European Journal of Material Science
Vol.7, No.1, pp.15-28, September 2020
Published by ECRTD-UK
Print ISSN: ISSN 2055-6551(Print), Online ISSN: ISSN 2055-656X (Online)

CHARACTERIZATION OF NANO-SIZED HYDROXYAPATITE PARTICLES SYNTHESIZED FROM FISH SCALES USING CALCINATION METHOD

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ABSTRACT: *Hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ material is one of the biomaterial used for orthopedic medicine which was successfully synthesized from Tilapia scales using calcination method in this study. The synthesized hydroxyapatite is white and in powder form. The synthesized hydroxyapatite was characterized by X-ray diffraction analysis which confirmed hydroxyapatite as major phase, crystalline and nano sized while Fourier Transform Infrared spectroscopy identified the functional groups of the hydroxyapatite. Scanning Electron Microscope analysis indicated the grain size of the synthesized hydroxyapatite particle in nano metric region. Thermogravimetric analysis confirmed the thermal stability of the hydroxyapatite. The in-vivo cytotoxicity test using Wistar Albino Rats did not indicate any toxic effects of the material at acute level. The research indicted potentiality of fish scales in producing HA material and also provides a means of cleaning up the environment as tons of fish scales considered of no use are being discarded daily by converting them into useful products.*

KEY WORDS; Tilapia, Albino Rats, Hydroxyapatite, thermal. nanosize

INTRODUCTION

Hydroxyapatite is a calcium phosphate chemically similar to human hard tissues (teeth and bone) in morphology and composition (Hui *et al.,* 2010). It is also called pentacalcium hydroxide triphosphate with a formula $Ca_{10}(PO_4)_6$ $(OH)_2$. It is naturally occurring mineral calcium apatite-calcium, phosphorous and oxygen that grows in hexagonal crystal structure. It is a major component and an essential ingredient of normal bone and teeth. Hydroxyapatite is the hydroxyl end member of the complex apatite group which crystallizes in the hexagonal crystal system and a pure hydroxyapatite powder is white (Jeong, 2012).

HA material in powder form is widely used for various biomedical applications such as bone substitute material, orthopedic and dentistry due to its excellent biocompatibility, bioactivity and osteoconduction properties (Sandeep, *et al.*, 2012). Biocompatibility is the ability of material to perform a specific function (when interacting with body tissue) with an appropriate host response under specific conditions, bioactivity is the ability of material to provide an appropriate scaffold for bone formation and osteoconductivity refers to the formation of bone-like apatite on their surface with a strong bone-calcium phosphate biomaterial interface (Teerawat, 2015). Recently, hydroxyapatite in nano size is more preferred as medical implants to hydroxyapatite in micro size due to its biocompatibility, surface area and similarities to natural apatite. Considering the numerous applications of calcium phosphate compounds in biomedical fields, several nanoHA synthesis techniques and variations have been developed (Alisyovana, *et al.,* 2013). Huge amounts

of Tilapia scales are discarded as biowastes daily after consumption of the protein part and these biowastes supports microbial action and leads to environmental pollution. The study seek to bride the gap for the increasing global clinical demand of the material due to high accidents rates which produces an estimated nine million fractures annually (Renata *et al*., 2018). Study also highlighted production of HA material from natural source using cheap, simple and uncomplicated procedure than the complex methods which will assist in reducing cost of orthopedic surgery (Bose and Saha, 2016) and to impact positively on the environment.

MATERIALS AND METHODS

Sample collection and preparation

Tilapia fishes were obtained from two different fish vendors, one from Kawo (Unguwar Dosa) and the other from Gamji Gate, all within Kaduna Metropolis where the scales were removed from the fishes on the spot and were labeled as TIL-KAW and TIL-GAM respectively. Tilapia scales were obtained by mechanically removing the scales from body of the fish and the bulk scales were washed thoroughly using a strong water jet and then boiled in water for an hour to remove organic substrates and adherent fish substances. The scales were dried at room temperature for two days and stored in clean porcelain container (Himanshu and Satya, 2016; Mustapha *et al.,* 2011). The dried Tilapia scales samples were crushed and pound into powder using agate pestle and mortar.

Synthesis of nano sized hydroxyapatite from Tilapia samples (Mustapha *et al.*,2011)

Nano- sized hydroxyapatite was synthesized from the ground Tilapia scales samples using calcination method. Thirty grams of raw sample powder was measured into three open aluminum crucibles and heated in furnace (protherm furnace SXL, model no; 1006) at 900^0C for 2 hours, after which the powder was allowed to cooled in the furnace. Nanosized hydroxyapatite was obtained as white powder after calcination at 900^0C. The white powder was further ground into fine powder using pestle and mortar.

Characterization of Synthesized HA

(a) Physical characterization

Refractive index was determined using Refractometer (Bellingam and Stanley England, Model No. 909271); colour was determined using visual comparism,; while conductivity was measured using conductivity meter (Jenway, England, model No. DDS.307).

(b) Chemical characterization

. X-ray diffractometer (Panalytical Empyrean Model) was used to study phase composition, degree of crystallinity and size of the crystal particles of HA at 25^OC and over a range of 10-80^0 2θ. The nano particle size was calculated using Scherrer's formula,

$$D = \frac{0.9\lambda}{b\cos\theta}$$

Where

λ = 0.154 nm for Cu

b = FWHM (Full width Half Maximum)

θ = Diffraction angle

D = particle size in nano meter.

Fourier Transform Infrared spectrophotometry (FTIR MACHINE MB 3000) was used to identify the functional groups in the material. Scanning Electron Microscope (SEM) (phenomenon prox model, model no. 4.5.3) was used to investigate the surface morphology and microstructural characteristics of the HA material while thermogravimetric analyser (Perkin Elmer TGA, model No. 400) was used to evaluate the thermal stability and percentage weight loss where the material was heated from 30 to 900. ^{0}C at heating rate of 10 ^{0}C/min.

In-Vivo Cytotoxicity test

In-vivo cytotoxicity study of the synthesized hydroxyapatite material was carried out using ninety (90) Wistar Albino Rats according to the method of Lorke's (1983). Different dose concentrations of 10 mg/kg, 100 mg/kg, 1000 mg/kg, 1600 mg/kg, 2900 mg/kg and 5000 mg/kg of the synthesized HA material was given to the Rats depending on their weights in two phases orally using syringe were evaluated after 24 hrs.

RESULTS AND DISCUSSION

Synthesis and Physical Characterization

The results for determination of physical properties of HA and its synthesis is presented in Table 1.where a total of 117 g of the HA powder was synthesized from 240 g of Tilapia scales used giving 50 % (percent) yield. The colour of the HA synthesized of all the samples was observed to be white and this agreed with Jeong (2012) whose report indicated that the colour of pure hydroxyapatite is white. The refractive index average value which indicate purity of synthesized HA ranged between 1.660-1.658 and conductivity values ranged between 0.69 -1.02. and the values reported here are in agreement with reports of Anthony *et al.,* (2000). The synthesis of HA from fish scales has expanded the horizon of research for his clinical material. The study further revealed that the scales can be used to synthesized mesoporous HA (2-50nm), which are material of interest in bone tissue engineering.

Table 1: Results of characterization of synthesized HA

S/N	Tilapia scale Sample	Quantity of hydroxyapatite Synthesized (g)	%yield	colour	Refractive Index	Conductivity (ms/cm)
1	TIL-KAW	59	66	white	1.660	0.69
2	TIL -GAW	58	65	White	1.658	1.02

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CHEMICAL CHARACTERIZATION

XRD ANALYSIS

Both crystallinity and phase of hydroxyapatite synthesized from fish scales were identified from XRD peaks of HA (Fig 1-2) as having narrow and sharp peaks. Brahim *et al.,* (2014) reported that hydroxyapatite phase in XRD analysis is identified by having narrow and sharp peaks. Major phase of the material was in this study was further confirmed as hydroxyapatite by comparing the diffraction pattern obtained with Joint Committee for Diffraction Pattern Standard (JCPDS Card (00-009-432). The XRD peaks of the synthesized nano hydroxyapatite in this study all have their peaks width becoming narrower and sharper and this also indicated crystallinity of hydroxyapatite material. The size of particles were calculated and presented in Table 2 which indicated the nano size of hydroxyapatite synthesized.

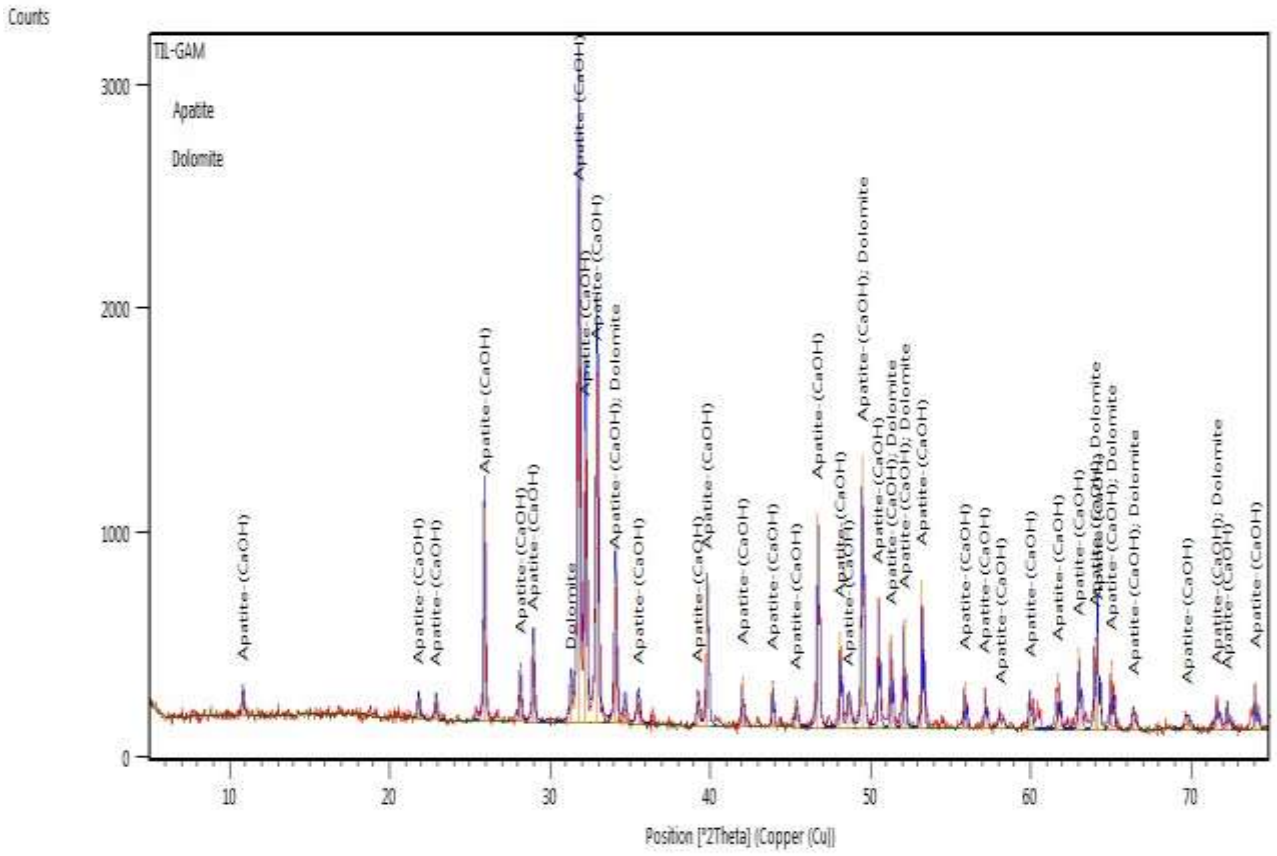


Fig. 1: XRD Result for TIL-GAM

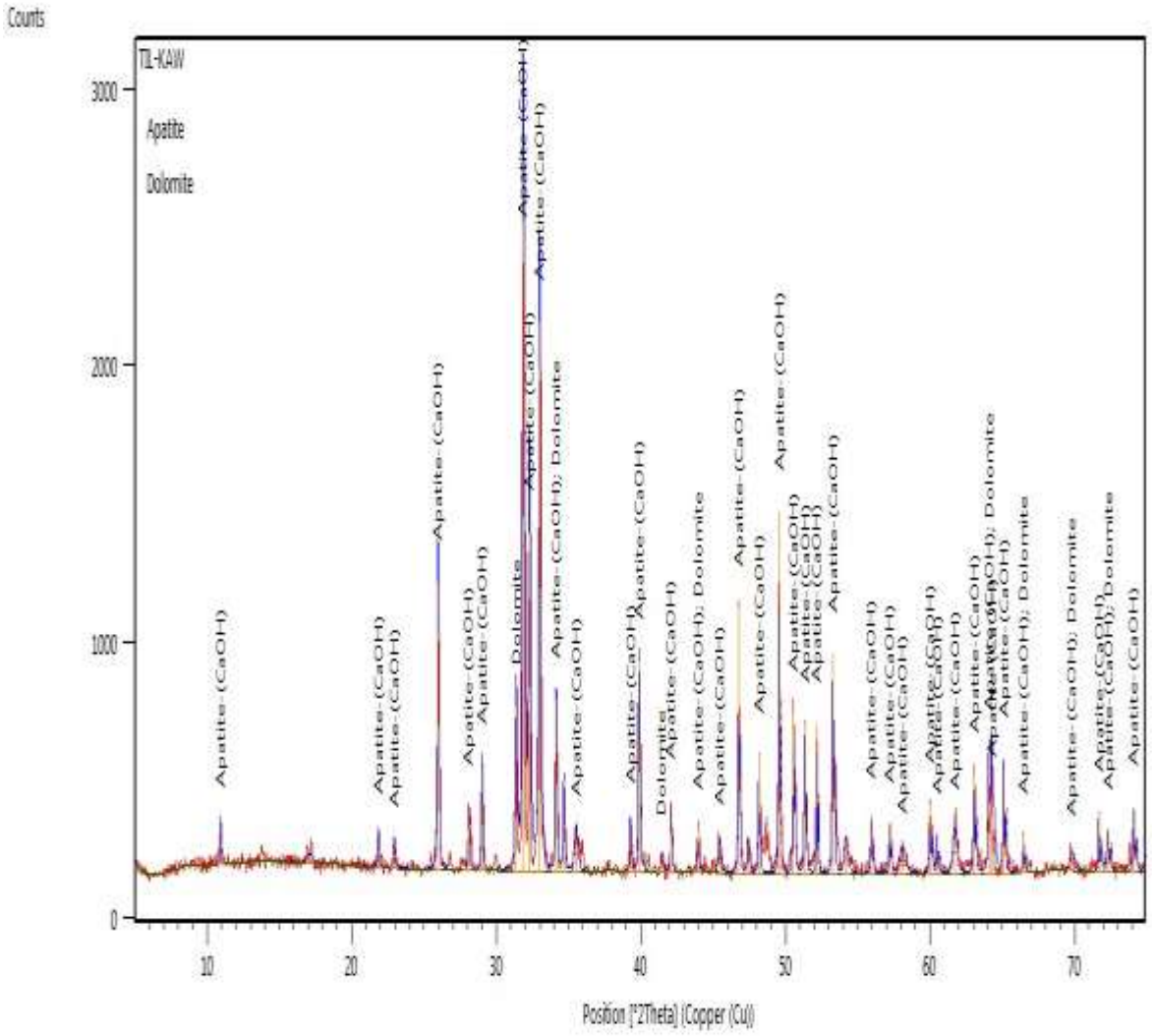


Fig. 2.: XRD Result for TIL-KAW.

Table 2: Particle size for nano-sized hydroxyapatite obtained from Tilapia scales.

SAMPLE	DIFFRACTION IN DEGREE (2θ)	B (FWHM) IN DEGREE	B (FWHM) in Radian (10^{-3})	PARTICLE SIZE IN nm
TIL-KAW	17.0924	0.5117	8.93	80 nm
	22.9517	0.2047	3.57	75 nm
	31.8700	0.1535	2.68	57nm
	32.2683	0.1535	2.68	95 nm
	35.5042	0.2535	3.57	75 nm
TIL-GAM	22.8900	0.1535	2.68	83 nm
	31.7988	0.1535	2.68	56 nm
	34.6781	0.1791	3.12	45 nm
	46.7183	0.1023	1.78	92 nm
	64.1950	0.0936	1.63	32 nm

TGA ANALYSIS

TGA results of nano size hydroxyapatite synthesized from Fish scales (Fig 3 – 4) was observed to be thermally stable between 700 ^{0}C up to 900 ^{0}C. This range of temperature indicating thermal stability agreed with the temperature range of 700 – 900 ^{0}C reported for thermally stable hydroxyapatite by Figuereido *et al., (*2010) and Zainol *et al.,* (2012). The material undergoes a major weight loss between 320 ^{0}C-460 ^{0}C and 310^{0}C – 470 ^{0}C for TIL- GAM and TIL- KAW respectively indicating over 70 % weight loss on TGA curve. However, a 3 % and 6 % weight loss indicating minor weight of the material was observed for and these values are similar with what was reported in the literature. The minor weight loss observed was due to the removal of both surface and absorbed water while the major weight loss was attributed to the removal of organic components.

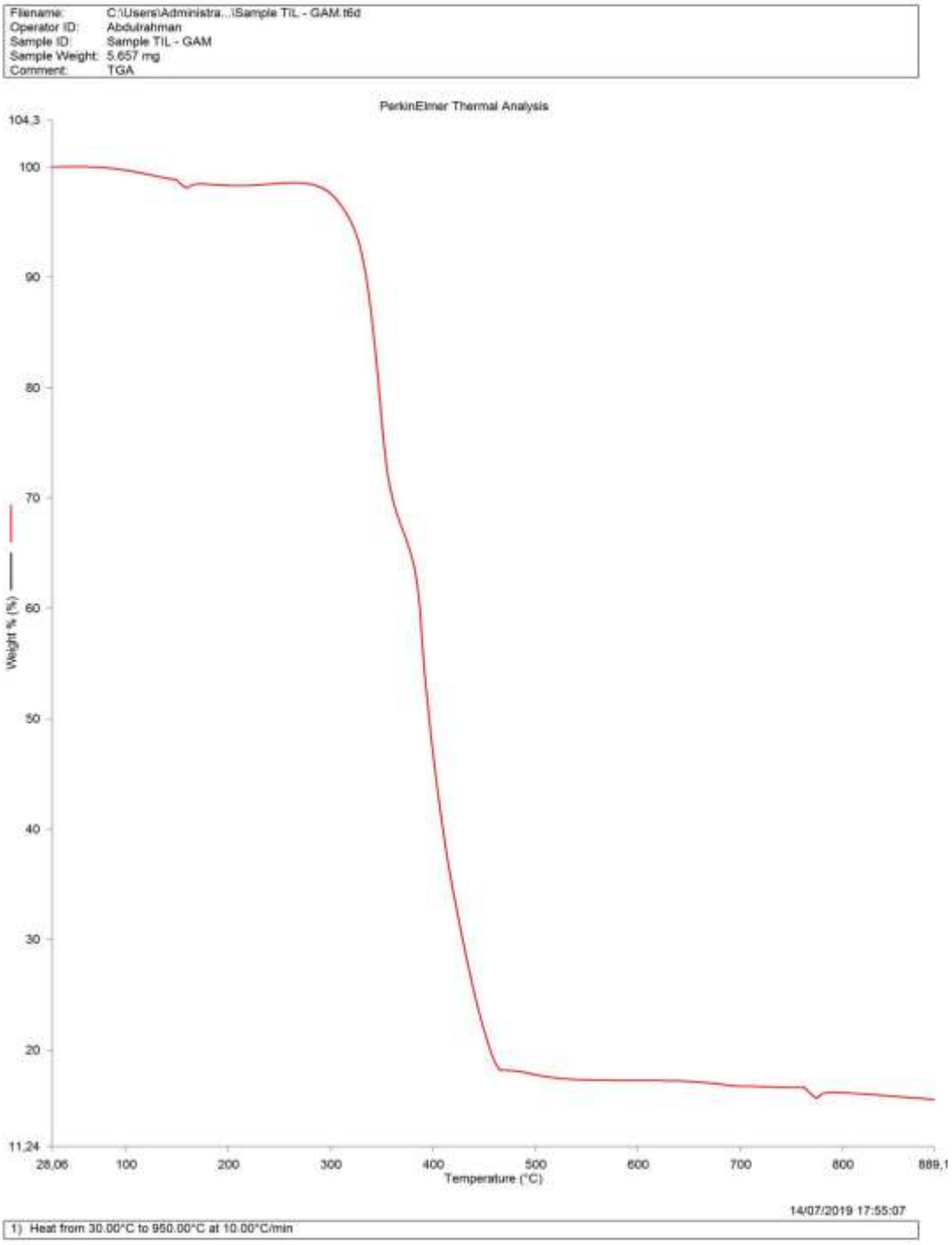


FIG 3 : TGA RESULT FOR TIL-GAM

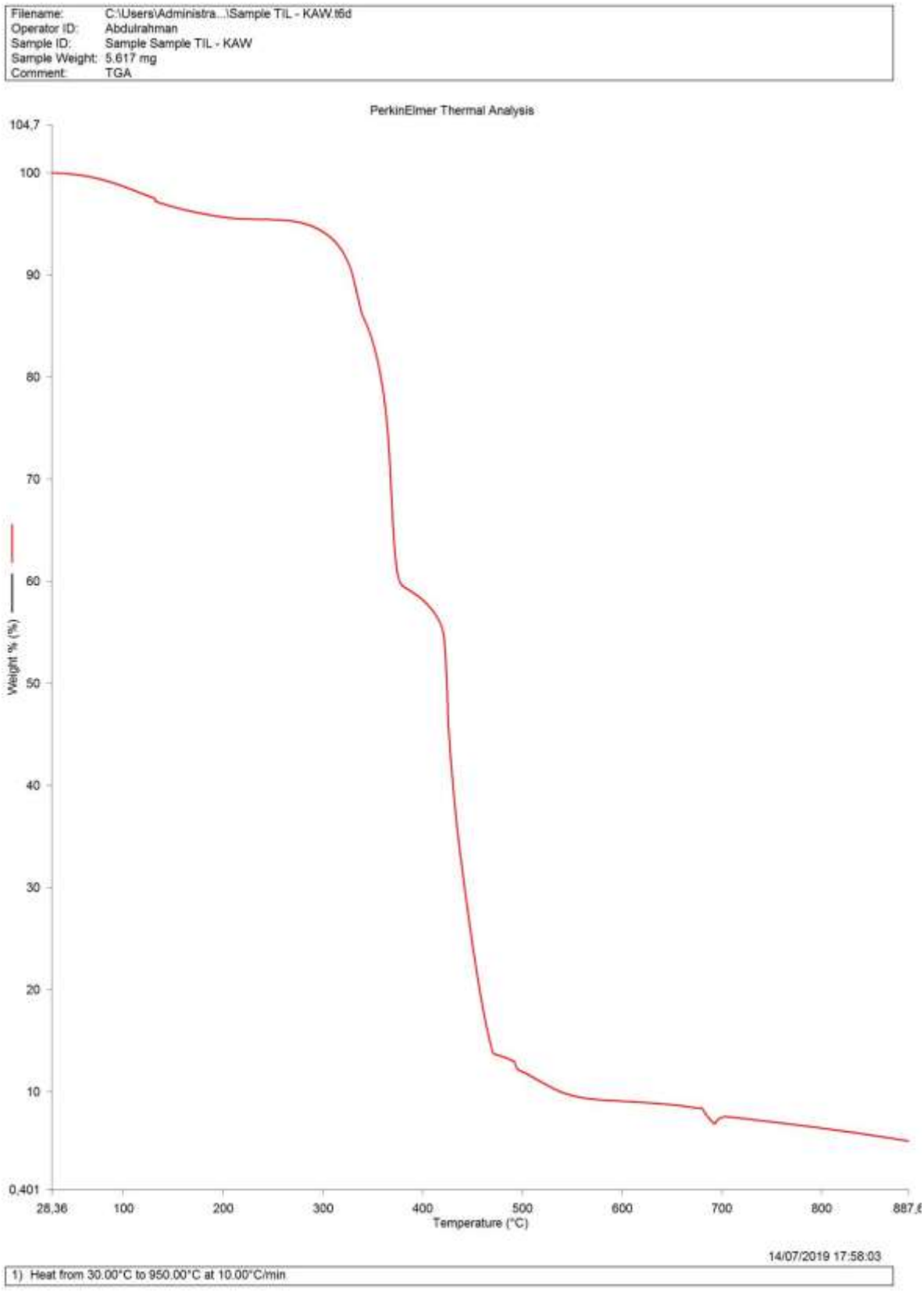
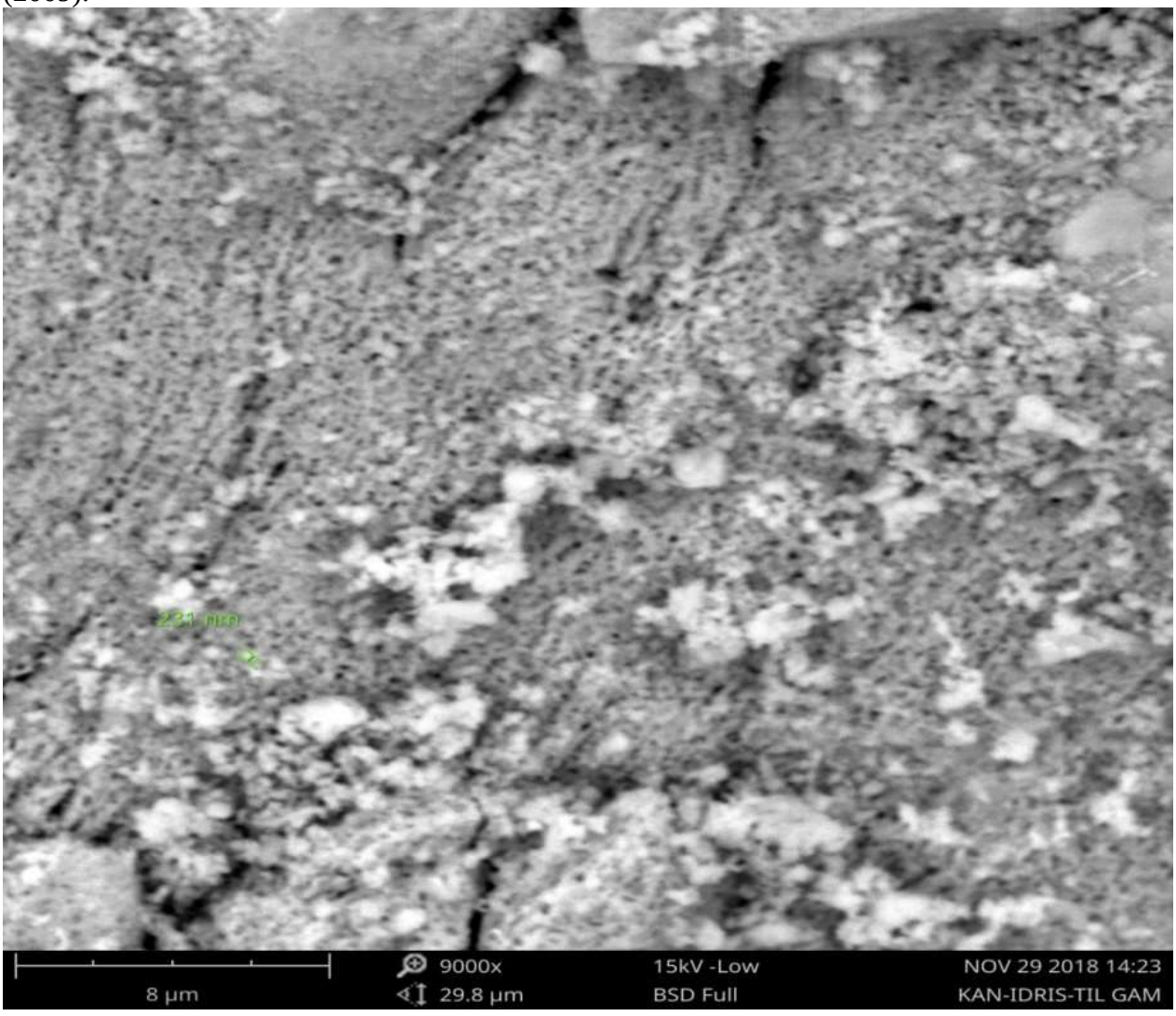


FIG 4.: TGA RESULT FOR TIL-KAW

SEM ANALYSIS

Analysis of SEM images (Fig 5-6) of nano hydroxyapatite from fish scales shows morphology of nano hydroxyapatite as spherical nano particles. The SEM image indicated that each single particles of nano hydroxyapatite is agglomerated with nano-sized grains. These agglomerate built up from single particles with potential of having a size between 300-600 nm. The range in the size of the agglomerates in this work was as reported by Enamul-hoque *et al.,* (2014) and Nasser *et al* (2009).



. Fig 5: SEM image for TIL GAM sample (9000x)

Fig 6.: SEM image for TIL-KAW Sample (9000x)

IN-VIVO CYTOTOXICITY TEST

The results of in-vivo cytotoxicity in Table 3 obtained after 24 hrs evaluation shows no record of mortality and this indicated that the material has no toxic effects at acute level and this finding agreed with reports of Alisyovana *et al.,* (2013) and Masud *et al.,* (2017). The study also agreed with Remiya and Mohanan (2017).whose chronic toxicity investigation of nano-HA using Wistar Albino Rats did not indicate any toxic effects

Table 3: Results of in-vivo cytotoxicity test of nano-HA using Wistar Albino Rats

Sample	Different Dose concentrations administered					
	Phase I (mg/kg)			Phase II (mg/kg)		
	10	100	1000	1600	2900	5000
TIL-GAM	0/3	0/3	0/3	0/1	0/1	0/1
TIL-KAW	0/3	0/3	0/3	0/1	0/1	0/1

KEY: Numerator is the number of death of animal recorded and Denominator is the number of animals use

FTIR ANALYSIS

The FTIR spectra of the nano hydroxyapatite synthesized from the two samples of Tilapia scales (Fig 7-8) indicated characteristic peaks due to OH^-stretching at 3570.8 cm^{-1} which are sharp and small. The values were close to the values reported by Sandeep *et al.* (2012) and Hui *et al.*, (2010) . The peaks at 1028.7cm^{-1}, 1025.0 $cm^{-1}$and 1088.4 cm^- are corresponding to vibration mode of PO_4^{2-} ion which were characteristically sharp and broad. The absorption peaks values were similar to the values of PO_4^{2-} ion reported by Greta *et al* (2010). The CO_3^{2-} ion vibrations was observed at 1427.6, 1420.1 and 715.6 cm^{-1} for hydroxyapatite synthesized from Tilapia Scales and these findings agreed with Sahin *et al.,* (2014) who detected CO_4^{2-} at 1457.0 cm^{-1}

Sample ID:KANGIWA SAMPLE TIL-GAM
Sample Scans:30
Background Scans:16
Resolution:8
System Status:Good

Method Name:Transmittance Method
User:Admin
Date/Time:2018-11-26T12:04:25.423+01:00
Range:4000 - 650
Apodization:Happ-Genzel

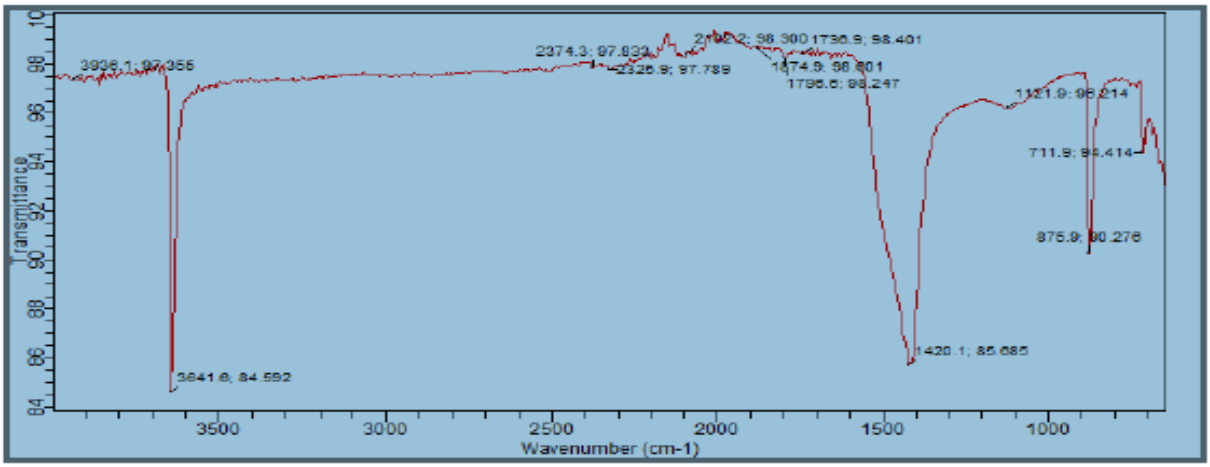


Fig 7: FTIR Result for TIL-GA

Sample ID:KANGIWA SAMPLE TIL-KAW
Method Name:Transmittance Method
Sample Scans:30
User:Admin
Background Scans:16
Date/Time:2018-11-26T12:00:31.933+01:00
Resolution:8
Range:4000 - 650
System Status:Good
Apodization:Happ-Genzel
File Location:C:\Program Files\Agilent\MicroLab PC\Results\KANGIWA SAMPLE TIL-KAW_2018-11-26T12-00-31.a2r

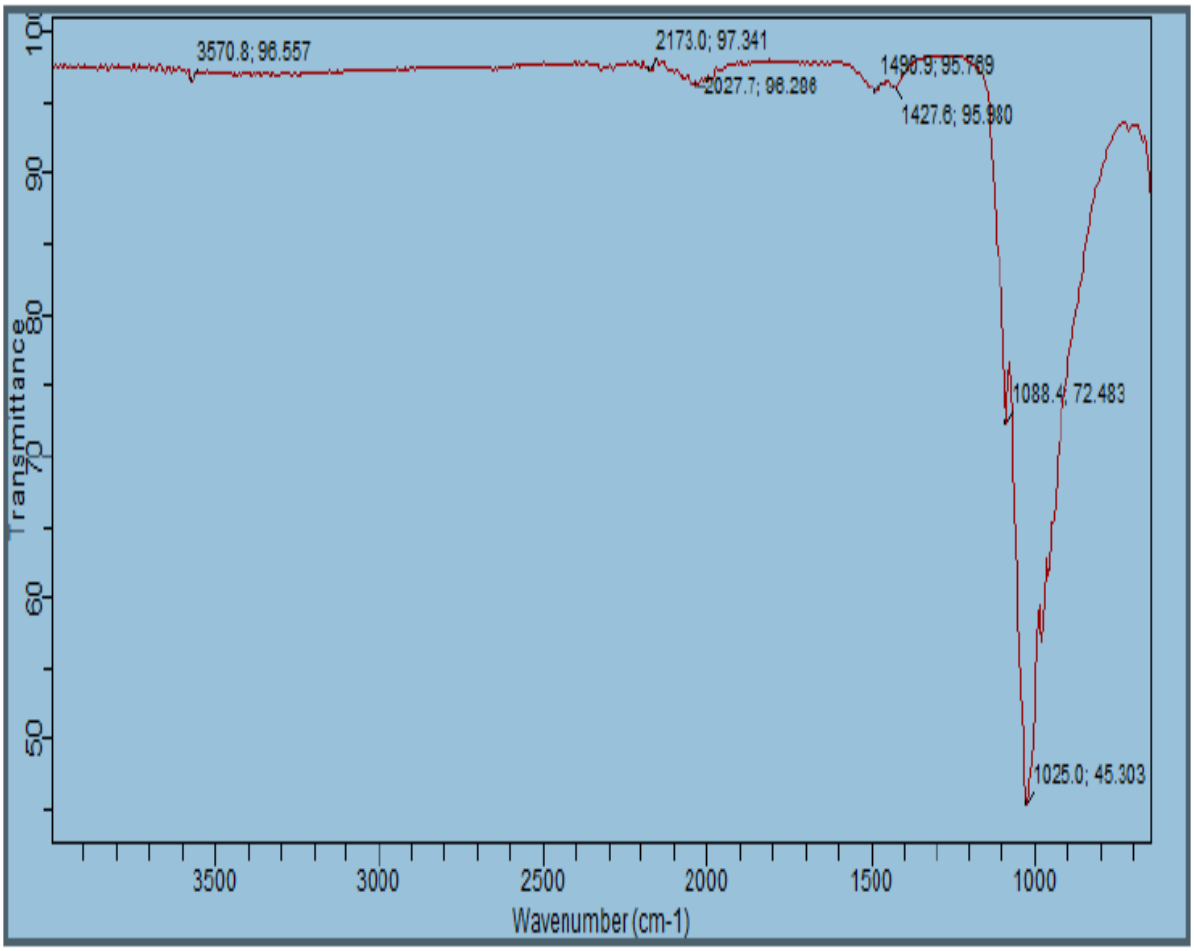


Fig 8: FTIR Result for TIL-KAW

RESEARCH IMPLICATIONS

Fish scales from this specie of fish aside being cheap and natural source for the synthesis of hydroxyapatite, it also produced mesoporous hydroxyapatite material (material whose nano size ranged between 2-50 nm) which are material of interest in maxillofacial reconstruction surgery. More so, the safety of the material for use in orthopedic medicine arising from in-vivo cytotoxicity test was also highlighted in the study.

CONCLUSION

Pure, natural and white crystalline nano sized hydroxyapatite (HA) material was extracted from Tilapia scales using calcination method. The synthesized nano hydroxyapatite exhibited thermal stability between 700 and 900 ^{0}C. The material did not show any toxicity effects from the in-vivo cytotoxicity study using Wistar Albino Rats. The study showed the potentials of fish scales in the production of nano biomaterial for orthopedic applications and to help the environment by converting these scales wastes into useful product.

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ACKNOWLEDGEMET

This work was supported by Nigerian Tertiary Education Trust Fund (TetFUND) grant of 2017

CONFLICT OF INTEREST

None declared