natural bitumen and ANB. Crude petroleum/oil or natural bitumen is not uniform in composition as its composition varies from well to well (Amit, 2010). The implication of this is that crude petroleum/oil or natural bitumen, depending on the source behaves differently to modifiers. Therefore, it was decided to investigate the viability of using PPA to modify the ANB with a view to improve its service life by making it less susceptible to relatively high road pavement temperature.

2.0 Methodology

2.1 Materials

2.1.1 Bitumen

The bitumen used in this study was natural bitumen sample collected from one of the observatory wells sunk by the Nigerian Bitumen Corporation (NBC -7) located opposite Saint Stephen's Primary School, Agbabu, Ondo State, Nigeria. Agbabu is located on the so called bitumen belt of south-western Nigeria. The belt has geographical location of Longitude $3^{o}45'E$ and $5^{o}45'E$ and Latitude $6^{o}00'N$ and $7^{o}00'N$ and spans across Edo, Ondo and Ogun States of Nigeria (Fagbote *et al.*, 2012).

2.1.2 Polymers

The modifier used in this study was Polyphosphoric acid (PPA) with concentration of 105% phosphoric acid (75.9% P_2O_5). The specific gravity is 2.05.

2.2 Extraction of Moisture from Bitumen Sample

Method described by Vernon and Katy (1999) was modified and employed for extracting moisture from ANB (Bakare *et al.*, 2015). Calcium chloride granular salt was mixed with bitumen sample and heated to $65^{o}C$ in an oven to get rid of moisture. Meanwhile, the moisture content was monitored using infrared moisture analyser (Sartorius AG Germany), MA 35M-00023012 until it was approximately zero.

2.3 Purification of Bitumen Sample

The Bitumen sample was purified using the modified method of Rubinstein and Strausz, (1979). The mixture of 1000 cm^3 chloroform and 100 g of natural bitumen sample was stirred with a glass rod to complete dissolution. The bitumen in the filtrate was then recovered by vacuum evaporation of the solvent using rotary evaporator.

2.4 Preparation of PPA modified ANB

The raw natural bitumen sample collected from Agbabu was dehydrated and then purified as described in our previous study (Olabemiwo *et al.*, 2015). Melt blend technique was used for preparation of the PPA modified samples via a high shear mixer. 400 g of ANB was heated to a temperature range of $150\text{-}155^{o}C$ in an iron container. Then, PPA was gradually added. Mixing continued at $150\text{-}155^{o}C$ temperature range with the speed of the mixer also maintained at 1200 rpm for 1hr to obtain a homogenous mixture. The percentage composition of PPA by weight of bitumen used for blending varied from 2 to 6%.

2.5 Aging Simulation of Unmodified and Modified ANB Samples

2.5.1 Thermal Aging Using Oven

Thermal ageing was simulated by weighing 5 g of the modified bitumen sample into a Pyrex glass Petri dish to a thickness of 0.1 cm. The dish was then set in a hot air oven at 60^{o}C for three weeks. 0.4 g of the sample was withdrawn periodically from the oven at interval of one week and conditioned to room temperature for FTIR analysis to study the sample's degradation due to heat at 60^{o}C (Olabemiwo *et al.*, 2010).

2.6 FTIR Analysis of Aged Unmodified and Modified ANB Samples

Fourier Transform Infrared (FTIR) Spectrophotometer FTIR-8400s, SHIMADZU, with Spectra range: 4,000-400 cm^{-1} was used. The IR spectra of aged modified and unmodified samples were generated by scanning each sample. 1.5 mg of the sample was ground with 150 mg of analytical grade potassium bromide. The mixture was pressed in a hydraulic press to form a pellet. The pellet was mounted on the instrument and the infrared spectra of modified and unmodified samples were generated by separately scanning each sample (4000-400 cm^{-1}) on Fourier Transform Infrared Spectrophotometer. Calculations of functional and structural indexes for the bands were based on the equations proposed by Mouillet *et al*., (2008).

- $Sulphoxide = \frac{A_{1030}}{\sum A}$; ……(*Equation 1*)
- *The sum of the area represents* $(\sum A) = A_{1700} + A_{1600} + A_{1460} + A_{1376} + A_{1030} + A_{864} + A_{814} + A_{743} + A_{724} + A_{(2953,2923,2862)}$ …. (*Equation 2*)

3.0 Results and Discussion

3.1 FTIR Analyses of PPA Modified and Unmodified ANB Samples

The FTIR spectra of unmodified and modified Agbabu natural bitumen were recorded in the range of 4000-400 cm^{-1} as shown in Figure 1.0 and Figures 2.0(a-c) respectively.

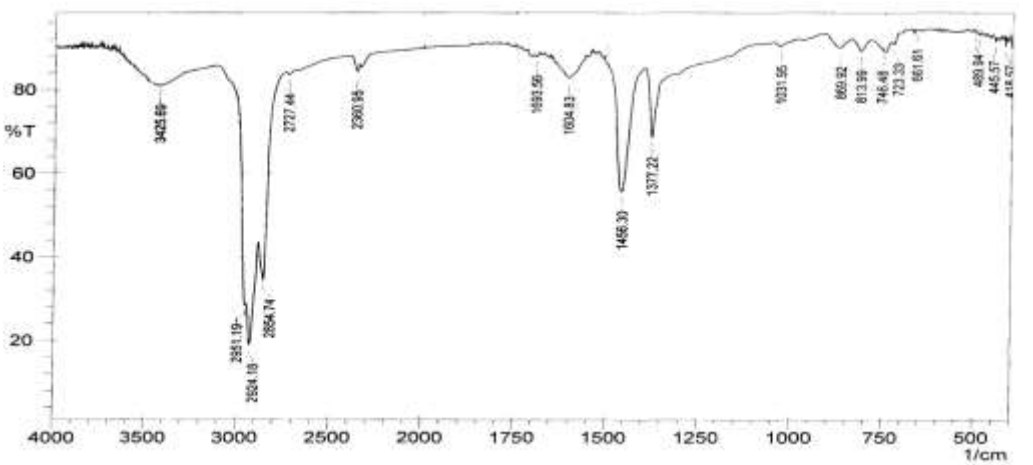

Figure 1.0: FTIR of Unmodified ANB

The peaks obtained in the FTIR spectrum of unmodified ANB are similar to the ones identified by Lamontagne *et al.,* (2001). In their study, they identified absorption bands related to asphalt binder of grade of PG 64-22 to include 2922 cm^{-1}(υ_{as} CH_2 CH_3), 2882 cm^{-1}(υ_s CH_2 CH_3), 1601 cm^{-1}(υ C=C), 1455 cm^{-1} (υ_{as} CH_3 deformation) , 1376 cm^{-1} (υ_s CH_3 deformation), 1031 cm^{-1} (υ SO2), 868 cm^{-1}, 813 cm^{-1}, 747 cm^{-1}(C-H aromatics), and 722 cm^{-1} (alkyl chain). Assignment of functional groups in the unmodified ANB is as described in our previous studies (Olabemiwo *et al.,* 2016).

The comparison of peak positions and intensities of various peaks appearing in the infrared spectra of the base bitumen, Figure 1.0 and PPA modified ANB (containing 2, 4, and 6% PPA compositions), Figure 2.0(a-c) revealed that there is appearance of additional new peaks with wave numbers in the ranges of 999-1006 cm^{-1} for P-O-P stretching and 497-500 cm^{-1} for P-O-P bending vibration in the spectra of PPA modified ANB samples. This indicates that the PPA has undergone some type of interactions with bitumen resulting in structural changes in the modified bitumen. There is no noticeable disappearance of prominent infrared peaks, in contrast to findings of Gupta *et al.,* (2012) who reported disappearance of some prominent infrared peaks in modified bitumen. However, peak shifting occurred in the infrared spectra of PPA modified ANB samples prepared in this study. Changqing *et al.* (2012) reported that asphalt experienced no obvious changes in functional groups before and after modification. This suggests that the modification of asphalt with PPA is a physical process.

Some similarities in the PPA modified ANB spectrum were noted with the submission of Feng and Jianyin, (2010) on major spectrum of PPA who reported infrared absorption peaks to include 1012 cm^{-1}, 933 cm^{-1}, 772 cm^{-1}, and 478 cm^{-1} as shown in Figure 3.0. However, P-O-P asymmetric vibration at 933 and 773 cm^{-1} were absent in the spectra of PPA modified ANB samples.

Furthermore, the infrared spectra of PPA modified samples showed that sulphoxide bands were absent, which is in agreement with the findings of Masson and Collins, (2009). Their finding showed that sulphide groups (aliphatic or aromatic), was inert when heated with PPA at 150°C for 1 hour i.e. the aliphatic or aromatic sulphur compounds did not react with PPA during blending.

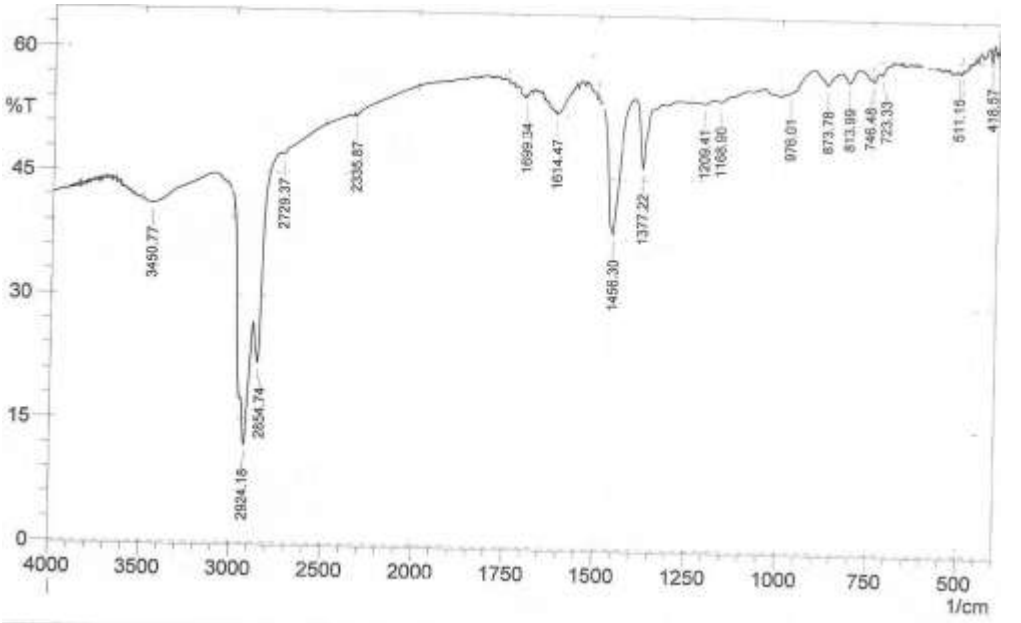

Figure 2.0 (a): FTIR Spectrum of 2% PPA Modified ANB

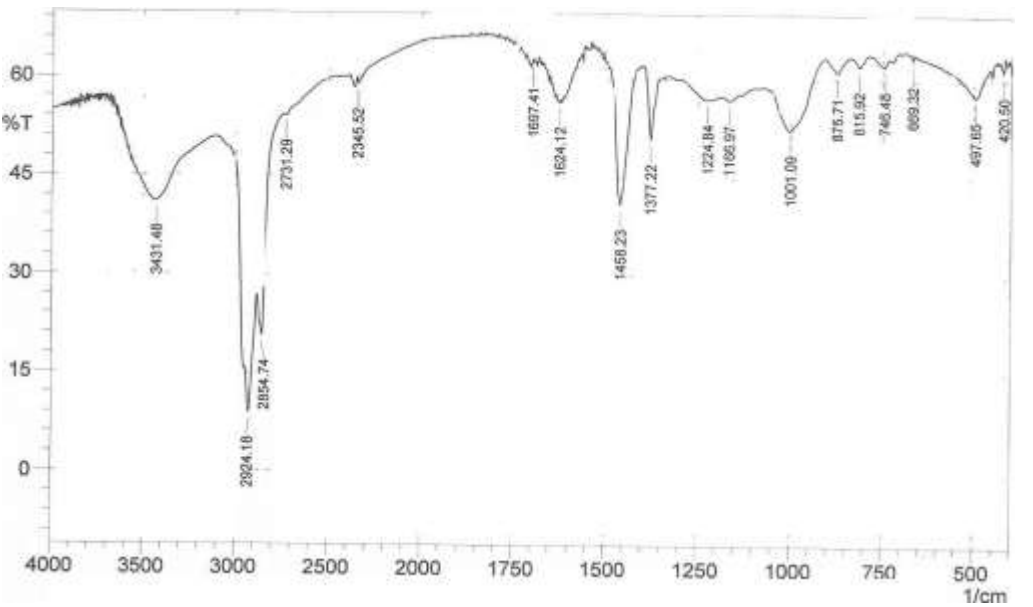

Figure 2.0 (b): FTIR Spectrum of 4% PPA modified ANB

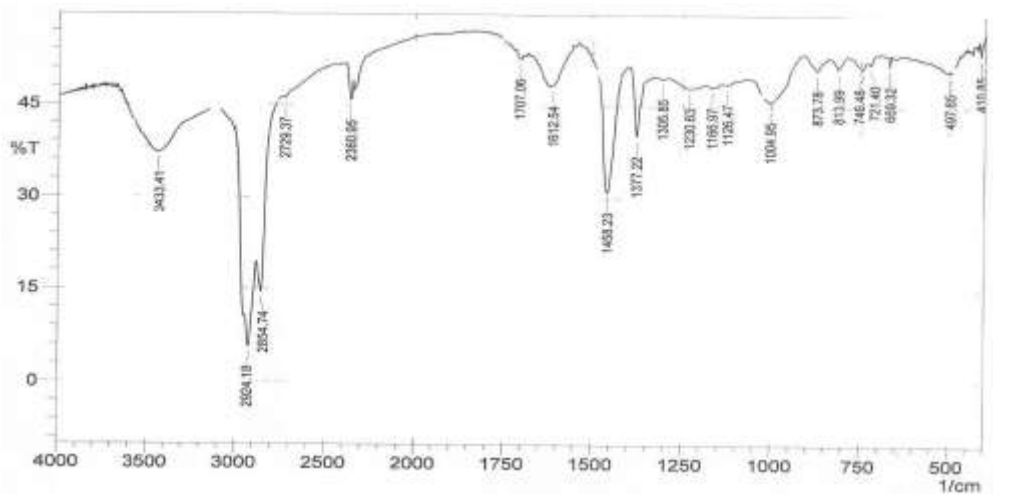

Figure 2.0 (c): FTIR Spectrum of 6% PPA Modified ANB Modified

3.2 FTIR Analyses of Thermal Aged Modified and Unmodified ANB Samples

The FTIR spectra of aged modified and unmodified ANB samples are as shown in Figures 3.0(a-l). The FTIR test was carried out to detect the effect of aging on the areas of absorption peaks as well as absorption peak wave numbers present in the unmodified and modified ANB samples. The PPA modified and unmodified ANB samples were subjected to thermal aging using oven at 60^{o}C for a period of three weeks

Petersen and Harnsberger, (1998) reported that aging was responsible for shifting in absorption peaks wave numbers observed in neat ANB with intensities/transmittances of these peaks varied with no regular pattern. The shift in some peak wave numbers and transmittances may be due to oxidation of benzylic carbon or benzylic compounds in bitumen. This formed hydroperoxide or peroxy radicals capable of reacting with aliphatic sulphides to produce sulphoxide or undergo non radical self (condensation) reaction to form ketones. The amount of ketones formed on non-benzylic carbon was reported to be small (Petersen and Harnsberger, 1998).

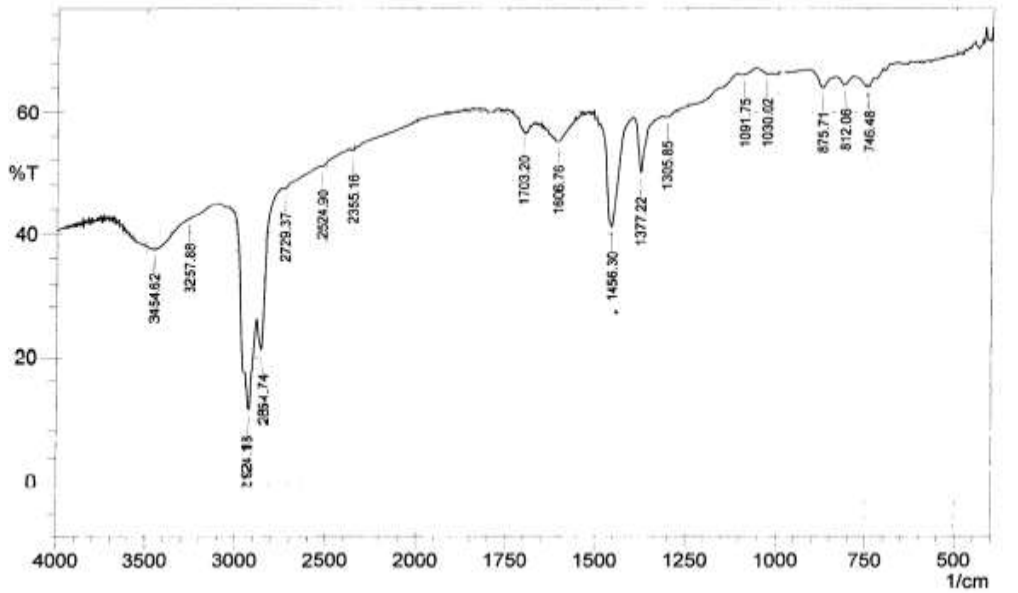

Figure 3.0 (a): FTIR Spectrum of Unmodified ANB Aged with Oven for one Week

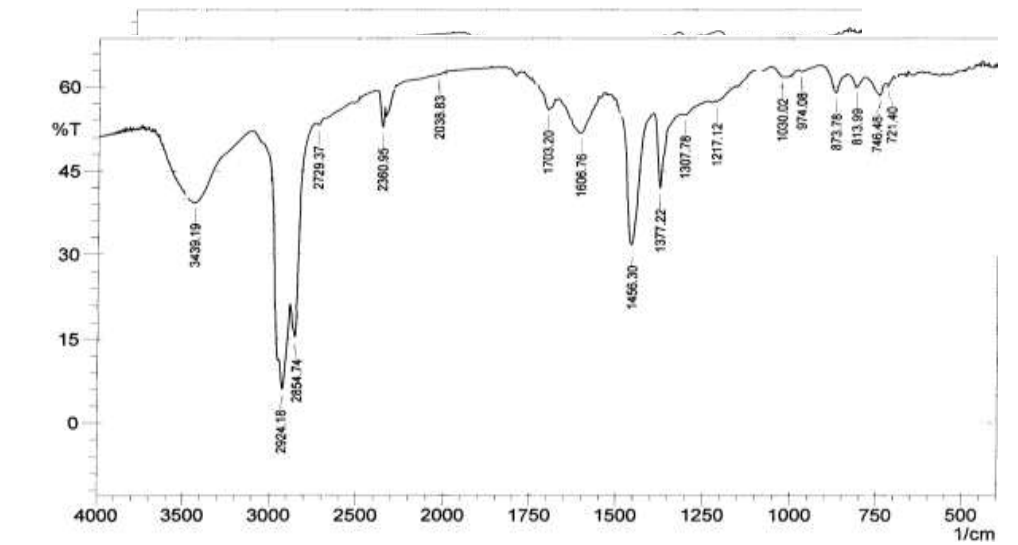

Figure 3.0 (b): FTIR Spectrum of Unmodified ANB Aged with Oven for Two Week

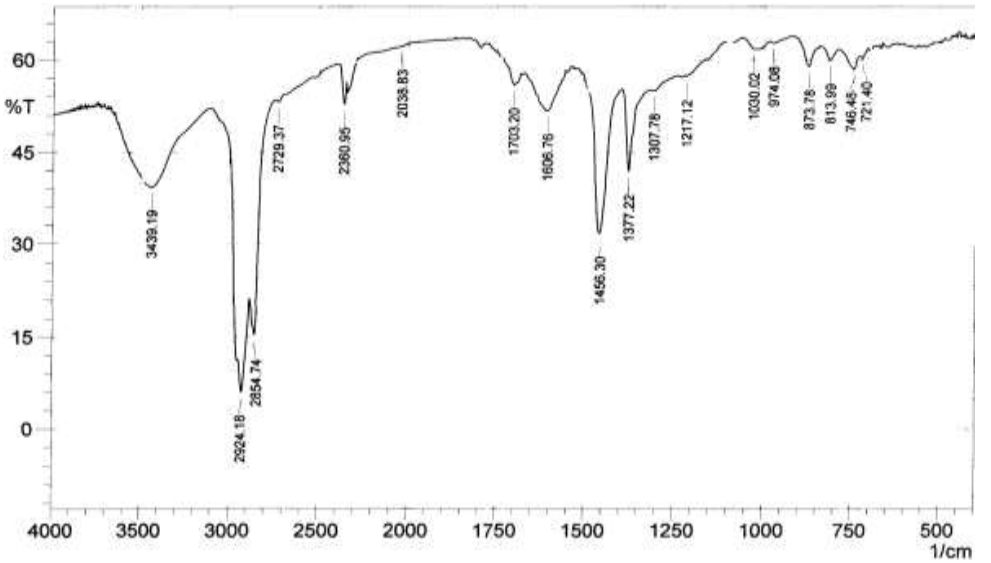

Figure 3.0 (c): FTIR Spectrum of Unmodified ANB Aged with Oven for Three Weeks

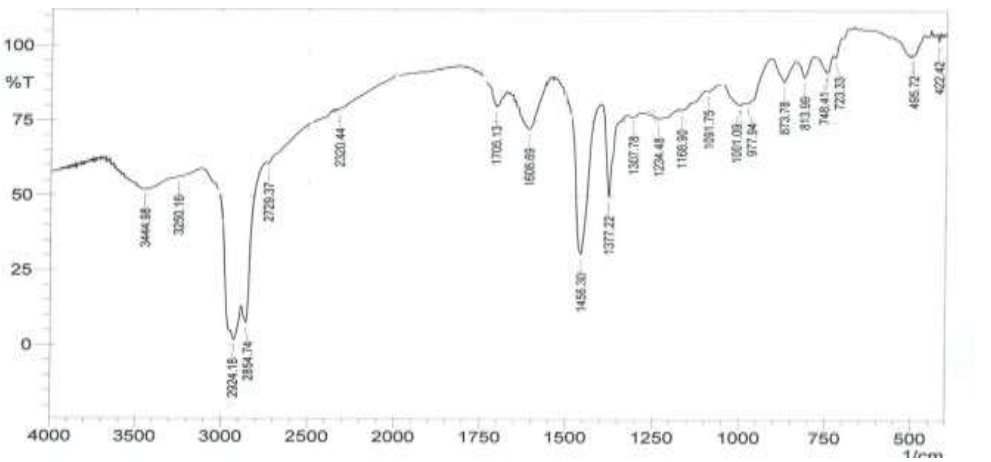

Figure 3.0 (d): FTIR Spectrum of 2% PPA Modified ANB Aged with Oven for one Week

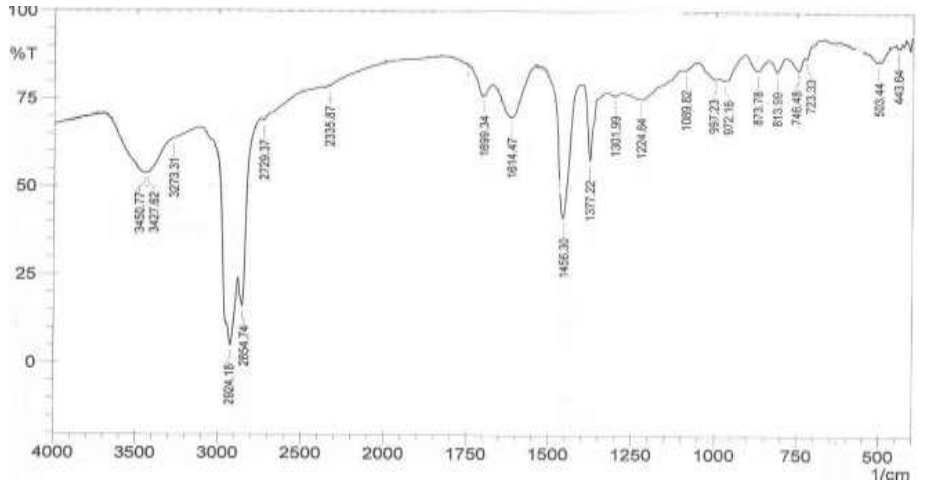

Figure 3.0 (e): FTIR Spectrum of 2% PPA Modified ANB Aged with Oven for Two Weeks

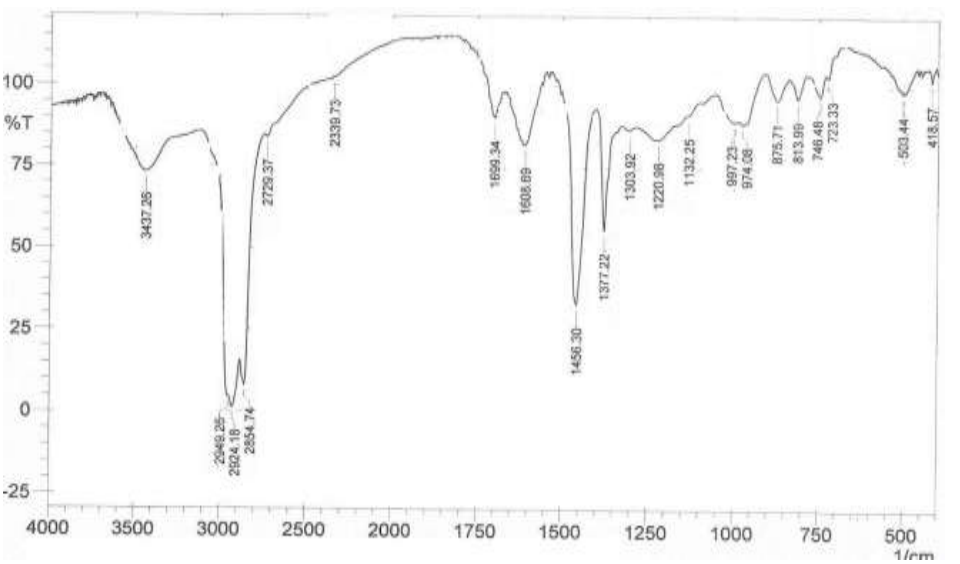

Figure 3.0 (f): FTIR Spectrum of `2% PPA Modified ANB Aged with Oven for Three Weeks

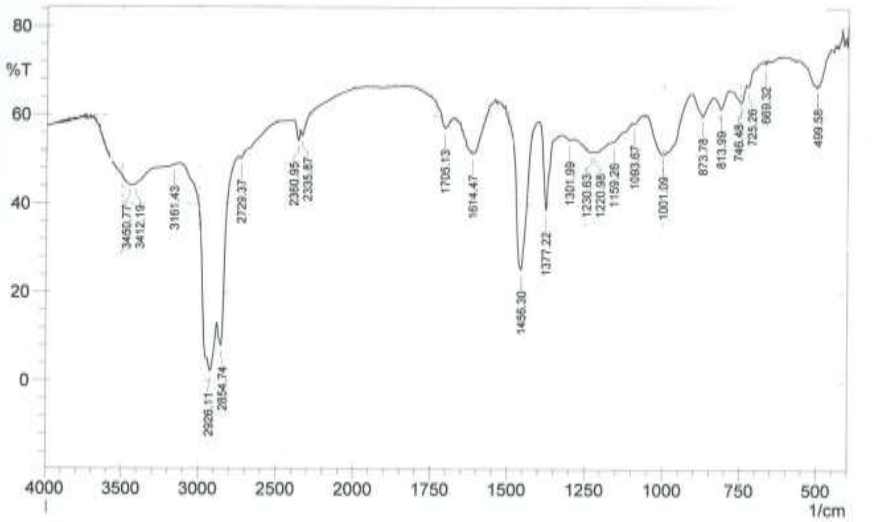

Figure 3.0 (g): FTIR Spectrum of 4% PPA Modified ANB Aged with Oven for One Week

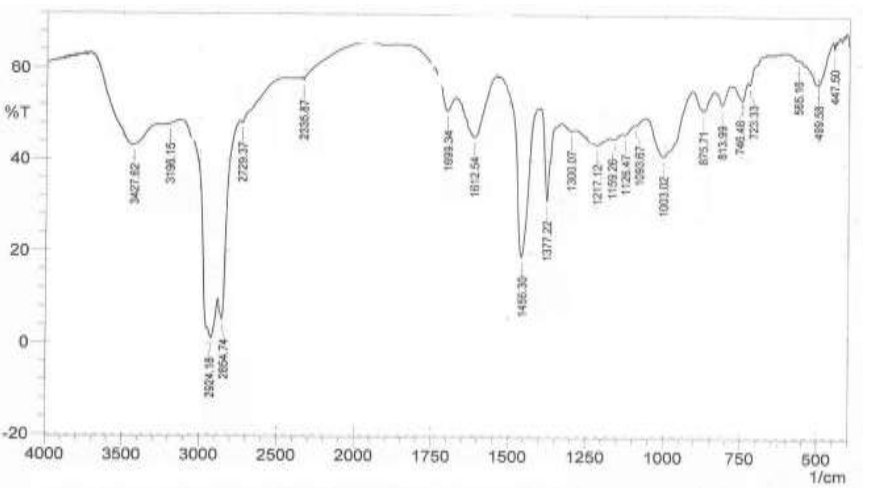

Figure 3.0 (h): FTIR Spectrum of 4% PPA Modified ANB Aged with Oven for Two Weeks

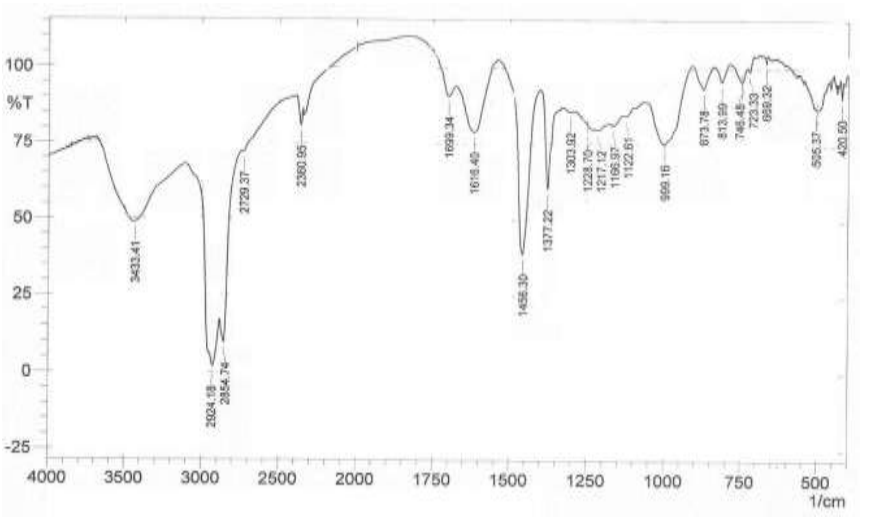

Figure 3.0 (i): FTIR Spectrum of `4% PPA Modified ANB Aged with Oven for Three Weeks

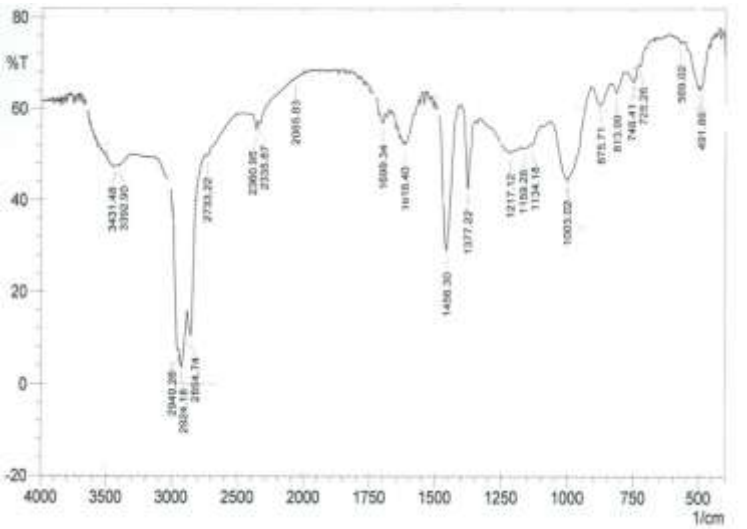

Figure 3.0 (j): FTIR Spectrum of 6% PPA Modified ANB with Oven for One Week

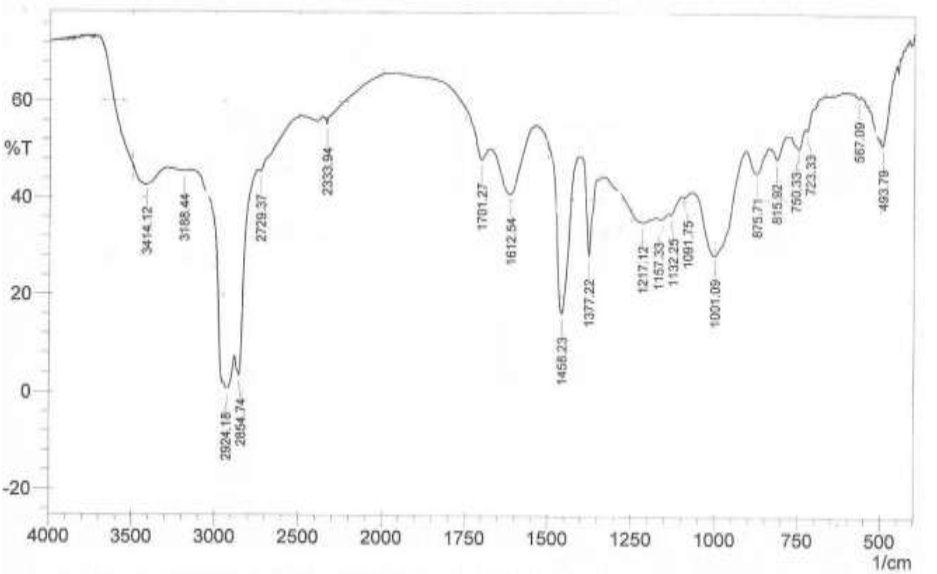

Figure 3.0 (k): FTIR Spectrum of 6% PPA Modified ANB with Oven for Two Week

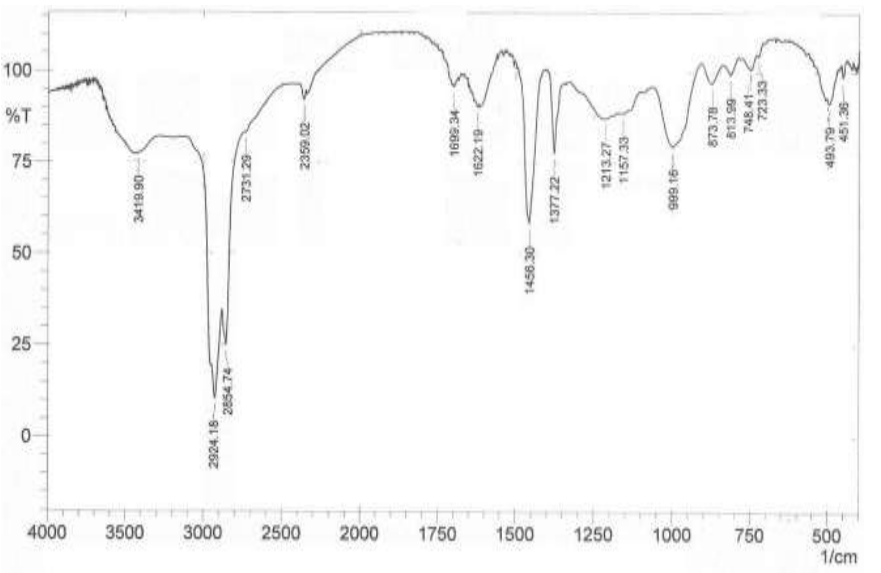

Figure 3.0 (l): FTIR Spectrum of 6% PPA Modified ANB Aged with Oven for 3 Weeks

3.3 Carbonyl indexes of thermal aged modified and unmodified ANB

The plot of carbonyl index as a function of the aging period of thermal aged PPA modified ANB is as shown in Figures 4.0. To quantify the effect of modified samples on reducing the aging of ANB, the carbonyl indexes were calculated for both modified and unmodified samples using the formula of Mouillet *et al*., (2008) in Equations 1 and 2. This is by measuring areas of the FTIR bands to assess the relative amounts of carbonyl compounds formed on aging. The infrared absorbance peak at about 1,700 cm^{-1} is attributed to C=O stretch in carbonyl compounds, such as ketones, carboxylic acids and anhydrides.

The plot of carbonyl index showed that there was growth in the sizes (areas) of carbonyl peaks after aging which was found to be dependent on the period of aging. However, the carbonyl index increased in both modified and unmodified ANB with the aging period due to reaction of bitumen molecules (carbon compounds) with oxygen. The rate of increase in the index (carbonyl) is higher in neat ANB than the modified sample. This is in line with the earlier submission by Lamontagne *et al.,* (2001) that the effect of aging was more considerable in the unmodified ANB compared to that of polymer modified ANB at different aging period as high values of carbonyl and sulphoxide indexes imply high degree of aging effect and vice versa. Similarly, Seyed, (2012) in his study reported that FTIR spectra of unmodified and SBS modified samples showed that aging caused oxidation of bitumen and formed the carbonyl and sulphoxide structures in bitumen. Aging in modified bitumen by SBS has been less than base bitumen.

Hardening of bitumen occurs as a result of oxidation and loss of volatile components. The degree of hardening decreases with increasing percentage of PPA content in the ANB in this study. The plot of sulphoxide index against aging period could not be established for the aged PPA modified samples because FTIR analysis for aged PPA modified samples did not show values for sulphoxide peak as reported by Masson and Collins, (2009). Baumgardner, (2009) also reported that PPA acid when used to modified bitumen actually has anti-oxidative characteristics.

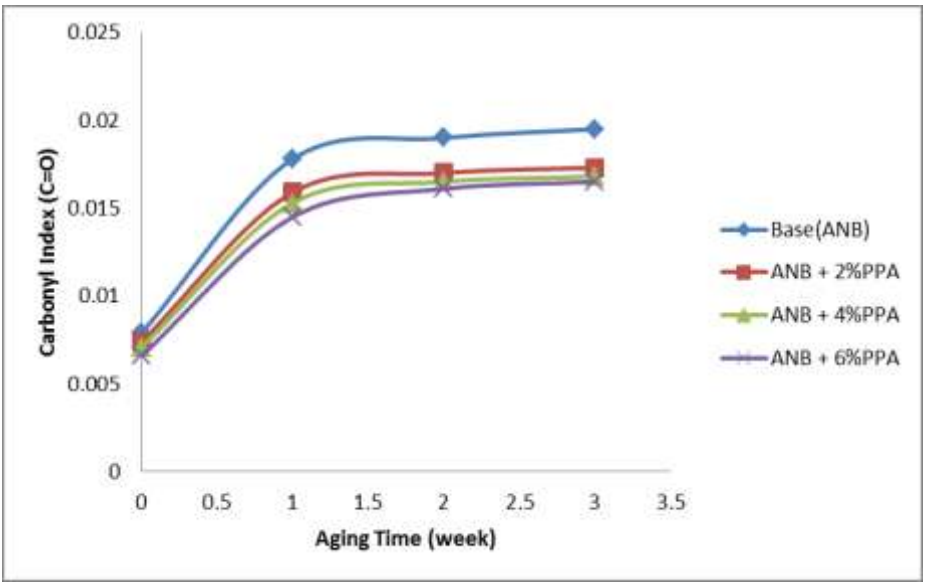

Figure 4.0: Variation of Carbonyl Index of PPA Modified and Unmodified ANB Samples with Period of Light Aging at 60°C

4.0 Conclusion

Samples of ANB modified with PPA were thermally aged (60°C) for a period of three weeks. The infrared spectra of PPA modified ANB samples show carbonyl peak but did not show sulphoxide peak. In addition, the carbonyl index values for PPA modified ANB samples are lower at different aging periods compared to that of unmodified ANB. These imply that sulphur compounds (e.g. sulphides) in ANB are completely inert to oxidants in presence of PPA and also the addition of PPA has improved the aging resistance of ANB to certain degree. The aging resistance of the modified ANB samples is found to increase as the amount of PPA incorporated into ANB increased.

5.0 Acknowledgment

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References

[1] Amit, K. (2010). Physical and Chemical Aging Behavior of Asphalt Cements from Two Northern Ontario Pavement Trials, *Queen's University Kingston, Ontario, Canada,* 1-109.

[2] Bakare, H. O.; Esan, A. O. and Olabemiwo, O. M. (2015). Characterisation of Agbabu Natural Bitumen and Its Fractions Using

Fourier Transform Infrared Spectrometry, *J. of Chemical and Mater. Res.*, 7(8), 1-16.

[3] Baumgardner, G. L. (2009). Polyphosphoric Acid Modified Asphalt Binders–Industry Perspective; Usage, Why, How", Paragon Technical Services, https://engineering.purdue.edu/NCSC/PPA%20Workshop/2009/index.html (viewed 09/09/10).

[4] Burke, R. E. and Whitacre, C. H. (1939). Manufacture of Improved Asphalts, U.S. Patent, no. 2,179,208.

[5] Changqing, F; Rulen, Y.; Ying, Z.; Jingbo, H.; Min, Z. and Xionghua, M. (2012). Combined Modification of Asphalt with Polyethylene Packaging Waste and Organophilic Montmorillonite, *Polym. Testing*, 31, 276 -281.

[6] Chen, J. S. and Huang, L. S. (2000). Developing an A ging M odel to E valuate Engineering Properties of Asphalt Paving Binders, *Mater. And Structures Const.*, 33,559-565.

[7] Daranga, C.; Clopotel, C.; Mofolasayo, A. and Bahia, H. (2009). Storage Stability and Effect of Mineral Surface on Polyphosphoric Acid (PPA) Modified Asphalt Binders. *Presented during Poster Session, 88th Annual Meeting of the Transportation Research Board; Washington, D.C.*, 2-11.

[8] Fagbote, E. O. and Olanipekun, E. O. (2011). Seasonal and Depth Effects on the Physico-chemical Parameters of the Soils of Farm Settlements with Bitumen Deposit, *Inter. Res. J. of Biotech.*, 2(9), 198-212.

[9] Feng Z. and Jianyin, Y. (2010). The Research for High-performance SBR Compound Modified Asphalt, *Const. and Build. Mater.*, 24(3), 410-418.

[10] Gupta, R. S. and Kaur, V. (2012). Characterization of Bitumen and Modified Bitumen (e-PMB) using FTIR, Thermal and SEM Techniques: International Science Congress Association, *Res .J. Chem. Sci.* 2(8), 31-36.

[11] Iliya Y. and Adam Z. (2011). Attenuated Total Reflection (ATR) Fourier Transform Infrared (FT-IR) Spectroscopy of Oxidized Polymer-Modified Bitumens, *Society for Applied Spectroscopy*, 65(7), 765-770. DOI: 10.1366/10-06217.

[12] Lamontagne, J.; Dumas, P.; Mouillet, V. and Kister, J. (2001). Comparison by Fourier Transform Infra-Red (FTIR) Spectroscopy of Different Ageing Techniques: Application to Road Bitumens, *Fuel*, 80, 483-488.

[13] Lins, V. F. C.; Araújo, M. F. A. S.; Yoshida, M. I.; Ferraz, V. P.; Andrada, D. M. and Lameiras, F. S. (2008). Photodegradation of Hot-mix Asphalt. *Fuel*, 87, 61-3254. http://dx.doi.org/10.1016/j.fuel.2008.04.039

[14] Masson, J. F. and Collins, P. (2009). FTIR Study of the Reaction of Polyphosphoric Acid and Model Bitumen Sulfur Compounds, *Energy and Fuels*, 23, 440-442.

[15] Mouillet, V.; Lamontagne, J.; Durrieu, F.; Planche, J. P. and Lapalu, L. (2008). Infrared Microscopy Investigation of

Oxidation and Phase Evolution in Bitumen Modified with Polymers, *Fuel*, 87, 80-1270. http://dx.doi.org/10.1016/j.fuel.2007.06.029

[16] Olabemiwo, O. M.; Adediran, G. O; Adekola, F. A. and Olajire A. A. (2010). Aliphatic and Polycyclic Aromatic Hydrocarbons Profiles of Photomodified Natural Bitumen of Agbabu, Southwestern, Nigeria, *Bull. Chem. Soc. Ethiop.*, 24(3), 46-466.

[17] Olabemiwo, O. M.; Akintomiwa, O. E. and Bakare, H. O. (2015). Preliminary Investigation on Modification of Agbabu Natural Bitumen with Some Polymeric Materials, *Inter. J. of Sci. and Eng. Res.*, 8(9), 1342-1349.

[18] Olabemiwo, O. M.; Akintomiwa, O. E.; GeorgeO.A., and Bakare, H. O. (2016). The performance of Agbabu Natural Bitumen Modified with Polyphosphoric Acid through Fundamental and Fourier Transform Infrared Spectroscopic Investigations, *Case Studies in Const. Mater.*,(5), 39–45.

[19] Petersen, J. C. and Harnsberger, P. M. (1998). Asphalt Aging: A Dual Oxidation Mechanism and its Interrelationships with Asphalt Composition and Oxidative Age Hardening, *In Transportation Research Record 1638*, TRB, *National Research Council, Washington, D.C*: 47–55.

[20] Rubinstein, I. and Strausz, O. P. (1979). Thermal Treatment of the Athabasca Oil Sand Bitumen and Its Component Parts, *Geo-chim. Cosmochim. Acta* 43, 1887-1893.

[21] Sa Araujo, M. A. F.; Leite L. F. M.; Pasa, V. M. D. and Lins, V. F. C. (2013). Rheological Behaviours and Thermal Behaviours of Weathering-Aged Polymer Modified Bitumen, *Brazillian J. Petrolm. and Gas*, 7(4), 155-167.

[22] Seyed, A. T. (2012). Evaluate Aging Effect of SBS Modified Bitumen, *World Academy of Sci., Eng. and Tech.*, 7, 209-212.

[23] Shulga, O., Maldonado, R., Lewandowski, L., and Romagosa, H. (2012). Application of Polyphosphoric Acid for HMA Modification. US and European Experience, *5th Eurasphalt and Eurobitume Congress, 13-15th, Istanbul*, A5EE-140.

[24] Tabatabaee, H. A; Velasquez, R. and Bahia, H. U. (2012). Predicting Low Temperature Physical Hardening in Asphalt Binders, *Const. and Build. Mater.*, 34, 9-162. http://dx.doi.org/10.1016/j.conbuildmat.2012.02.039

[25] Vernon, H. S. and Katy, T (1999). Processing for Removing Water from Heavy Crude Oil, *United State Patent. Patent no: 6,007,702.*

[26] Xiaohu, L. U.; Yohann, T. and Per, R. (2008). Aging of Bituminous Binder–Laboratory Tests and Field Data, Conference Paper, 1-12. DOI:10.

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Preparation, Release Pattern and Antibacterial Activities of Chitosan-Silver Nanocomposite Films

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Abstract: The present study examined the preparation of chitosan-silver nanocomposite films as carriers for silver release pattern. Chitosan a biopolymer having immense structural possibilities for chemical and mechanical modifications to generate novel properties, functions and applications. Chitosan–silver nanocomposite films has been synthesised by reduction method, which is a simple and inexpensive method. The chitosan-silver nanocomposite films was characterized in terms of its surface plasmon resonance and crystalline structure by using UV-Visible spectroscopy, X-ray diffraction, Fourier transform infrared and Scanning electron microscope. Swelling and release studies were carried out on crosslinked and un-crosslinked nanocomposite films. Antibacterial activities of chitosan-silver nanocomposite films were investigated on some pathogens: Staphylococcus aureus, Shigella dysenteriae, Escherichia coli, Salmonella typhii and Klebsiella pneumonia using agar well diffusion method. crosslinked chitosan-silver nanocomposite demonstrated a slower release pattern relative to un-crosslinked chitosan-silver nanocomposite. The crosslinked and un-crosslinked nanocomposite became dislodged and completely released at 120 minutes and 90 minutes respectively. The results of the antibacterial activities revealed that the cross-linked chitosan-silver nanocomposite films has higher antibacterial properties than un-crosslinked chitosan-silver nanocomposite films. This study provides nanocomposite films potentially useful for delivery system.

Keywords: Biopolymer, Carrier, Nanocomposite, Antibacterial.

1. Introduction

In the past years, several synthetic as well as natural polymers have been examined for pharmaceutical applications. A basic requirement for these polymers to be used in humans or animals is that they have to degrade into molecules with no toxicity to biological environments. In recent years, biodegradable polymers have attracted attention of researchers as carriers for drug delivery system [8].

Chitosan is a polysaccharide that occurs naturally. Its units are composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). Chitosan interact very easily with bacterium and binds to DNA, glycosaminoglycans and most of the proteins, thereby enhancing the antimicrobial effect of metal nanoparticles [7]. Chitosan properties allow it to rapidly clot blood and have recently gained approval in the United State of America for use in bandages and other hemostatic products. Chitosan is used in pharmaceutical manufacturing as fillers in tablets and to mask bitter tastes in solutions taken by mouth. Films and coatings based on biopolymers functions as barriers against moisture, oxygen, aroma flavor as well as oil are the materials for future applications [3].

Metal nanoparticles embedded into polymer matrix can be used as sensor, materials with solvent switchable electronics properties, optical limiters and filters, and for optical data storage. They can be applied for catalytic applications and antimicrobial coatings. Nanocomposite of polymer/metal nanoparticles has attracted great attention because of its potential applications in the field of catalysis, bioengineering, photonics, and electronics [9]. Polymers are considered good host materials for metallic nanoparticles as well as other stabilizing agents, including citrates, organic solvents, and organometallics [4]. Some examples of metal/polymer nanocomposites being synthesized include poly (vinylalcohol), poly (vinylpyrolidone) [6], chitosan [1], gelatin [6]. This accounts for the different properties of nanocomposites that are synthesized.

2. Experimental

2.1. Materials

All reagents in this work were of analytical grade and were used as received without further purification. AgNO3 (99.98% Sigma -Aldrich) was used as the silver precursor, Sodium citrate, (crosslinking agent), Ammonia solution, Sodium hydroxide and glacial acetic acid (99%) were also obtained from Sigma–Aldrich. All the aqueous solutions were prepared with double-distilled water.

2.2. Methods

2.2.1. Preparation of Crosslinked Chitosan-Silver Nanocomposite Films

5 g of silver nitrate was dissolved by stirring in 100 mL of distilled water. 20 mL of the solution was taken and aqueous NaOH was added dropwise to the vigorously stirred solution till precipitation occurred. The dirty green precipitate formed was dissolved in few drops of dilute ammonia. Two layers were formed after centrifugation and addition of acetic acid. It was decanted

and added in chitosan matrix (6 mL, 1% (w/v) in acetic acid). Film was made by casting the solution on the petri dish and dried at room temperature for 24 hours. When the film has been harvested cross-linking was done by dipping the film in a 10 mL solution of sodium citrate (5 % w/v) after adjustment to pH 5. The film was washed with water to remove excess sodium citrate.

2.2.2. Swelling Studies

The completely dried, pre-weighed chitosan, crosslinked chitosan-silver and un-crosslinked chitosan-silver nanocomposite films were immersed in 250 mL phosphate buffer (pH 7.4) at 25 oC. The water uptake of the films was measured at 20 minutes interval using analytical balance to determine the mass. The swelling ratio (Q) of the films was calculated using the following equation: Q = Ws/Wd,

Where Ws is the weight of the swollen film at different time intervals (20, 40, 60, 80, 100, 120 and 140 minutes) and Wd is the weight of dry film.

2.2.3. Release Study

In Vitro release study of silver from chitosan-silver nanocomposite films was determined. A known weight of the dried nanocomposite films was put in the 20 mL phosphate buffer solution in a 50 mL tube. The temperature and rotation were adjusted to 37 oC and 90 rpm respectively. At predetermined time of 30, 60, 90, 120, 150, 180 and 210 minutes, 10 mL of sample was withdrawn and ultra-centrifuged for 30 minutes. The samples were further analyzed using UV-Visible spectrophotometer. The absorbance of each solution of the nanocomposite films was measured at λmax 425 nm.

2.2.4. Antibacterial activity of Chitosan-silver nanocomposite films

The antimicrobial activities of the synthesized films were tested against human pathogens like Staphylococcus aureus, Shigella dysenteriae, Escherichia coli, Salmonella typhii and Klebsiella pnueumoniae by agar well diffusion method. An overnight culture of each pathogen grown in nutrient broth at 37 oC was diluted to a turbidity equivalent of 3.0×108 cfu/mL (1.0 Mcfarland standard) with a sterile normal saline. The cell suspension was then swabbed on the surface of Mueller-Hinton agar plates. A sterile cork borer of diameter 7 mm was used to make wells on the agar plates. Each well was filled with 50.0 µL of each solution of chitosan-silver nanocomposite. The plates were left for one hour to allow the test materials to diffuse in the agar and then incubated at 37 oC for 24 hours without inversion. The antimicrobial activity was determined by measuring the clear zone (zone of inhibition) around the wells. The diameter (mm) of the zone of inhibition of 1.00 mm or greater was considered as a significant inhibition [11].

2.3. Characterization Methods and Instruments

The synthesized chitosan-silver nanocomposite films were confirmed using UV-visible spectroscopy (UV-visible), X-ray diffraction (XRD), Scanning electron microscope (SEM) and Fourier transform infrared spectroscopy (FTIR). The UV-visiblespectra of the nanocomposite were detected over the range of 300–700 nm using a Shimadzu (UV.1650) UV-visible spectrophotometer. Crystalline structures of the synthesized chitosan-

silver were examined using Rigaku D/Max-2550Pc (Tokyo. Japan). Morphology of the films was studied by SEM through a JEOL JSM 840A.

3. Result and Discussion

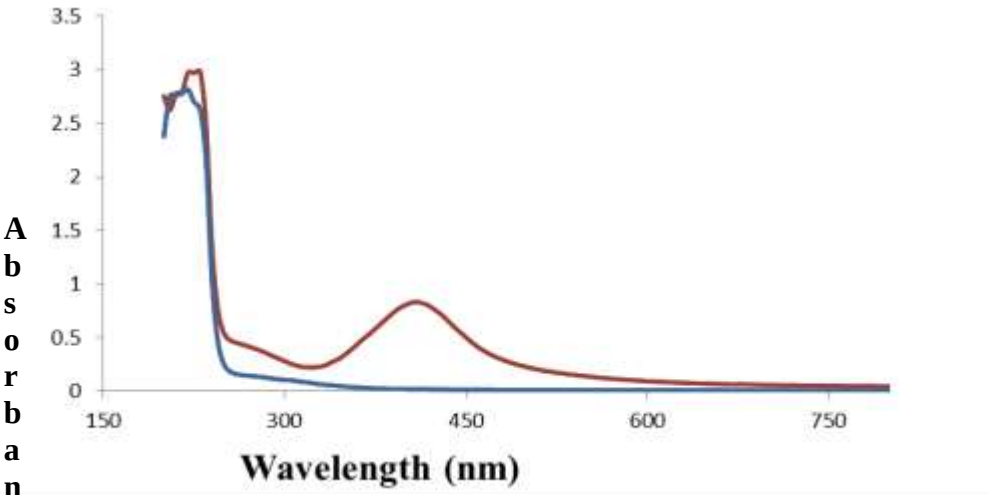

Figure 1: UV-visible spectrum of chitosan-silver nanocomposite films.

3.1 Characterization

The UV-Visible spectrum of chitosan-silver nanocomposite films is shown in Figure 1. The spectrum of chitosan-silver nanocomposite films shows a single peak at maximum wavelength 420 nm which confirmed the presence of silver; this peak arises due to surface plasmon resonance vibrations of silver atoms. The peak at maximum wavelength 230 nm shows the presence of chitosan.

Figure 2 shows the X-ray diffraction pattern chitosan-silver nanocomposite films. Several distinct diffraction peaks at 2θ values of 20.5, 21.0, 27.2, 36.8, 42.8, 50.5, 60.2 and 67.7 (crystalline peaks) is attributed to the presence of silver while the non-crystalline part indicate the presence of Chitosan as shown on the chitosan-silver nanocomposite XRD pattern which confirmed the formation of the nanocomposite.

Figure 3 shows the FTIR spectra of chitosan-silver nanocomposite films. The absorption peaks in the chitosan-silver nanocomposite spectra at 2930.93$cm^{-1}$and 1342.50 $cm^{-1}$are characteristic of O-H stretching and O-H bending of chitosan respectively. The stronger the hydrogen bond the longer the O-H bond, the lower the vibration frequency, and the broader and the more intense the absorption band. The peak observed at 1619.29 cm^{-1}indicates hydrogen bonding nature of N-H bending and the peak at 3369.75 cm^{-1} represents H bonded-OH of chitosan, the lower the frequency the stronger the H bond. The SEM image in Figure 4 reveals rough surfaces with whitish particles (silver nanoparticles) surrounded with black particles

(chitosan), a clear evidence of swelling on the surface.

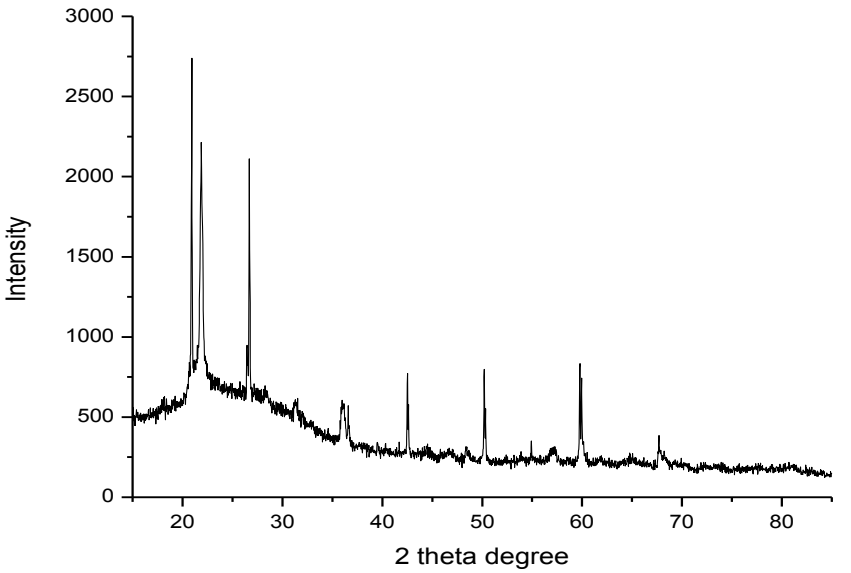

Figure 2: XRD pattern of Synthesized Chitosan – Silver nanocomposite film

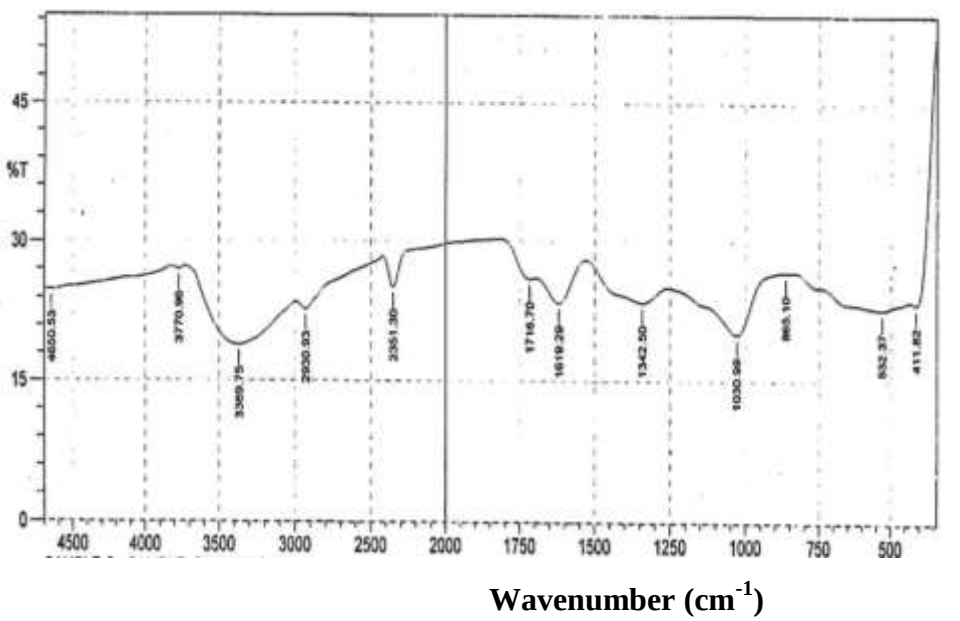

Figure 3: FTIR Spectra of Synthesized Chitosan-silver Nanocomposite film

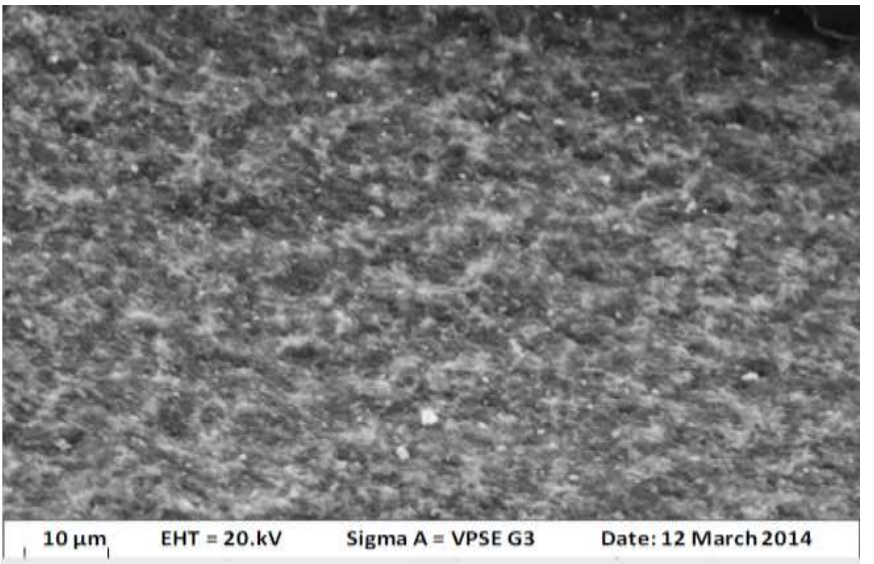

Figure 4: SEM image of chitosan-silver nanocomposite film

3.2 Swelling Test

Swelling test is useful for correlating drug release characteristics from polymeric films and barriers. It can be judiciously used in predicting and modifying drug release from dosage forms. The cross-linked chitosan-silver nanocomposite is known to be dependent on the availability of the cationic sites and the negatively charged species.

3.3 Release Study

Phosphate buffer (pH 7.4) has been considered as release medium which simulates body fluid according to literatures. However, crosslinked chitosan-silver demonstrated a slower silver release pattern relative to un-crosslinked chitosan-silver. Moreover, it was apparently noted from the graphs that almost all the silver nanoparticles within cross linked and uncross-linked became dislodged and completely released at 120 minutes and 90 minutes respectively. This result perfectly agrees with our swelling studies, where swelling property of cross-linked chitosan-silver exhibited onset of dormancy at 120 minutes which made it coincided with un-crosslinked chitosan-silver. From swelling and release studies, it is easy to explain that silver nanoparticles is gradually released into the system (according to figure 6 below). This is a clear evidence that polymer composite like chitosan can serve as carrier in delivery system.

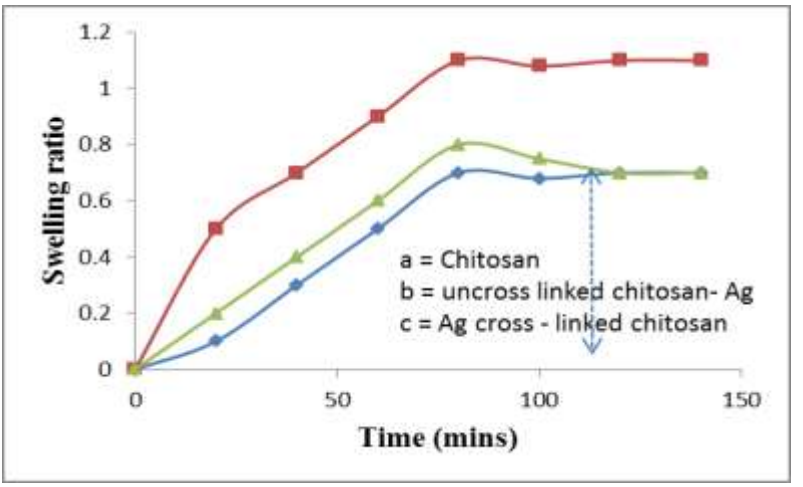

Figure 5: Swelling ratio against time.

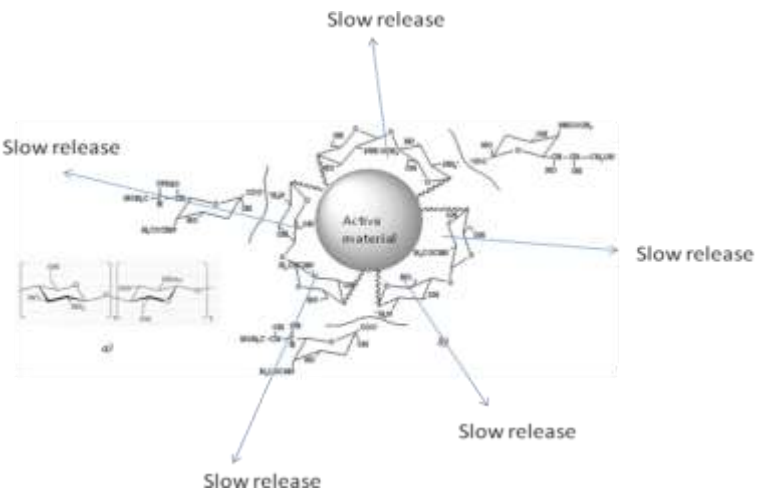

Figure 6: Shows the release of Silver from Chitosan

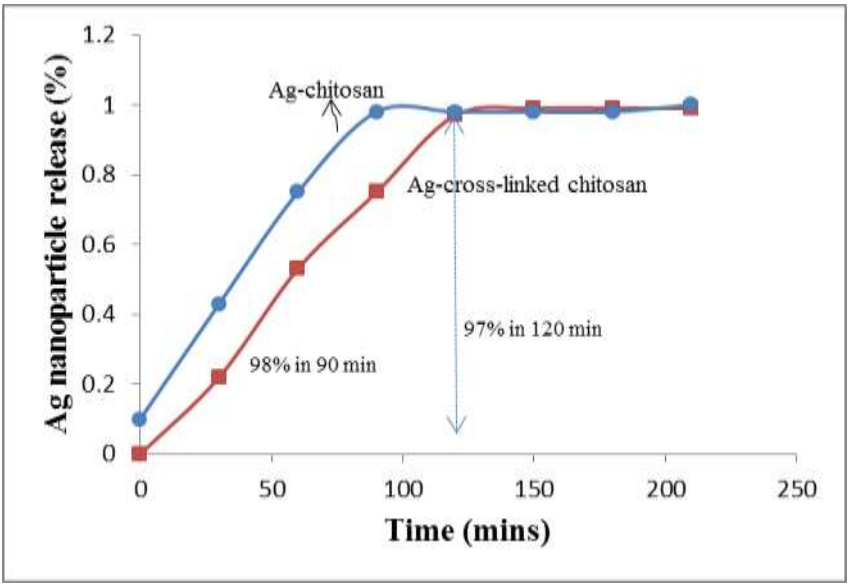

Figure 7: Shows silver released pattern of crosslinked chitosan-silver film compared with un crosslinked chitosan-silver.

Table 1: Antibacterial Activity of Films against test pathogens

Pathogens	**Zones of inhibition (mm)**												
	A (mg/ mL)			**B (mg/ mL)**			**C (mg/mL)**			**D (mg/mL)**			***Control**
	100	50	25	100	50	25	100	50	25	100	50	25	
Staphylococcus aureus	16	10	5	12	8	Nil	12	7	Nil	11	6	Nil	21
Shigella sp.	15	10	6	11	5	10	11	5	Nil	8	Nil	Nil	21
Escherichia coli	10	5	Nil	9	4	Nil	8	4	Nil	7	Nil	Nil	16
Salmonella sp.	9	6	Nil	8	5	Nil	8	4	Nil	6	Nil	Nil	19
Klebsiella sp.	8	6	Nil	9	7	Nil	7	Nil	Nil	Nil	Nil	Nil	21

Control: Ciprofloxacin, Sample A: crosslinked chitosan- silver, Sample B: un-crosslinked chitosan-silver, Sample C: crosslinked chitosan, Sample D: chitosan

The antibacterial activity of the synthesized nanocomposite films was evaluated by agar well diffusion method. The inhibitory growth was measured based on the diameter of the clear inhibition zone. The results of the antibacterial activity of the synthesized nanocomposite films are shown in Table 1. With the exception of Chitosan, films at higher concentration (100mg/mL) showed higher antibacterial activity against *Staphylococcus aureus*, *Shigella sp*. *Escherichia coli*, *Salmonella sp*. and *Klebsiella sp*, but they were not as active as ciprofloxacin (control) which could be due to the concentrations used. The results further revealed that the higher the concentrations of the films, the higher their antibacterial activities against test pathogens.

4. Conclusion

In this study, Chitosan-silver nanocomposite films were synthesized by reduction method. Various characterisation techniques also confirmed the synthesis of chitosan-silver nanocomposite films. Crosslinked chitosan-silver nanocomposite film showed a slow silver release pattern relative to the un-crosslinked nanocomposite. The synthesized nanocomposite demonstrated higher antibacterial activity at higher concentration against some pathogens. Thus, Chitosan-silver nanocomposite films can be useful in different biological research and biomedical applications including delivery system (wound dressing).

References

[1] Cao, X. L., Cheng, C., Ma, Y. L., & Zhao, C. S. (2010). Preparation of silver nanoparticles with antimicrobial activities and the researches of their biocompatibilities. *J. Mater. Sci. Mater. Med.*, 21, 2861–2868.

[2] Jolanta, K., Malgorzata, C., Zbigniew, K., Anna, B., Krzysztof, B. (2010). Application of spectroscopic methods for structural analysis of chitin and chitosan. *Marine drugs.* Vol 8, pp 1567-1636.

[3] Kelner, A., & Schacht, E. (2005). Tailor-made polymers for local drug delivery: Release of macromolecular model drugs from biodegradable hydrogels based on poly (ethylene oxide). *J.Control. Release.*, 101, 13–20.

[4] Kiesow, A., Morris, J. E., Radehaus, C., & Heilmann, A. (2003). Switching behavior of plasma polymer films containing silver nanoparticles. *J. Appl. Lett.*, 94, 6988–6990.

[5] Lu, S., Gao, W., &Gu, H. Y. (2008). Construction, Application and Biosafety Silver Nanocrystalline Chitosan Wound Dressing. *Burns*, 34(5), 623–628.

[6] Ochei, J. & Kolhatkar, A. (2008) Y. pp. 778-817, Medical Laboratory Science: Theory and Practice.

[7] Sarkar, A., Kapoor, S., & Mukherjee, T. (2005). Preparation, characterization, and surface modification of silver nanoparticles in formamide. *J Phys Chem,*109, 7698– 7704.

[8] Shin, H. S., Yang, H. J., Kim, S. B., & Lee, M. S. (2004). Mechanism of growth of colloidal silver nanoparticles stabilized by polyvinyl pyrrolidone in γ-irradiated silver nitrate solution. *J. Colloid Interface Sci.*, 274, 89–94.

[9] Xu, X., & Zhou, M. (2008). Antimicrobial gelatin nanofibers containing silver nanoparticles. *Fibers Polym*, 9, 685–690.

[10] Zeng, W., & Fang, C. X. (2004). Novel Method of Preparing

Microporous Membrane by Selective Dissolution of Chitosan/Poly Ethylene Glycol Blend Membrane. *Journal of Applied Polymer Science*, 91(5),2840–2847

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The Effect of Drying Methods on the Chemical Composition of the Essential Oil of *Caesalpinia Pulcherrima* Growing in Lagos, Nigeria

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Abstract: This research studied the effects of different drying methods on the yield and chemical composition of hydrodistilled essential oil from the red variety of leaves of Caesalpinia pulcherrima. A total of 26, 23, 30 and 25 compounds were identified in the oils of the fresh, air-dried, sun-dried and oven-dried plant materials, respectively. In general, the air-dried plant yielded more essential oils than the fresh, sun-dried and oven-dried plant. The air-dried, sun-dried and oven-dried plant materials yielded 0.90 %, 0.20 % and 0.58 % of the essential oils, respectively whereas the fresh plant materials yielded oils of 0.63 %. The essential oils of Caesalpinia pulcherrima were composed mainly of sesquiterpenoids. The fresh leaf oil comprises caryophyllene, 15.51 %; α-cadinol 14.36 %; γ–muurolene, 13.28 %; nerolidol 8.32 % as the most prominent components. While, the major components common to the different drying methods are air-dried; (phytol, 12.28 %; copaene, 9.07 %; γ-pyronene, 8.95 %; neryl propanoate, 6.55 %), sun-dried (neryl propanoate, 8.18 %; copaene, 5.49 %; phytol, 4.72 %; γ-pyronene, 0.87 %), oven-dried (copaene, 18.77 %; neryl propanoate, 7.61 %; γ-pyronene, 4.59 %; phytol - 3.25 %). This results showed that similar major compounds were present in all the dried leaf oils but at varying quantities, whereas, they all differ from the major compounds of the fresh leaf oil. This disparity may be due to chemical transformation of the components in the different environments exposed to for moisture removal.

Keywords: Caesalpinia pulcherrima, Leaf oil, Sesquiterpene, Phytol, Copaene, γ-Pyronene

1.0 Introduction

Caesalpinia pulcherrima is a species of flowering plant in the pea family, Fabaceae, it is native to the tropics and subtropics of the Americas. It could be native to the West Indies, but its exact origin is unknown due to widespread cultivation [1]. Common names for this species include Poinciana, Peacock Flower, Red Bird of Paradise, Mexican

Bird of Paradise, Dwarf Poinciana, Pride of Barbados, and flamboyant-de-jardin [2]. In Nigeria, it is commonly known as Eko-omode by Yorubas, Waken Bature by Hausas and Nwayi/Nwoke Ibem by Igbos [3]. The seeds of Caesalpinia species are poisonous at maturity but some are edible before they reach maturity (e.g. immature seeds of C. pulcherrima) or after treatment (4). In India, leaves of C. pulcherrima are traditionally used as purgative, tonic and antipyretic, while roots extracts are used in the treatments of convulsions, intermittent fevers, lung and skin diseases [5]. In the Amazon rain forest, the juice from the leaves and flowers are used for the treatments of fever and sores while the seeds are used to cure bad cough, breathing difficulty and chest pain [6]. Four grams from the root is also reported to induce abortion in the first trimester of pregnancy [7][8]. It is a striking ornamental plant, widely grown in domestic and public gardens in warm climates with mild winters, and has a beautiful inflorescence in yellow, red and orange. In this work, we present the effect of drying methods on the chemical composition the leaf essential oil of the red variety of C. pulcherrima growing in Lagos state, Nigeria.

2.0 Materials and Methods

2.1 Isolation of the essential oil

Fresh leaves of Caesalpinia pulcherrima were collected at mid-day in the wet season of 2015 on the campus of University of Lagos, Lagos State, Nigeria. The plant was taxonomically identified and authenticated at the Herbarium of the Department of Botany of the University of Lagos (LUH 6392). Prior to hydrodistillation, the plant was dried by air, sun and the oven and pulverized. About 350 g each of the fresh and dried leaves of the plant were separately subjected to hydrodistillation for 4 h, using a Clevenger-type apparatus [9]. The oils were dried over anhydrous sodium sulfate and stored in a sealed vial prior to analysis.

2.2 GC-MS analysis of the essential oil

The analysis of the oils was carried out using a GC (Agilent Technologies 7890A) interfaced with a mass selective detector (VLMSD, Agilent 5975C) equipped with a non-polar Agilent HP-5MS (5 %-phenyl methyl polysiloxane) capillary column (30 m × 0.32 mm i.d. and 0.25 μm film thickness) with Injector series (Agilent, 7683B). The carrier gas was helium with linear velocity of 1 ml/min. Oven temperature was set at 80 °C for 2 minutes, then programmed until 120 °C at the rate of 5 °C/min withhold time of 2 minutes, and finally increased to 240 °C at the 10 °C/min rate, isothermal at the temperature for 6 min hold time. Injector and detector temperatures were 300 °C and 200 °C respectively. Injection mode, split less, volume injected, 1 μl of the oil. The MS operating parameters were as follows: Ionization potential, 70 eV; interface temperature, 200 °C and acquisition mass range; 50-800. Relative percentage amounts of the essential oil components were evaluated from the total peak area (TIC) by apparatus software.

2.3 Identification of components

The components of the oils were identified by matching their mass spectra and retention indices with those of the Wiley 275 library (Wiley, New York) in the computer library and literature [10]. The yield of each

component was calculated per kg of the plant material, while its percentage composition was calculated from summation of the peak areas of the total oil composition.

3.0 Results and Discussion

The fresh, air-dried, sun-dried and oven-dried leaves yielded 0.63 %, 0.90 %, 0.20 % and 0.58 % of the essential oils, respectively. GC-MS analysis of the leaf essential oil of C. pulcherrima (Table 1) revealed a total of 26, 23, 30, and 25 compounds in the fresh, air-dried, sun-dried and oven-dried leaf oils, respectively.

The four essential oils were dominated by sesquiterpenoids (54.20 % – 70.0 %). The predominant compounds in the fresh leaf oil were caryophyllene (15.51 %), α-cadinol (14.36 %), γ–muurolene (13.28 %), nerolidol (8.32 %). The major components of the air-dried leaf oil were phytol (12.28 %), coapaene (18.77 %), neryl propanoate (6.55 %) and caryophyllene oxide (6.54 %), γ-elemene (6.39 %). The sun-dried leaf oil comprises of neryl propanoate (8.18 %), α-muurolene (6.20 %), coapaene (5.49 %), (-)-neoclovene-(I), dihydro (4.98 %), phytol (4.72 %) and γ-elemene (4.70 %). And in the oven-dried leaf oil copaene (9.07 %), neryl propanoate (7.61 %), viridiflorol (7.42 %), γ-pyronene (4.59 %) and α-Fenchene (4.08 %) predominates. The result revealed that the significant components identified in all the oils were phytol, copaene, caryophyllene and neryl propanoate, although in varying quantities (Table 1, Figure 1). The most abundant components differ, air dried (Phytol, 12.28 %), sun dried (neryl propanoate 8.18 %), and oven dried (copaene, 18.77 %), while the fresh leaf oil had caryophyllene (15.51 %) as the major compound.

In this research, the sun dried leaves had the most compounds, but the least yield. This could be due to the conversion of the components in the fresh plant materials to other components not present naturally in the plant. The low yield may be due to losses which may have taken place due to the high drying temperature. Asekun et al., (2005/2006) [11] reported that the volatile oil from fresh L. leonurus aerial part (0.39%) increased during air-drying and oven-drying processes to 0.62 % and 0.71 % respectively, whereas sun-drying of the plant part caused a decrease in yield to 0.30 %.

According to literature, Ogunbinu et al., (2010) reported that of the 17 components identified in the air-dried leaf oil of C. pulcherrima, α-phellandrene (36.5 %), p-cymene (15.3 %) and γ-terpinene (7.9 %) were the major components [12]. Usman et al., (2012) [13] analysed air-dried leaves of red and yellow varieties of Caesalpina pulcherrima, the yields were 0.50 and 0.52 %, the GC and GC-MS analyses of the oils revealed the abundance of α-terpinene (44.4 %) and citronellal (58.0 %) in the oils of red and yellow varieties, making the oils γ-terpinene and citronellal chemotypes respectively. The essential oil of C. pulcherrima composed majorly of sesquiterpenoids in contrast to other reports that has the dominance of monoterpenoids. The presence of phytol, a diterpenoid in significant quantity in the oils is noteworthy as it was absent in the oils earlier reported [12] [13].

There is reduction in the concentration of copaene from 18.77 % to 2.31 % and an increase in content of phytol from 3.25 % to 12.28 % in the fresh, air, oven and sun dried oils. The different drying methods exposes the components to conditions that enable oxidation, reduction, cyclisation and direct conversion of these components to other forms. The reduction of the composition of copaene and increase in phytol content may be due to their chemical transformation into other components as a result of varying ways of moisture removal. The results show that the composition of the oils was significantly affected by the drying methods employed.

Table 1. Chemical composition of the essential oils from *Caesalpinia pulcherrima* leaves using different drying methods.

S/N	COMPOUND	Fresh (%)	Air dried (%)	Sun dried (%)	Oven dried (%)
1	Isopentyl hexanoate	-	0.83	1.19	-
2	Santolina triene	-	0.24	-	-
3	Elemene	0.33	-	-	-
4	γ-Pyronene	-	4.59	0.87	8.95
5	*Allo* aromadendrene	0.29	-	-	-
6	γ-Elemene	-	-	4.70	6.39
7	Copaene	2.31	**18.77**	5.49	9.07
8	β-Copaene	4.36	-	-	-
9	Calarene	-	-	3.64	0.69
10	Cis-β-farnesene	4.77	-	-	-
11	α-Muurolene	-	-	6.20	-
12	γ –Muurolene	13.28	-	2.10	-
13	δ-Cadinene	4.78	1.74	1.61	1.52
14	Cubebene	0.32	-	2.18	0.09
15	Valencene	-	-	0.45	-
16	Elixene	0.33	-	-	-
17	α –Calacorene	-	1.78	4.68	1.00
18	Cubenol	3.42	-	-	-
19	Ledane	-	0.25	-	-
20	γ-Terpinene	0.43	-	-	-
21	γ-Caryophyllene	-	3.15	-	-
22	Nerolidol	8.32	0.82	2.64	5.24
23	γ-Gurjunene	2.01	-	0.53	-
24	(+)-2-Carene, 4-.alpha.-isopropenyl-	-	2.03	-	-
25	α-Cadinol	14.36	-	2.97	-
26	α-Himachalene	-	1.79	-	-
27	Terpinen-4-ol	0.08	-	-	-
28	Aromadendrene	-	1.34	0.78	-
29	Bicyclo[5.3.0]decane,2-methylene-5-(1-methylvinyl)-8-methyl-	-	-	-	1.24
30	L-α-Terpineol	0.11	-	-	-
31	8-(2-Acetyloxiran-2-yl)-	-	4.10	-	2.44

	6,6-dimethylocta-3,4-dien-2-one.				
32	Linalool	0.28	-	-	-
33	β-Selinene	-	-	1.44	-
34	3-Thujene-2-one	0.04	-	-	-
35	β-Caryophyllene	-	-	-	2.17
36	Caryophyllene	**15.51**	-	-	1.17
37	3-Buten-2-ol, 2-methyl-4-(1,3,3-trimethyl-7-oxabicyclo[4.1.0]hept-2-yl)-	-	6.69	-	-
38	Caryophyllene oxide	1.71	-	2.33	6.54
39	Viridiflorol	-	7.42	2.99	-
40	Pinane	-	1.96	-	-
41	β-Ylangene	1.12	-	-	-
42	Sativene	1.00	-	-	-
43	Farnesol (E), methyl ether	-	-	5.18	2.37
44	Tricyclo[4.3.1.1.(3,8)undecane-3-carboxylic acid,methyl ester	-	3.25	-	-
45	Spatunelol	2.95	-	-	-
46	Isoaromadendrene epoxide	-	-	3.70	2.23
47	Neryl propanoate	-	7.61	**8.18**	6.55
48	Aromadendrene oxide(2)	-	5.30	2.29	1.73
49	Aromadendrene(1)	-	4.80	-	-
50	1-Methyl-6-(3-methylbuta-1,3-dienyl)-7-oxabicyclo[4.1.0]heptane	-	3.89	4.21	-
51	Patchoulane	-	-	3.53	5.31
52	Cedrene epoxide	-	-	4.13	-
53	2-Pentadecanone,6,10,14-trimethyl-	-	-	1.93	-
54	Humulane-1,6-diene-3-ol	0.96	-	-	-
55	γ-Neoclovene	-	-	-	2.85
56	(-)-Neoclovene-(I), dihydro	-	-	4.98	3.78
57	(-)-Isolongilofol methyi ether	-	-	-	1.31
58	Fenchol	-	-	-	3.81
59	Fenchone	-	-	4.82	-
60	Globulol	1.57	-	-	-
61	Epiglobulol	1.19	-	-	-
62	Phytol	-	3.25	4.72	**12.28**
63	Acetic acid, 2-acetoxymethyl-1,2,3-trimethyl ester	-	-	-	1.19
64	(Z)6,(Z)9-Pentadecadien-1-ol	-	1.15	-	1.27
65	1,5-Cyclodecadiene, (E,Z)-	-	-	0.56	-

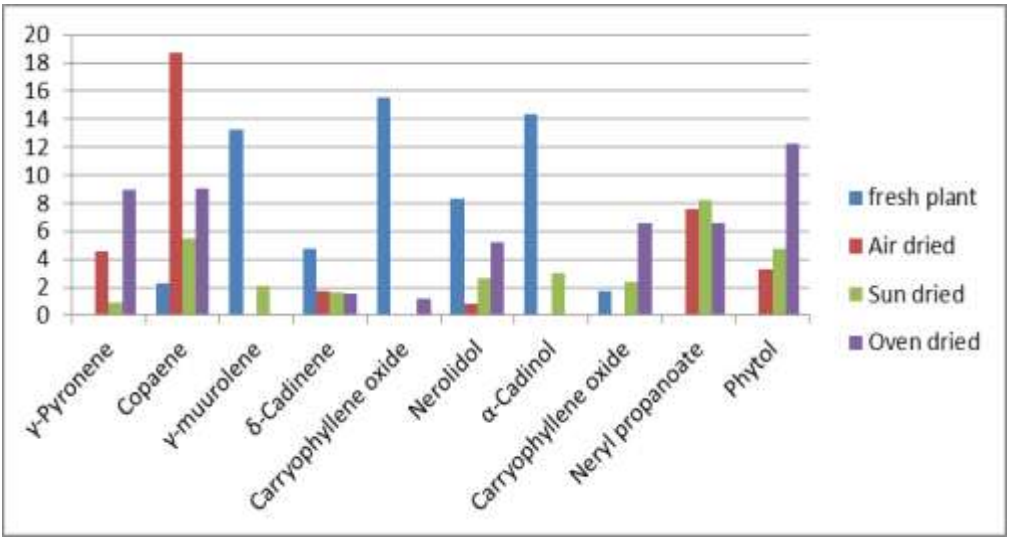

Figure 1: Chemical composition of the major essential oil components from *Caesalpinia pulcherrima* leaves using different drying methods.

Conclusion

The different drying methods affected the yield and chemical composition of the essential oils of *C. pulcherrima.* Air drying method gave the highest oil yield and all the major components of the leaf oil were also identified, hence it may be considered as the best method of drying the leaves of *C. pulcherrima.*

References

1. "Taxon: *Caesalpinia pulcherrima* (L.) Sw.". Germplasm Resources Information Network. United States Department of Agriculture. 2004-03-26. Retrieved 2010-12-03.
2. Burkill H. M. (1997). *The useful plants of West Tropical Africa*, Vol *6*, Royal Botanical Garden, Kew, UK.
3. Londa S. (2004). Plants and empire: colonial bio prospecting in the Atlantic world. Cambridge, mass; Harvard University press, p.4
4. Nelson, L. Shih, R. D. and Balick, M. J. (2007). Handbook of Poisonous and Injurious Plants. Co-published with The New York Botanical Garden. ISBN 978-0-387-31268-2.
5. Gautum, R, Sakiani A and Jachak, S.M. (2007). Indian medicinal plant as a source of antimicrobial agent. *Journal of Ethnopharmacolology*, **110**:200-234.
6. Kumar, D., Singh, J., Baghotia, A. and Kumar, S. (2009). Anti convulsant effect of ethanol extract of *C. pulcherrima* ,(L.) Sw.fabaceae, leaves. *Brazilian Journal of Pharmacology*, **20**(5) :1410-1414.

7. Schiebinger, Londa L. (2004). Plants and empire: Colonial bioprospecting in the Atlantic world. Cambridge, Mass.: Harvard University Press. p. 4. ISBN 978-0-674-01487-9.
8. Frisch, J.D. and Frisch, C.D., (2005). *Aves Brasileiras e Plantas que as atraem,* São Paulo: Dalgas Ecotec, 398, ISBN 978-85-85015-07-7.
9. U.S Pharmacopeia National Formulary (2007). The Official Compendia of Standards ***1***, 200-202.
10. Adlard, E. R. and Alan, J. (2001). Gas Chromatographic Techniques and Applications. Sheffield Academic, London, pp: 3-7.
11. Asekun, O. T. Grierson, D. S. and Afolayan, A. J. (*2005/2006)*. Influence of Drying Methods on the Chemical Composition and Yield of the Essential Oil of *Leonotis Leonurus Journal Sci. Res. Dev., **10**: 61-64*
12. Ogunbinu, A. O., Okeniyi, S., Flamini, G., Cioni, P. L., and Ogunwande, I. A. (2010). Monoterpenoid constituents of the volatile oils of *Cynometra megalophylla* Harms., *Caesalpinia pulcherrima* L. Swartz and *Pachylobus edulis* G. Don., growing in Nigeria. *Journal of Essential Oil Research*, **22**(6), 536-539.
13. Usman, L. A., Zubair, M. F., Olawore, N.O., Ashamu, M., Ismaeel, R.O. and Oladosu, I.A.. (2012). Chemical constituents of leaf essential oils of two varieties of *Caesalpinia pulcherrima* Linn growing in north central Nigeria. *Elixir of Organic Chemistry* **44,** 7085-7087.

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Phytochemical Screening and Antimicrobial Studies of *Crateva adansonii* Leaf Extract

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Abstract: Diverse challenges of microbial infections and upsurge of multi-drug resistant microbes informed the investigation into the phytochemical and antibacterial properties of *Crateva adansonii*. Cold extraction was carried out using methanol solvent. The crude extract of *Crateva adansonii* was fractionated into the *n*-hexane, methanol and chloroform layers successively. The phytochemical screening indicated the presence of alkaloids, saponins, terpenoids, flavonoids and cardiac glycosides. The antimicrobial assay showed that, for *Bacillus* spp, the organism was sensitive to the chloroform fraction of leaf extract at 1.562 mg/ml. For *Micrococcus varians*, result showed organism was sensitive to the crude extract at 3.125 mg/ml. According to the result of antifungal screening, the *n*-hexane fraction and crude extract showed activity against *Aspergillus niger* at 12.500 mg/ml and 3.125 mg/ml respectively. From these results, the crude extract of the leaf of *Crateva adansonii* shows activity against both bacteria and Fungi; hence, it may might be a good source of new drug for treating infections caused by these pathogens.

Keywords: Phytochemicals, antimicrobial, antibacterial assay, inhibitory concentration.

1.0 Introduction

Plants have been a major source of food and energy for man. The various ways to which plants have been of immense benefit ranges from feeding, tonic, treatment of illnesses, laxative, energy booster, preventive as well as curative (Moharam and Ali, 2012). The specific plants to be used and methods of application for particular ailments were passed down through oral history (Robert *et al.*, 2003). *Crateva adansonii* is a deciduous tree with grey, very smooth bark. It is called the sacred

garlic pear and temple plant. The plants' common name in Hausa is 'Ungundu' in Yoruba, it is 'eegun-orun'; in Igbo, it is called 'Amakarode' (Peter, 1997). The plant belongs to the family *Capparidaceae* (Ryan and Ray, 2004). The wood is soft and yellow, having a strong smell when exposed via cut. It is a small tree that grows up to 3-10 m tall with 3 foliated leaves. Studies have shown that *C. adansonii* has tonic properties; has a counter-irritant effect for headache; applied for rheumatic conditions after powdering and boiling in oil (Ainslie, 1937). The powdered leaves are applied to cysts and swellings, while the bark is used for treating sterility (Kerhero & Adam, 1974). According to Burkill (1985) and Tsado *et al*. 2015) its leaves are used to treat ear infections. In Senegal, the roots are used in the treatment of syphilis, jaundice and yellow fever Ayodeji *et al.* (2011). The aim of the work is to identify medicinal plants that are active against infectious diseases. The phytochemical screening will reveal the secondary metabolites present while the antibacterial studies will give insight into the range of microorganisms which the plant are active against.

2.0 Materials and Methods

2.1 Collection of Plant Samples

Fresh leaves of C. *adansonii* plant was collected in Iyesi village, within Ota environment, Ogun State, Nigeria, in the early hours of the day, during the rainy season between June and October 2015. Taxonomic identification and confirmation were carried out by a botanist at the Forestry Herbarium, Ibadan with No. FHI 110016. The test bacteria, which were environmental organisms, *Bacillus* spp, *E.coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Micrococcus varians*, Serratia spp, *Aspergillus niger* were sourced from the Department of Biological Sciences College of Science and Technology, Covenant University, Ota, Ogun State, Nigeria. The bacteria strains were maintained at 0.5% on Nutrient agar at 4 ^{o}C.

2.2 Preparation of Plant Extracts

Fresh leaves were hand-plucked and cleaned for debris using tap water while distilled water was used in rinsing the leaves. The leaves were air-dried in the shade at room temperature for two weeks. Dried leaf samples were pulverized. 100 g of pulverized plant materials was weighed and soaked in 500 ml of methanol and left for 72 hours in order to prepare the extracts. Whatman number 1 filter paper was used to filter the extracts and filtrates were concentrated under vacuum below 40 ^{o}C using rotary evaporator. The crude extract was partitioned into aqueous, chloroform, hexane and methanol fractions by liquid-liquid extraction.

2.3 Determination of Phytochemical Constituents

The phytochemical constituents of the plant leaves were screened qualitatively using the Standard steroids procedures described by Sofowora (1993), Trease and Evans (1989) and Harborne (1973). Screening involved tests for alkaloids, saponins, tannins, flavonoids and terpenoids.

2.4 Antibacterial Activity Bioassay

The extracts were evaluated for antibacterial activity against *Bacillus* spp, *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Micrococcus varians*, *Serratia* spp, *Aspergillus niger*,

using the methods of Ogbulie *et al.* (2007); Doughari and Manzara (2008).

2.5 Determination of Minimum Inhibitory Concentration (MIC)

Determination of minimum inhibitory concentration (MIC) was carried out on the leaf of *Crateva adansonii,* using the method described by Mahesh and Satish (2008). The procedure was carried out on the different fractions of *C. adansonii* leaves that showed sensitivity against the growth of some selected organisms from the microbiology laboratory and Applied Biology and Biotechnology unit of Covenant University, Ota, Ogun State, Nigeria. The medium used was nutrient agar which was prepared according to the manufacturers' standard. Extracts concentrations, the chloroform, methanol and hexane fractions obtained from fractionation were adjusted to 50, 25, 12.5, 6.25, 3.125 and 1.562 mg/ml by serial dilution method. The sterile nutrient agar plates were seeded using swab sticks with the test organisms or isolates of 0.5% Mcfarland standard and were properly labeled. Wells were bored into the sterile nutrient agar plates, with the aid of a sterile cork borer of 9 mm diameter. With sterile 1ml pipettes, 0.2 ml of each extract of different concentrations was dispensed into the already bored wells on the inoculated nutrient agar plates and plates were incubated at 37^{o}C for 24 hours, after which they were observed for growth or death of the test isolates or organisms. The lowest concentration inhibiting growth was taken as the MIC. The antibiotic used was Gentamycin.

3.0 Results

The result of the qualitative phytochemical screening revealed the presence of Alkaloids, Saponins, Terpenoids, Flavonoids and Cardiac glycosides (Table 1). This report revealed that the aqueous, methanol and crude extracts contain alkaloids, while all solvents showed the presence of saponins. However, the *n*-hexane extract revealed the trace presence of terpenoids, flavonoids and cardiac glycosides.

Table 1: Phytochemical Analysis for the Leaves of Crateva adansonii

Phytochemical	Aqueous	Chloroform	Methanol	*n*-Hexane	Crude
Alkaloids	++	-	++	-	++
Saponins	+	++	++	++	++
Terpenoids	-	-	-	+	-
Flavonoids	-	-	-	+	-
Cardiac glycosides	-	-	-	+	++

Key: - is Absent, + is Present, ++ is Significant and +++ is Very significant

Table 2: Antimicrobial Activity of Leaf Extracts of *C. adansonii* (mm)
(Zones of inhibition measured in mm)

Fraction	*Bacillus* spp	*E. coli*	*S.aureus*	*P. aeruginosa*	*Serratia* spp	*M. varians*	*A. niger*	Yeast
n-Hexane	16	-	-	-	-	10	20	-
Methanol	-	-	-	25	-	-	-	-
Chloroform	25	-	-	23	-	10	-	-
Aqueous	-	-	-	16	-	-	-	-
Crude	-	15	-	17	-	25	20	-

B. spp = Bacillus sp.; E. coli = Escherichia coli; S. aureus = Staphylococcus aureus; P. aeruginosa = Pseudomonas aeruginosa; M. varians = Micrococcus varians; A. niger = Aspergillus niger.

The result from Table 2 showed that the methanol, chloroform and crude extracts exhibited the highest zone of inhibition (25 mm) for *P. aeruginosa*, *Bacillus spp.* and *Micrococcus varians* respectively. The chloroform fraction showed zone of inhibition (23 mm) for *P. aeruginosa*, while the *n*-hexane, and crude fraction exhibited zone of inhibition at 20 mm for *A. niger*. The antimicrobial activity demonstrated by the leaf extract against some bacterial pathogens could be due to some bioactive constituents of the extracts.

Table 3: Result of Minimum Inhibitory Concentration in mg/ml of Leaf Extracts of *C. adansonii* in *(mg/ml)*

Fractions	*P. aeruginosa*	*S. aureus*	*M. varians*	*E. coli*	*Bacillus* spp	*Serratia* spp	*A. niger*
Chloroform	-	-	-	-	1.562	-	-
Methanol	-	-	-	-	-	-	-
n-Hexane	-	-	-	-	-	-	12.5
Crude	-	-	3.125	-	-	-	3.125

B. spp = Bacillus sp.; E. coli = Escherichia coli; S. aureus = Staphylococcus aureus; P. aeruginosa = Pseudomonas aeruginosa; M. varians = Micrococcus varians; A. niger = Aspergillus niger

The result from Table 3 showed that the MIC value of *C. adansonii* was 1.562 mg/ml for the chloroform extract against *Bacillus spp*. and 12.5 mg/ml for the *n*-hexane extract against *Aspergillus niger,* while the crude extract was 3.125 mg/ml against *M. varians* and *Aspergillus niger.* The crude, hexane and chloroform fractions have shown some lethal effect on the test organisms as indicated by the values of the median lethal dose, $LD_{50.}$

Table 4: Antimicrobial Activity of the Organic Chemicals Used (mm)
(Zones of inhibition measured in mm)

Fraction	*Bacillus spp*	*E.coli*	*S.aureus*	*P. aeruginosa*	*Serratia spp*	*M. varians*	*A. niger*	Yeast
n-Hexane	-	10	-	10	-	-	-	-
Methanol	-	11	-	10	-	-	-	-
Chloroform	-	12	-	11	-	-	-	-
Aqueous	-	-	-	-	-	-	-	-

B. spp = Bacillus sp.; E. coli = Escherichia coli; S. aureus = Staphylococcus aureus; P. aeruginosa = Pseudomonas aeruginosa; M. varians = Micrococcus varians; A. niger = Aspergillus niger

The results from table 4 shows that the test organisms used were not sensitive to the organic chemicals when used alone without the fractions from the leaf of *C. adansonii*. The observed values shown for *E. coli* and *P. aeruginosa* indicated resistance to the organic chemicals. This implies that it is only the dissolved fractions that showed activity on the test organisms.

4.0 Discussion

Phytochemicals are known to be responsible for the pharmacological and toxic activities of plants (Lawal *et al*., 2005). Plants are known to produce phytochemicals so as to defend them against predators; however, studies have also shown that they can be used to protect human against diseases. The results from the phytochemical analysis of *Crateva adansonii* is in agreement with previous studies: Agbankpe *et al*, (2015); Borokinni and Omotayo, (2012). Report has shown flavonoids to exhibit anticarcinogenic potentials due to their antioxidant and anti-inflammatory properties. Likewise, saponins are used as adjuvant in the production of vaccines (Asl and Hosseinzadeh, 2008). Alkaloid has been used as CNS stimulant and powerful painkillers, among other uses. The cardiac glycoside has been used for over two centuries as stimulant in cases of cardiac failure and diseases. Igboko, (1983) reported that plants containing tannin have been used for healing of wounds, hemorrhoids and burns in herbal medicine.

The antibacterial analysis shows that the crude, chloroform and methanolic fractions contains bioactive constituents which can effectively inhibit the growth of some microorganisms. This result confirms previous studies by Agbankpe *et al*, (2016) and Tsado *et al*, (2016).

5.0 Conclusion

This study reveals that *C. adansonii* is a highly potent plant which lends credence to its traditional use as a medicinal plant. However, further studies are currently being carried out on the anti-pyretic, anti-hypertensive, anti-malaria, anti-tubercular and analgesic activities of *C. adansonii* for a better bioactivity profiling and documentation for possible future drug design.

References

Agbankpé A.J., Bankolé S.H., Dougnon T.J., Yèhouénou B., Hounmanou Y. M.G., Baba-Moussa L.S. (2015). Comparison of Nutritional Values of *Vernonia amygdalina*, *Crateva adansonii* and *Sesamum radiatum*: Three Main Vegetables Used in Traditional Medicine for the Treatment of Bacterial Diarrhoea in Southern Benin (West Africa), *Food and Public Health*, 5(4): 144-149.

Agbankpé A.J., Dougnon T.V., Bankole S.H., Houngbegnon O., Dah-nouvlessounon D., and Baba-Moussa L. (2016) In Vitro Antibacterial Effects of *Crateva adansonii*, *Vernonia amygdalina* and *Sesamum radiatum* used for the Treatment of Infectious Diarrhoeas in Benin. *J. Infect. Dis. & Ther.* 4(3): 281.

Ainslie J.R. (1937). A list of Plants Used in Native Medicine in Nigeria. Oxford University Press, London, p. 54.

Ayodeji A.A., Anthony A.A and Mumuni A.M. (2011). Evaluation of the antimicrobial activities of crude extract of *Cryptolepiss anguinolenta* and *Crateva adansonii* leaves and their interactions. *J. Applied Pharmaceut. Sci.*, 1: 85-89.

Asl M.N. and Hosseinzadeh H. (2008). Review of pharmacological effects of *Glycyrrhiza*sp. and its bioactive compounds. *Phytother. Res.*, 22: 709-724.

Burkill H.M. (1985). The useful plants of West Tropical Africa. 2nd Edition. Volume 1, Families A–D. Royal Botanic Gardens, Kew,
Richmond, United Kingdom. 960 pp.

Doughari J.H. and Manzara S. (2008). In vitro antibacterial activity of crude leaf extracts of *Mangifera indica Linn. African Journal of Microbiology Research*, 2: 067–072.

Harborne J.B. (1973). Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis. 3rd Edn., Chapman and Hall, London, pp: 279.

Igboko D.O. (1983). Phytochemical studies on *Garcinia kola.* M.Sc. Thesis, University of Nigeria, Nsukka.

Kerharo J., Adam J.G. (1974). La Pharmacopée sénégalaise traditionnelle-Plantes medicinal esettoxiques. Paris Edition, Vigot–Frères, Volume 1, 1011 pages.

Lawal M., Wasagu R.S.U. and Ladan M. J. (2005). Hepatotoxicity risk assessment of neem (*Azadirachta indica*) seed extract using albino rats. *Biol. Environ. Sci. J. Tropics*, 2: 36-38.

Moharam M.H.A., Ali H.A.O. (2012). Preventative and Curative Effects of Several Plant Derived Agents against Powdery Mildew Disease of Okra. *Notulae Scientia Biologicae* 4(3): 76-82.

Ogbulie J.N., Ogueke C.C. and Nwanebu F.C. (2007b). Antibacterial properties of *Uvariachamae*, *Congronema latifolium*, *Garcinia kola*, *Venonia amygdalina* and *Aframomium melegueta*. *African Journal of Biotechnology*. 13: 1549 – 1553.

Ogbulie J.N., Ogueke C.C., Okoli L. C. and Anyanwu B.N. (2007a). Antibacterial activities and toxicological potential of crude ethanolic extract of *Euphorbia hirta*. *African Journal of Biotechnology*. 6(13): 1544 – 1548.

Peter N., Villages M. (1997) Plants as a source of medicine. *Herbal network.*14:1-8.

Roberts C.W., McLeod R., Rice D.W., Ginger M., Chance M.L., Goad L.J. (2003). Fatty acid and sterol metabolism: potential antimicrobial targets in apicomplexan and *trypanosomatid* parasitic protozoa. *Molecular Biochemical Parasitology*. 126(2): 129-142.

Ryan K.J., Ray C.G. (2004). *Sharris Medicinal Microbiology*, 4th Edn., Mc. Graw Hill, New York. 8-85.

Sofowora A. (1993). Medicinal Plants and Traditional Medicines in Africa. Chichester John Wiley & Sons New York, pp: 97-145.

Trease G.E. and Evans W.C. (1989). Pharmacognosy. 11th Edn., Brailliar Tridel and Macmillian Publishers, London, pp: 48-65.

Tsado N.A., Lawal B., Santali E.S., Mohammed A.S., Balarabe M.M., Ibrahim H.A. and George J.J. (2015). Phytochemicals and Acute Toxicity Profile of Aqueous and Methanolic Extracts of *Crateva adansonii* Leaves in Swiss Albino Rats. *Asian Journal of Biochemistry*. 10: 173-179.

Tsado A. Ndarubu, Lawal Bashir, Ossai P. Chukwudi, Jagaba Aliyu, Gwadabe N. Kontagora, Jiya A. Gboke, Umar A. Muhammad and Oladunjoye J. Olabode, 2016. Antioxidants and Antimicrobial Activities of Methanol Extract of *Newbouldia laevis* and *Crateva adansonii*. *Journal of Pharmacy and Allied Health Sciences,* 6: 14-19.

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Assessment of Micro Flora, Deoxynivalenol (Don) and Fumonisin Contamination of Grains sold in Local Markets, Nigeria

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***Abstract*:** Fusarium the major deoxynivalenol (DON) and fumonisin producing species of fungi grow as a corn endophyte often without causing disease symptoms in plant. Climate changes resulting in appropriate weather conditions helps in Fusarium growth causing seedling blight, stalk rot and ear rot. Thus most grain contamination of Fusarium comes from the farm. This study was carried out to assess the micro flora and level of DON and fumonisin contamination in Zea mays, Sorghum bicolor, Triticum aestivum and Pennisetum americanum sold in Ota. Forty samples comprising ten of each grain type was analyzed, Fumonisin at concentration ≥4.0 mg/kg (ppm) was detected in eight samples of Zea mays and two samples of Triticum aestivum while DON at concentrations ≥1.25mg/kg (ppm) was detected in all the wheat samples using the Rida® Quick Fumonisin and DON test kits. The total aerobic plate count for the samples ranged from 2.0×104 to 8.4×106 cfu/g, fungal count ranged from 1.0×104 to 6.0×106 cfu/g while coliform count ranged from < 10 to 2.0×103 cfu/g. The predominant microbial isolates from the grains included species of Fusarium, Aspergillus, Mucor, Penicillium, Bacillus, Klebsiella, and Pseudomonas. Infection of grains by fungal species and contamination with mycotoxins can generally be influenced by favourable weather conditions. Measures to address climate changes, effective hazard analysis and critical control point (HACCP) and good storage system are advocated to prevent mould contamination and deleterious mycotoxin production in grains.

Keywords: Fumonisin, Deoxynivalenol, Micro flora, Mycotoxins, Coliform, Climate changes, HACCP

Introduction

Fungal contamination of food and feeds causes considerable economic losses due to damage to crops, discoloration, off-odors, off-flavors, reduced yields, loss of nutritive value and mycotoxin production. Mycotoxins are secondary metabolites of fungi some of which are

harmless and even helpful, serving as antibiotics and other clinical and industrial chemicals (Magen and Aldred, 2005). Some mycotoxins are toxic to animals and man having predilection for some organs of the body. The Food and Agriculture Organization estimates that 25% of the world food crops are affected by mycotoxins during growth and storage under a diverse range of climatic conditions and situations (FAO, 1979; Miller and Trenholmed, 1994). The accumulation of mycotoxins in foods and feeds represents a major threat to human and animal health as they are responsible for much different toxicity including the induction of cancer, mutagenicity, estrogenic and gastrointestinal disorders, urogenital, vascular, kidney, liver and nervous disorders (Pitt and Hocking, 2009).

Fumonisins constitute a group of carcinogenic metabolites mainly produced by Fusarium verticilloides, Fusarium proliferatum and Fusarium nygamai, as well as Alternaria spp, It has been associated with toxicities such as hepatotoxin and nephrotoxin in all animal species tested, it is implicated in elevated human esophageal cancer incidence in Africa, Asia and Central America (Franceschi et al., 1990; Marasas et al., 1991; Rheeder et al., 1992; Chu and Li, 1994) and with high incidence of liver cancer, neural tube defects and apoptosis in the liver. Fumonisins is associated with several diseases in animals including liver and kidney tumors in rodents, equine leukoencephalomalacia (ELEM) in horses, and acute pulmonary edema in pigs (Wild and Gong, 2010).

Deoxynivalenol toxicity in man is associated with abdominal pain, vomiting, headache, dizziness, fever, intestinal wall necrosis, anorexia and impaired antibody and immunoglobulin levels. In animals DON toxicity is usually linked with feed refusal, decreased feed conversion, altered nutritional efficiency, weight loss, vomiting, severe dermatitis, abortion, bloody diarrhea, hemorrhaging, and abnormal feathering, lesions at the edges of bird beaks, decreased egg production, decreased weight gain, anorexia, and death (Pestka et al., 2005;Yoshizawa and Morooka, 1977; Herrman et al., 2002; Forsyth et al., 1977; Rotter et al., 1994; Trenholm et al., 1984; Young et al., 1983).

The main objective of this research is to determine the microbial contaminants specifically mycoflora and fumonisin and deoxynivalenol contamination levels of some grains sold in Ota and also create awareness to the public at large.

Materials and Methods

Sample collection and preparation

Samples were collected from the popular Oja-Ota and Sango Ota markets, these outlets were chosen because they are the major market and are highly patronized by members of the public in the sampling area. Ten samples each of Corn (Zea mays), Millet (Pennisetum americanum), Wheat (Triticum aestivum) and Sorghum/Guinea corn (Sorghum bicolor) were purchased randomly from food vendors. The samples were aseptically collected in sterile polyethylene bags and transported same day to the laboratory for further analysis. Two hundred and fifty gram (250 g) of each grain samples

was ground using warring blender, this served as stock for all other analyses.

Microbiological analysis

One gram of the samples was homogenized in normal saline and diluted 10-1 to 10-5. Aliquot 0.1mL of the sample homogenates was spread inoculated onto Saboraud Dextrose agar plus Chroramphenicol (SDA+C) for fungal isolation; Nutrient agar was inoculated for total aerobic plate count (TAPC) and MacConkey agar for coliforms. One gram each of the ground samples was inoculated into lactose broth in caped test tubes with inverted Durham's tube, for coliform test. SDA + C was incubated at room temperature 28± 2°C for 3 to 5 days while other cultures were incubated for 24-48 h at 37°C.

Assay for Deoxynivalenol (DON) and Fumonisin

The RIDA® QUICK Deoxynivalenol (DON) and Fumonisin kits (R-Biopharm, UK) was employed. Following the manufacturer's instruction, the test kits were brought out of fridge and allowed to assume room temperature of 28±2°C. Aliquot 5 g and 1g of ground samples was respectively weighed into test tubes and 20 mL and 40 mL of extraction buffer (as contained in each test kits either for Fumonisin or DON) added and then manually shaken vigorously for 1-2 minutes and in a laboratory shaker (Jenway, UK) for another 2-3 minutes. The solution was filtered after 3-5 minutes sedimentation. The filtrates were used for the assays as applicable (Fumonisin or DON) and following the manufacturer's test instruction manual. The RIDA® QUICK DON and Fumonisin test is an antigen- antibody reaction, an immunochromatographic assay based on the principle of specific DON / Fumonisin antibody recognizing DON / Fumonisin molecules in the samples. The results are read visually following development of coloured bands. The test band is only visible in the presence of DON / Fumonisin in the sample. The control band is not influenced by DON / Fumonisin in the sample and should be present in all cases in order to prove that the test strip is valid. Results are considered positive that is the sample is contaminated with Fumonisin/DON if the control band/line is visible and the test band is also visible, mycotoxin level $\geq$ detection limit. Negative result is recorded if that the sample is free of DON/Fumonisn or below detection limit, if only the control band/line is clearly visible but test band invisible. Result is invalid if test band is clearly visible but control band is invisible.

Determination of Moisture Content of Samples

The standard procedure as described by AOAC (2000) was used. Samples were oven dried at 105°C for 2 hours to constant weight. Triplicate readings were recorded and the mean value was obtained.

Results

The mean microbial count of the samples and contamination of the samples by DON and Fumonisin is presented in table 1. It shows that all the samples had microbial contaminants. TAPC ranges from 2.0x 104 in Millet to 8.4x106 in Sorghum. Fungal count ranges from 1.0x104 in millet to 6.0x106 in Wheat. Coliform count ranged from < 10 to 2.0x103. However, only one maize sample had faecal

coliform contamination. The table also shows that eight samples of maize and two samples of wheat were contaminated with Fumonisn at concentrations ≥4.0 mg/kg while ten wheat samples had DON contaminations at levels ≥1.250 mg/kg. The sorghum and millet samples were neither contaminated with DON nor fumonisins. Table 2 shows the microbial isolates from the samples. Fungi specifically Aspergillus and Fusarium are the most prevalent in all the samples, while bacillus spp is the predominant bacterial contaminants. The percentage moisture contents of the grains ranged from 10-13% in Pennisetum americanum, 12-15% in Sorghum bicolor, 12-18% in Triticum aestivum to 16-23% in Zea mays.

Discussion

The microbial count in the samples which ranged from 104 to 106 for both TAPC and Fungal count cannot be considered as a challenge or treat because it is a raw food which will be processed by heat before consumption. Heat treatment is a control measure and could be a critical control point that will reduce the contaminants to insignificant level before consumption. Bacillus species are spore formers and common environmental contaminants, which could explain their presence in the grains. The presence of coliforms specifically faecal coliforms is an indication of contamination of some of the grains by faecal matter. This could be from the food vendors or from the environment in which grains are often spread to dry or displayed for sales. The low moisture contents of the grains could account for low contamination of the grains by bacteria except for bacillus which are known to produce resistant spores and Klebsiellae which are known to be hardy organisms. The presence of fungi in all the samples could be attributed to the normal flora of the plants. Fungi are spore bearers and common environmental contaminants that could have contaminated the grains from the field or during storage and display for sales.

The microbial contamination of a product has been reported to be dependent on the environment it passed through and to the sanitary quality observed during processing (WHO, 2008; Oranusi and Braide, 2012). Fungi are known to tolerate low water activity levels; this could also explain why they could survive in the grains with low moisture contents.

Fumonisins was detected in eight maize and two wheat samples at concentrations higher than accepted limits in food materials (FDA, 2001), this could be associated with the presence of Fusarium spp which are known to produce this mycotoxin. Fusarium spp are known to contaminate grains in the farm or in storage and under favourable conditions elaborate Fumonisins (Eugenia et al., 2014; Shephard et al., 2005).

The presence of DON in all the wheat samples in this research could be as a result of contamination of samples with Fusarium spp which are known to produce DON. Kushiro (2008) observed that Deoxynivalenol mycotoxin (DON) is one of several mycotoxins produced by certain Fusarium species (Fusarium graminearum, Fusarium culmorum, Fusarium proliferatum), that frequently infect corn, wheat, oats, barley, rice, and

other grains in the field or during storage. This makes it one of the most encountered mycotoxins. Pestka and Smolinski (2000); Herrman et al. (2002) reported the presence of DON in food and feeds. They observed that mycotoxin produced can withstand high temperatures of about 170◦C–370◦C; the danger resulting from this is that the toxin still remains in foods and feeds after basic culinary treatment.

Conclusion

Although the levels of microbial counts of the samples are considered to be at levels that will not pose any treat to consumer because the samples are raw materials that must be processed before consumption. The detection of Fusarium spp and consequent Fumonisin and DON at concentrations ≥ 4.0 mg/kg and 1.25 mg/kg respectively is a cause for concern since these mycotoxins can withstand processing temperature. Education of farmers and food vendors on the need for proper management of grains in the farm and during storage to prevent mould contamination is imperative. Consumers/ public enlightenment on the need to purchase good quality food items and application of GMP and HACCP in food production will be a step in the right direction.

Table 1 Mean microbial count cfu/g and assay for Fumonisin and DON mg/kg

Outlet/ Sample		Fungal count	Total aerobic Plat count	Coliform count	Fumonisin ≥4.0 mg/kg	DON ≥1.25 mg/kg
Outlet 1						
1)	Sorghum	5.0×10^4	3.0×10^6	$1.2x10^2$	-	-
2)	Maize	3.7×10^5	2.7×10^5	1.0×10^2	+	-
3)	Millet	3.6×10^5	2.0×10^4	NG	-	-
4)	Wheat	5.0×10^5	2.4×10^6	NG	-	+
Outlet 2						
5)	Sorghum	8.0×10^5	5.6×10^6	NG	-	-
6)	Maize	4.6×10^5	5.0×10^5	NG	+	-
7)	Millet	3.8×10^5	3.7×10^6	NG	-	-
8)	Wheat	5.3×10^5	2.4×10^6	$1.1x10^1$	-	+
Outlet 3						
9)	Sorghum	3.0×10^4	3.4×10^6	$1.3x10^2$	-	-
10)	Maize	3.5×10^5	3.5×10^5	NG	+	-
11)	Millet	1.5×10^5	3.2×10^5	NG	-	-
12)	Wheat	4.0×10^4	9.0×10^4	NG	-	+
Outlet 4						
13)	Sorghum	1.5×10^5	3.2×10^6	$2.0x10^3$	-	-
14)	Maize	3.3×10^5	7.0×10^4	NG	+	-
15)	Millet	5.0×10^5	4.0×10^6	NG	-	-
16)	Wheat	5.0×10^5	8.3×10^5	NG	-	+

Outlet 5						
17)	Sorghum	2.0×10^5	4.8×10^6	NG	-	-
18)	Maize	1.1×10^5	3.3×10^6	NG	+	-
19)	Millet	2.0×10^5	6.5×10^5	NG	-	-
20)	Wheat	4.5×10^5	5.4×10^5	NG	-	+
Outlet 6						
21)	Sorghum	1.0×10^5	3.4×10^6	NG	-	-
22)	Maize	1.6×10^5	2.4×10^5	NG	+	-
23)	Millet	2.8×10^6	4.5×10^6	< 10	-	-
24)	Wheat	6.3×10^4	6.4×10^5	NG	-	+
Outlet 7						
25)	Sorghum	2.6×10^5	7.9×10^4	NG	-	-
26)	Maize	2.9×10^5	2.4×10^5	NG	+	-
27)	Millet	1.0×10^4	2.1×10^6	$3.5x10^1$	-	-
28)	Wheat	1.6×10^5	8.0×10^6	NG	+	+
Outlet 8						
29)	Sorghum	4.0×10^5	8.4×10^6	NG	-	-
30)	Maize	4.7×10^4	3.8×10^5	NG	-	-
31)	Millet	2.0×10^4	2.6×10^6	NG	-	-
32)	Wheat	6.0×10^6	2.3×10^6	NG	-	+
Outlet 9						
33)	Sorghum	2.0×10^5	6.7×10^5	NG	-	-
34)	Maize	4.5×10^5	7.0×10^5	NG	+	-
35)	Millet	1.0×10^6	4.6×10^6	NG	-	-
36)	Wheat	5.0×10^4	3.5×10^6	$2.3x10^2$	+	+
Outlet 10						
37)	Sorghum	4.0×10^5	4.5×10^6	$2.9x10^2$	-	-
38)	Maize	2.5×10^4	5.2×10^5	NG	-	-
39)	Millet	1.2×10^4	2.2×10^6	NG	-	-
40)	Wheat	5.8×10^5	8.0×10^5	NG	-	+

KEY: NG: No Growth; -- = Not detected

Table 2 Microbial isolates from different grain samples

Grain sample	Microbial isolates
Maize	*Aspergillus niger*, *Fusarium* species, *Mucor mucedo*, *Penicillium* species Klebsiellae, *E. coli*, Bacillus spp
Millet	*Aspergillus* species, *Fusarium* species, Penicillium species, *Pseudomonas*, Bacillus spp.
Wheat	*Aspergillus* species, *Fusarium* species, Bacillus spp, *Pseudomonas*, Klebsiellae
Sorghum	*Aspergillus* species, *Fusarium* species, Bacillus spp, Klebsiellae

References

AOAC (2000). Association of Official Analytical Chemists. Official Methods of Analysis of AOAC International, 17th edition, AOAC Gaithersburg, MD, USA.

Chu, F. S. and Li, G. Y. (1994). Simultaneous occurrence of fumonisin B1 and other mycotoxins in moldy corn collected from People's Republic of China in regions with high incidences of esophageal cancer. *Applied and Environmental Microbiology* **60**: 847-852.

Eugenia, C. A., Maria, P. M., Sofia, A. P., Stella, M. C., Adriana, M. T. and Maria, C. F. (2014). Fumonisin occurrence in naturally contaminated wheat grain harvested in Argentina. *Journal of Food Control* **37**: 56-61.

FAO- Food Agriculture Organization of the United Nations (1979). Recommended practices for the prevention of mycotoxins. Rome. pp. 53-55.

FDA (2001). Guidance for Industry: Fumonisin Levels in Human Foods and Animal Feeds; Final Guidance. Center for Food Safety and Applied Nutrition Center for Veterinary Medicine. Federal Register, November 21, 2001, vol. 66, pp 56688-9.

Forsyth, D. M., Yoshizawa, T., Morooka, N. and Tuite, J. (1977). Emetic and refusal activity of deoxynivalenol to swine. *Applied Environmental Microbiology* **34**: 547–552.

Franceschi, S., Bidoli, E., Buron, A. E. and La-Vecchia, C. (1990). Maize and risk of cancer in the oral cavity, pharynx and esophagus in northeastern Italy. *Journal of the National Cancer Institute* **82**: 1407-1411.

Herrman, T. J., Trigo-Stockli, D. and Pedersen, J. R. (2002). Mycotoxins in feed grains and ingredients.

Manhattan, K. S: Cooperative Extension Service, Kansas State University.

http://www.ksu.edu/publications.com. Accessed May 25, 2015.

Magen, N. and Aldred, D. (2005). Conditions of formation of Ochratoxin A in drying transport and in different commodities. *Food Addit. Contamination* **22** (11): 10-16.

Miller, J. D. and Trenholmed, H. L. (1994). Mycotoxin in grain: Compound other than aflatoxin. *Animal Research and development* **19:** 7-37.

Pestka, J. and Smolinski, A. T. (2005). Deoxynivalenol: Toxicity and potential effects on humans. *Journal of Toxicology and Environmental Health* **8**: 39–69.

Pitt, J. I. and Hocking, A. D. (2009). Fungi and food spoilage (Third edition).Springer Dordrecht Heidelberg, London and New York. p. 50.

Oranusi, U. S. and Braide, W. (2012). Microbiological safety assessment of Apple fruits (*Malus domestica* Borkh) sold in Owerri Imo State Nigeria. *Advance J. Food Science and Technol*., 4 (2): 97-102.

Rheeder, J. P., Marasas, W. F. O., Thiel, P. G., Sydenham, E. W., Shephard, G. S. and Van-Schalkwyk, D. J. (1992). *Fusarium moniliforme* and fumonisins in corn in relation to human oesophageal cancer in Transkei. *Phytopathology* **82**: 353–357.

Rotter, B.A., Thompson, B.K., Lessard, M., Trenholm, H.L. and Tryphonas, H. (1994). Influence of low-level exposure to *fusarium* mycotoxins on selected immunological and hematological parameters in young swine. *Fundamental Applied Toxicology* **23**: 117–124.

Shephard, G. S., Westhuize, L. V., Gatyeni, P. M., Katerered, R. and Marasas, W. F. (2005). Do fumonisin mycotoxin occur in wheat. *Journal Agriculture Food Chemistry* **16** (23): 9293-9296.

Trenholmet, H.L., Hamilton, R. M., Friend, D. W., Thompson, B. K. and Hartin, K. E. (1984). Feeding trials with vomitoxin (deoxynivalenol)-contaminated wheat: Effects on swine, poultry, and dairy cattle. *Journal of American Vetinary Medical Association* **185**: 527–531.

WHO, Microbiological Harzards in fresh Leafy Vegetables and Herbs: Meeting Report. Microbiological Risk Assessment series No 14, Rome, 2008, 151-152.

Yoshizawa, T. and Morooka, N. (1977). Studies on the toxic substances in infected cereals; acute toxicities of new trichothecene mycotoxins; Deoxynivalenol. *Journal of Food Hygiene Society Japan* **15**: 261–269.

Young, L. G., Mc-Girr, L., Valli, V. E., Lumsden, J. H. and Lun, A. (1983). Vomitoxin in corn fed to young pigs. *Journal of Animal Science* **57**: 655–664.