British Journal of Pharmaceutical Research
14(1): 1-10, 2016; Article no.BJPR.30152
ISSN: 2231-2919, NLM ID: 101631759

SCIENCEDOMAIN *international*
www.sciencedomain.org

In vitro Antimicrobial Activity of Fruit Pulp Extracts of *Azanza garckeana* (F. Hoffm.) Exell & Hillc. and Isolation of One of its Active Principles, Betulinic Acid

Y. J. Dikko[1], M. E. Khan[1], T. A. Tor-Anyiin[1*], J. V. Anyam[1] and U. A. Linus[1]

[1]Department of Chemistry, University of Agriculture, Makurdi, P.M.B. 2373 Makurdi, Benue State, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Authors TATA and MEK designed the study, wrote the protocol. Author YJD wrote the first draft of the manuscript. Authors JVA and YJD managed the literature searches and the review process. Authors YJD and UAL managed the experimental process under the supervision of authors MEK and TATA. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BJPR/2016/30152
Editor(s):
(1) Wenbin Zeng, School of Pharmaceutical Sciences, Central South University, Hunan, China.
Reviewers:
(1) Ayodele Akinterinwa, Modibbo Adama University of Technology Yola, Nigeria.
(2) Aili Jiang, Yantai University, China.
(3) M. Vijayabaskaran, The Tamilnadu Dr. M.G.R.Medical University, India.
(4) Foluso O. Osunsanmi, University of Zululand, South Africa.
Complete Peer review History: http://www.sciencedomain.org/review-history/17172

Original Research Article

Received 21st October 2016
Accepted 4th December 2016
Published 8th December 2016

ABSTRACT

Aims: To perform anti-microbial activity guided isolation and to characterize compound(s) from fruit pulp (extracts) of *Azanza garckeana*.
Place and Duration of Study: Department of chemistry, University of Agriculture, Makurdi, Nigeria, and University of Strathclyde in Scotland, United kingdom, from October 2015 to September 2016.
Methodology: Crude extracts were screened for phytochemical components. *In vitro* Anti-microbial assay was performed using Agar Diffusion Method and Broth Dilution Method. Vacuum Liquid Chromatography and gel filtration (Sephadex LH20) were used for the isolation of medicinal

**Corresponding author: E-mail: toranyiint@yahoo.com;*

principles. Characterization of the isolated compound was carried out using proton Nuclear Magnetic Resonance Spectroscopy.
Results: Extracts contained steroids, flavonoids, terpenoids, saponins, alkaloids, reducing sugars and tannins. Ethyl acetate extract had the highest diameter of zones of inhibition (27 mm). Ethyl acetate and methanol extracts had the same Minimum inhibitory concentrations of 1.25 mg/ml against *Methicillin Resistant Staphylococcus aureus, Staphylococcus aureus, Pseudomonas aeruginosa* and *Candida krusei* but for *Escherichia coli* which had Minimum inhibitory concentration of 0.625 mg/ml for ethyl acetate extract. Ethyl acetate extract had the best minimum bactericidal/fungicidal concentration (2.5 mg/ml) on pathogens. It was determined from Proton Nuclear Magnetic Resonance Spectrum that the isolated compound contained features conclusively characteristic of Betulinic acid.
Conclusion: Fruit extracts of *Azanza garckeana* show promising *in vitro* anti-microbial activity. This is the first report of isolation and characterization of betulinic acid from fruits of this species.

Keywords: Azanza garckeana; betulinic acid; n-hexane extract; phytochemical screening; antimicrobial assay.

1. INTRODUCTION

Plants are potential sources of antimicrobial compounds and several researchers have investigated the antimicrobial activities of medicinal plants utilized in traditional or alternative healthcare systems [1,2]. Human pathogenic microorganisms have been reported to develop resistance to commonly vended antibiotic drugs used in therapy [3]. Consequently, there has been renewed interest in herbal remedies in several parts of the world with many of the herbal remedies being incorporated into orthodox medical practice [4]. Some African countries: Egypt, Burkina Faso, Ghana, Nigeria, Zambia and South Africa have also made good advances in the area of the use of plants for production of new drugs [4]. Acceptance of traditional medicine as an alternative form of health care and development of microbial resistance to available antibiotics has provided impetus to the search for new antimicrobial substances from various sources including medicinal plants [5,6].

Azanza garckeana plant locally called *goron tula* (Hausa, Nigeria); variously it is called *snotappel* (Afrikaans); *chinga, mukole* (Bemba); *azanza, tree hibiscus, snot apple, quarters, wild hibiscus, African chewing gum* (English); *muneko* (Lozi); *mukole* (Lunda); *uxhakuxhaku* (Ndebele); *mkole* (Nyanja); *mutohwe* (Shona); *mtobo* (Swahili); *muneko* (Tongan); *morajwa* (Tswana) [7]. *Azanza garckeana* is a tree, 3-13 m high, with a diameter at breast height of up to 25 cm [7]. *Azanza garckeana* grows naturally in semi-arid areas receiving lowest annual rain fall of 250 mm and highest rain fall of 1270 mm [8]. In Nigeria, *Azanza garckeana* is found abundantly found in Tula, a town in Kaltungo Local Government Area, Gombe State, Nigeria. It is also found around Kankiya, Katsina State and Daggish Kali hills of Zah district, Michika local government area of Adamawa State [9]. The plant is resistant to and can withstand mild frost [7]. Flowering occurs from May to October and fruiting/ ripening from November to April [10]. The fruit epicarp turns from a greenish to a brownish colour on ripening with the capsule showing clear division into 4 or 5 segments [11,7].

Among the Tula people of North-Eastern Nigeria, ripened fruits are consumed as an aphrodisiac and for treatment of infertility in men. Other parts of the plants have been formidably studied with numerous reports of isolated compounds [12, 13]; in Zimbabwe, an Infusion of the roots is dropped into the ear to treat earache or orally as an antiemetic or to treat cough, chest pain, menstruation and in large doses as an abortifacient [14-16]. A tea of the stems and leaves is taken to treat liver problems [10]. A poultice of the pounded fruit is applied to abscesses to sufficiently thin out inflamed tissue encompassing the infected cavity or draw pus to a head so the abscess may rupture [10,16]. The fruit of this important ethno-medicinal plant has not been previously investigated with view of isolating its medicinal principles. This study was aimed at isolating and characterizing phyto-constituent(s) from n-hexane extract of the fruit pulp of *A. garckeana* and by so doing to establish or otherwise refute folkloric claims on the fruits of *Azanza garckeana*.

2. MATERIALS AND METHODS

2.1 Plant Collection and Authentication

Fruit pulps of *A. garckeana* were collected in October, 2015 from Tula, Kaltungo L. G. A,

Gombe State, Nigeria. The plant was identified and authenticated by Mr. Mark Uleh of Department of Social Forestry and Environmental Protection, University of Agriculture, Markurdi, where a specimen with voucher number MVM 2013/UoN 02 was also deposited. Fruit pulps were air-dried for three weeks in the laboratory and subsequently pulverized using a wooden mortar and pestle set.

2.2 Sequential Extraction

Pulverized Fruit pulp (350 g) were defatted with hexane (500 ml) for five hours at ambient temperature and filtered. The marc was sequentially treated to microwave assisted extraction using 500 ml each of ethyl acetate and methanol. Extracts were filtered and excess volume reduced *in vacuo* using rotary evaporator at 40℃ (50℃ for methanol), and evaporated to constant weight in a fume cupboard before subsequent analysis.

2.3 Phytochemical Screening

Crude extracts were subjected to phytochemical tests for presence of saponins, tannins, flavonoids, and steroids, reducing sugar, terpenes and alkaloids, using standard procedures as previously reported by [17-19]. Test for Alkaloids (Wagner's test): The extract (10 ml) was taken and few drops of Wagner's reagent were added; the formation of a reddish brown precipitate indicated the presence of alkaloids.

Test for Flavonoids (Lead acetate test): The extract (10 ml) was taken and few drops of lead acetate solution (10%) was added. Appearance of yellow colour precipitate was taken to indicate presence of flavonoids.

Test for phenols (Sodium hydroxide test): The extract (5 ml) dissolved in 20% sulphuric acid solution (1.5 ml) followed by addition of three drops of aqueous solution of sodium hydroxide; a blue solution indicated the presence of phenols.

Test for Tannins (Feric chloride test): the extract (5 ml) was taken and 5% ferric chloride (1.5 ml) was added. The development of a bluish-black colour indicated presence of tannins.

Test for steroids (Salkowski's test): The extract (0.5 g) was dissolved in chloroform (2 ml) and an equal volume of concentrated H_2SO_4 was added along the sides of the test tube. The upper layer turned red and lower layer turned yellow, indicating presence of the steroids.

Test for Carbohydrates (Fehling's test): A mixture of Fehling solutions A and B (5 ml) was added to the extract (3 ml) and boiled over a water bath. Formation of a brick red precipitate indicated a positive test for reducing sugars.

Test for Cardiac Glycosides (Keller-killiani test): The plant sample (2 g) was dissolved in of glacial acetic acid (2 ml) containing a drop of ferric chloride solution and concentrated H_2SO_4 (1 ml) added. A brown ring at the interface indicated a positive test for desoxy-sugars [17-19].

2.4 Antimicrobial Screening

Antimicrobial activities of the plant extracts were determined using the following pathogens (clinical isolates): *Methicillin Resistant Staphylococcus aureus, Vancomycin Resistant Enterococci, Staphylococcus aureus, Escherichia coli, Helicobacter pylori, Proteus mirabilis, Pseudomonas aeruginosa, Candida albicans, Candida krusei* and *Candida tropicalis*. A portion of each extract (0.05 g) was weighed and dissolved in Dimethylsulphoxide (DMSO) (10 ml) to give a concentration of 5 mg/ml; this served as initial concentration of extracts used to determine antimicrobial activities.

Mueller Hinton and Sabourand Dextrose Agar were the growth media used for bacteria and fungi, respectively. All media were prepared according to manufacturer instructions, sterilized at 121℃ in an autoclave for 15 minutes, poured into sterile petridishes and allowed to cool and solidify. Diffusion method was used for screening the extracts. Mueller Hinton Agar was seeded with standard inoculum (0.1 ml) of bacteria and Sabourand Dextrose Agar with the fungi. The inocula were spread evenly over the surface of the media with sterile swabs. A standard cork borer (6 mm in diameter) was used to cut a well at the centre of each inoculated medium. Solutions of extracts (0.1 ml each) of concentration 5 mg/ml were then introduced into each well on the medium. Inoculated plates were incubated at 37℃ for 24 hours for the bacteria and at 30℃ for 7 days for the fungi, after which each plate was observed for inhibition zone of growth. Zones were measured with a transparent ruler and the results recorded in millimeters [20].

The minimum inhibition concentration (MIC) of extracts was carried out using broth dilution

method. Mueller Hinton and Sabourand Dextrose Broth were prepared; the broth (10 ml) was dispensed into test tubes and sterilized at 121℃ for 15 minutes, then allowed to cool.

McFarland's turbidity standard scale number 0.5 was prepared to give a turbid solution. Normal saline (10 ml) was dispensed into sterile test tubes and the test microbes inoculated and incubated at 37℃ for 24 hours. Dilution of the test microbes in the normal saline was done until the turbidity matched that of the McFarland's scale by visual comparison at which point, the test microbes had a concentration of about 1.5 x 108 CFU/ml.

A two-fold serial dilution of the extracts in the sterilized broth were made to obtain the concentrations of 5, 2.5, 1.25, 0.625 and 0.313 mg/ml. Initial concentrations were obtained by dissolving extracts (0.01 g) in sterile broth (10 ml). Having obtained the different concentrations of the extracts in the sterile broth, the test microbes in the normal saline (0.1 ml) were then inoculated into the different concentrations. Incubation was made at 37℃ for 7 days for the fungi and 37℃ for 24 hours for bacteria, after which the broth was observed for turbidity. Lowest concentration of extracts in the broth which showed no turbidity was recorded as the MIC [20].

Minimum bactericidal concentration/ Minimum fungicidal concentration (MBC/MFC) were carried out to determine whether the test microbes were killed or only their growth was inhibited. Mueller Hinton and Sabourand Dextrose Agar were prepared and sterilized at 121℃ for 15 minutes then poured into sterile petri-dishes and allowed to cool and solidify. The content of the MIC in the serial dilution were sub-cultured into the prepared media, incubation were made at 37℃ for 24 hours for bacteria and at 30℃ for 7 days for fungi, after which plates were observed for colony growth. MBC/MFC were the plates with lowest concentration of extract without colony growth [20].

2.5 Isolation of the Compound

Vacuum Liquid Chromatography was used in isolation from the n-hexane crude extract. The extract (4.8 g) was pre-adsorbed unto Celite (analytical filter aid) and introduced on the stationary phase [silica gel 60 G (35 g)] hitherto loaded and compacted under vacuum in a sintered glass funnel (porosity 3). The column was first run with only n- hexane (200 ml in 10 ml fractions) in order to reduce waxes and lipophilics; then using different ratios of the solvents hexane and ethyl acetate, beginning with 100% hexane: 0% ethyl acetate and subsequently with 1% increases in the ethyl acetate component up to 0% hexane: 100% ethyl acetate, the column was run at a flow rate of approximately 8 ml/min. A total of 25 fractions of this second set were collected in separate vials of 10 ml each.

A total of forty five fractions (including the initial hexane wash) (labelled AZH 1 to 45) were collected and allowed to evaporate to dryness at room temperature (38 - 40℃). The purity of each of the collected fractions was monitored using thin layer chromatography (TLC). The TLC plate were developed with a mixture of 15% H_2SO_4 in 85% methanol. The solvent system hexane: diethyl ether (4:1) gave the best resolution. Fractions 19 and 20 were pooled together on the basis of TLC similarity. The fractions (19 and 20), which showed pink single spots on development were concentrated by evaporation and labeled AZH- 001. Fraction AZH-001 produced white amorphous crystals (20 mg). The amorphous crystals were collected in a glass vial for further analysis (spectroscopy and melting point).

The Proton Nuclear Magnetic Resonance (^{1}HNMR) experiment was carried out on the purified fraction (20 mg) using a Bruker DPX 400 MHz NMR spectrometer with $CDCl_3$ as solvent and Tetramethylsilane (TMS) as internal standard. Its melting point was determined using an Electro thermal IA 9300 (Gallenkhamp) capillary melting point apparatus with a mercury in glass thermometer.

3. RESULTS AND DISCUSSION

3.1 Phytochemical Screening

Steroids, flavonoids, terpenoids, and tannins were found in the ethyl acetate and methanol extracts while saponins, alkaloids and reducing sugars were found exclusively in the methanol extract. N-Hexane was found to contain only steroids and terpenes (Table 1). The presence of these phytochemicals in the extracts follow roughly their degrees of polarity; steroids and terpenes being the least polar of the listed classes of compounds naturally were present in the least polar solvent. The more polar classes were found in the more polar solvents (ethyl acetate and methanol). Corollary, the most polar

classes (saponins, alkaloids and reducing sugars) were extracted by the most polar solvent (Methanol). The result of methanol extract is in agreement with that of Adamu et al. [9] on fruit pulps of *Azanza garckeana.* Ajayi, [21], stated that tannins, saponins and flavonoids have been linked or suggested to be involved with antibacterial and anti-viral activity. Investigations of the mode of action have indicated that tannins and flavonoids increase colonic water and electrolyte reabsorption and other phytochemicals by inhibiting intestinal mobility, while some components have been shown to inhibit particular entero-pathogens [22]. The slightly bitter taste of *Azanza garckeana* may be due to the presence of tannins and alkaloids; these have bitter characteristic tastes [23]. The beneficial effects of fruits, vegetables, and tea or even red wines have been attributed to flavonoid compounds rather than to known nutrients and vitamins, they show anti-allergic, anti-inflammatory, anti-microbial and anti-cancer activities [9]; have broad spectrum of biological properties such as antibacterial, antiviral and anti-inflammatory effects [24].

Table 1. Phytochemical screening results of crude extracts obtained via microwave assisted extraction

Phytochemicals	HE	EA	ME
Reducing sugar	-	+	+
Saponins	-	-	+
Steroids	+	+	+
Terpenoids	+	+	+
Tannins	-	+	+
Alkaloids	-	+	+
Flavonoids	-	+	+

Keys: + = Present, - = Below detectable levels, HE = Hexane, EA = Ethyl acetate, ME = Methanol

3.2 Antimicrobial Screening

The antimicrobial results obtained revealed that ethyl acetate extract had the highest diameter of zones of inhibition (27 mm) against test microbes with values of 24, 26, 27, 26 and 23 for *Methicillin Resistant Staphylococcus aureus*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida krusei* respectively followed methanol: 22, 21, 23, 23 and 20 in the same order. The hexane extract had the smallest diameters of zone of inhibition. Ethyl acetate and methanol extracts had the same Minimum inhibitory concentrations of 1.25 mg/ml against *Methicillin Resistant Staphylococcus aureus, Staphylococcus aureus, Pseudomonas aeruginosa* and *Candida krusei* but for *Escherichia coli* which had Minimum inhibitory concentration of 0.625 mg/ml for ethyl acetate extract. Ethyl acetate extract had the best minimum bactericidal/fungicidal concentration (2.5 mg/ml) on pathogens. *Vancomycin Resistant Enterococci, Helicobacter pylori, Candida albicans,* Candida *tropicalis* and *Proteus mirabilis* were resistant to all extracts (Table 2). Five of the organisms showed sensitivity to the extracts (Table 2). In this regard, ethyl acetate extract showed the widest zones of inhibition against *Escherichia coli* and *Staphylococcus aureus,* at 27 mm and 26 mm, respectively (Table 2), minimum inhibition concentration (MIC) of 0.625 mg/ml and minimum bactericidal concentrations (MBC) of 2.5 mg/ml on *Escherichia coli*. The hexane extract showed MIC (2.5 mg/ml) against *Methicillin Resistant Staphylococcus aureus* while against *Staphylococcus aureus, Proteus mirabilis* and *Candida krusei* it had MIC of 1.25 mg/ml (Table 3).Tested pathogens showed MBC/

Table 2. The antimicrobial activities of *Azanza garckeana* fruit pulp extracts

Pathogen	Sensitivity/ zone of inhibition (mm) control (5 µg/ml)				
Extract	**EA**	**HE**	**ME**	**CF**	**FZ**
MRSA	S(24)	S(18)	S(22)	R(32)	-
VRE	R(0)	R(0)	R(0)	-	-
S. aureus	S(26)	S(20)	S(21)	S(34)	-
E. coli	S(27)	S(21)	S(23)	S(37)	-
H. pylori	R	R	R	-	-
P. mirabilis	R	R	R	S(32)	-
P. aeruginosa	S(26)	S(20)	S(23)	-	-
C. albicans	R	R	R	-	S(35)
C. krusei	S(23)	S(18)	S(20)	-	S(37)
C. tropicalis	R	R	R	-	R(34)

KEY: S = Sensitivity, R = Resistance, - = No zone of inhibition, Numeric value in brackets = Diameter of zone of inhibition in millimetres (mm), Drug concentration (Positive Control) = 5 µg/ml, Negative control = Normal saline, EA= Ethylacetate, HE=Hexane, ME=Methanol, CF= Ciprofloxacin, FZ= Fluconazole, VRE= Vancomycin Resistant. Enterococci, MRSA=Methicillin Resistant Staphylococcus aureus

MFC activity at 2.5 ml except hexane extract that was not active against any of the pathogens at 2.5 ml (Table 4). Fruit pulps extracts from *Azanza garckeana* reportedly have broad spectrum antibacterial and antifungal activities against tested pathogens, especially those associated with skin infection, bloody diarrhea, tuberculosis, acne and anti-vulvovaginal candidiasis [25].

3.3 Characterization

Fraction AZH-001 was isolated as a white amorphous solid (20 mg) with a melting point of 278-279℃. The pink spot on TLC plate on spraying with concentrated H_2S0_4 showed its terpenoidal nature; It had an Rf value of 0.4 in hexane diethyl ether (4:1) which was similar to that reported for betulinic acid by [26-28].

Table 3. Minimum inhibition concentrations (MIC) of extracts against test microorganisms (mm)

Test pathogen	EA					HE					ME				
	5 mg/	2.5 mg/ml	1.25 mg/ml	0.625 mg/ml	0.312 mg/ml	5 mg/ml	2.5 mg/ml	1.25 mg/ml	0.625 mg/ml	0.312 mg/ml	5 mg/ml	2.5 mg/ml	1.25 mg/ml	0.625 mg/ml	0.312 mg/ml
MRSA	-	-	*µ*	+	++	-	*µ*	+	++	+++	-	-	*µ*	+	++
VRE	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
S. aureus	-	-	*µ*	+	++	-	-	*µ*	+	++	-	-	*µ*	+	++
E. coli	-	-	-	*µ*	+	-	-	*µ*	+	++	-	-	*µ*	+	++
H. pylori	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
P. mirabilis	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
P. aeruginosa	-	-	*µ*	+	++	-	-	*µ*	+	++	-	-	*µ*	+	++
C. albicans	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
C. krusei	-	-	*µ*	+	++	-	-	*µ*	+	++	-	-	*µ*	+	++
C. tropicalis	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

Key: - = No turbidity (no growth), µ = MIC, + = Turbidity (light growth), ++ = Moderate turbidity, +++ = heavy turbidity, R = pathogen is resistant to extract, VRE= Vancomycin Resistant. Enterococci, MRSA = Methicillin Resistant Staphylococcus aureus

Table 4. Minimum bactericidal concentrations (MBC) / minimum fungicidal concentrations (MFC) of extracts against test microorganisms (mm)

Test pathogen	EA					HE					ME				
	5 mg/ml	2.5 mg/ml	1.25 mg/ml	0.625 mg/ml	0.312 mg/ml	5 mg/ml	2.5 mg/ml	1.25 mg/Ml	0.62 mg/ml	0.312 mg/ml	5 mg/ml	2.5 mg/ml	1.25 mg/Ml	0.625 mg/ml	0.312 mg/ml
MRSA	-	*µ*	+	++	+++	*µ*	+	++	+++	++++	*µ*	+	++	+++	++++
VRE	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
S. aureus	-	*µ*	+	++	+++	*µ*	+	++	+++	++++	*µ*	+	++	+++	++++
E. coli	-	*µ*	+	++	+++	*µ*	+	++	+++	++++	-	*µ*	+	++	+++
H. pylori	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
P. mirabilis	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
P. aeruginoas	-	*µ*	+	++	+++	*µ*	+	++	+++	++++	-	*µ*	+	++	+++
C. albicans	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
C. krusei	-	*µ*	+	++	+++	*µ*	+	++	+++	++++	*µ*	+	++	+++	++++
C. tropicalis	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

Key: - = No turbidity (no growth), µ = MBC/MFC, + = Turbidity (light growth), ++ = Moderate turbidity, +++ = high turbidity, ++++ = Heavy turbidity, R = pathogen is resistant to extract, VRE= Vancomycin Resist. Enterococci, MRSA = Methicillin Resistant Staphylococcus aureus

Table 5. Proton spectral data of AZH-001 in $CDCl_3$ compared with published results

Experimental data ^{1}H (δ)	[27] ^{1}H (δ)	[29] ^{1}H (δ)	[28] ^{1}H (δ)	[26] ^{1}H (δ)
4.73(1H,d,J=2.34 Hz, Ha- 29)	4.73 (1H,br. s, Ha-29)	4.65 (1H, d, J=0.4 Hz)	4.66 (s) H- 29	4.68 (2H, d, J = 32 Hz, H-29)
4.60(1H,d,J=2.02 Hz, Hb- 29)	4.60 (1H, br.s, Hb-29)	4.55 (1H, d, J=0.4 Hz)		
3.18(1H,dd,J=11.32,4.98 Hz, H-3)	3.18(1H, dd, J=11.2 Hz,4.9 Hz, H-3)	3.16 (dd, J=9.5, 6.0 Hz)	4.47 (dd), H-3, 10.2, 5.5 Hz	3.17 (m)
2.98 (1H,m, H-19)	2.98 (1H,m,H-19)	2.95 (ddd, J=9.5, 6.0 Hz, 0.5 Hz)	3.0 (t), 10.2, 5.5 Hz, H-19	3.0 (m), 2.29 (q)
1.68(3H,d,J=3.62 Hz, Me-30)	1.68 (3H,s,Me-30)	1.65 (3H, s, Me -30)	1.68,s,H-30	1.69 (s, 3H, H-30)
1.55 (m, H-18)				0.75-1.61 (m, rest of Protons
0.96 (3H,d, J=4.10 Hz, Me-23)	0.96 (3H, s, Me -23)	0.96 (3H, s, Me -23)	0.95 (s)	
0.93 (3H,S, Me-27)	0.93 (3H,s,Me-27)	0.90 (3H, s, Me -27)	0.93 ,s, H-27	
0.75 (3H,d, J=2.65 Hz, Me-24)	0.74 (3H,s, Me-24)	0.65 (3H, s, Me -24)	0.91 ,S, H-24	
0.81 (3H,S, Me-25)	0.81 (3H, s, Me-25	0.75 (3H, s, Me -25)	0.81 (s) H-25	
	0.97 (3H,s,Me-26)	0.98 (3H,s,Me-26)	0.95 (3H, s, Me -26),	

δ = Chemical shift

It was determined that the fraction was a triterpene from the proton NMR spectrum specifically with a carbon skeleton of the lupane type. Signals attributable to an exo-methylene group at 4.73/4.60 (d, H-29) which together with an allylic methyl at δ1.68 (3H, d, *J*=3.62 Hz; Me-30) which indicated an isopropenyl function. The double doublet δ3.18 with couplings of 11.32 and 4.98 Hz centered at C-3 could be assigned to H-3. The large coupling of this proton (H-3) with the vicinyl methylene protons suggested a β (beta) orientation of the hydroxyl group at C-3. The up-field region between δ 0.7 to δ 2.4 of AZH-001 spectrum was expanded. The up-field signals at δ 0.96 (3H, d, *J*= 4.10 Hz; Me-23) and δ 0.75 (3H, d, *J*=2.65 Hz; Me-24) were due to methyl protons at C- 23 and C-24, respectively. In addition, the spectrum also showed a multiplet at δ2.98 (m, H-19) and δ 1.55 (m, H-18) for the methine protons at position 18 and 19, respectively; methyl group resonances at δ 0.93 (3H, s, Me-27) and δ 0.81 (3H, s, Me-25) (Table 5).

These spectral features are in close agreement to those documented and reported by Igoli and Gray; Nazma et al. Ghais et al. Shumaia et al. [26-29] (Table 5) for betulinic acid. These results are therefore suggestive that the compound present in AZH-001 could most probably be betulinic acid based on its melting point, TLC and comparison of its ^{1}H- NMR spectrum data (Table 5) with documented reports. Based on the above spectral information, the structure of AZH-001 is as shown (Figs. 1 and 2).

Betulinic acid has been reported to exhibit a variety of biological and medicinal properties such as inhibition of human immunodeficiency virus (HIV) [30], anti-bacterial [31], anti-malarial [32], anti-inflammatory [33], anthelmintic [34], antinociceptive [35], and anti- cancer activities [36].

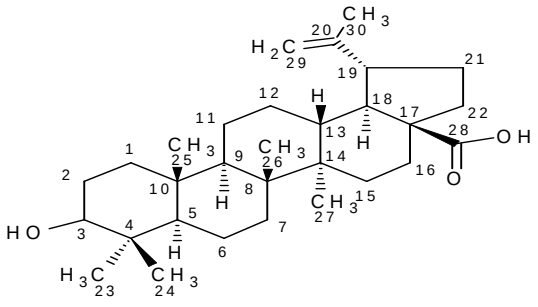

Fig. 1. Structure of betulinic acid (3β-hydroxy-lup-20(29)-en-28-oic acid)

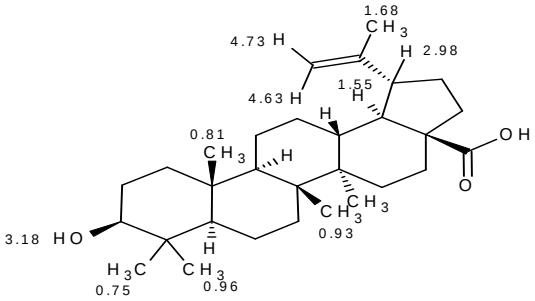

Fig. 2. Structure of betulinic acid showing proton NMR chemical shifts values

4. CONCLUSION

In summary, this investigation into the chemical constituents of the fruit pulp of *A. garckeana* has led to isolation of a triterpene; betulinic acid. Bioassay of crude extracts lends credence to some ethno-medicinal claims on fruits of the plant.

It is recommended that further studies be carried to ascertain the quantity of the important phytochemical, betulinic acid, in the plant.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

ACKNOWLEDGEMENT

Authors are grateful to Professor J. O. Igoli for his assistance in acquiring the ^{1}H-NMR spectra.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Srinivasan D, Nathan S, Suresh T and Perumalsamy O. Antimicrobial activity of certain Indian medicinal plants used in folkloric medicine. Ethnopharmacol. 2001; 74:217-220.
2. Hamill FA, Apio S, Mubiru NK, Ziraba RB, Mosango M, Maganyi OW, Soejarto DD. Traditional herbal drugs of Southern

Uganda. Journal of Ethnopharmacol. 2003; 84:57-78.

3. Agada GOA, Chollom SC, Gotep JG, Gambo NN, Tyem AD, Okeke IO, Nwankiti OO, Okwori AEJ. Evaluation of antimicrobial potential of ethanolic leaf and stem bark extracts of *Tamarindus indica*. International Journal of Applied Microbiology Science. 2012;1(3):26-34.
4. Sofowora EA. Recent trends in research into African medicinal plants. Journal Ethnopharmacol. 1993;38(3):209-214.
5. Clark WS. Antimicrobial activities of phenolic consistuents of *Mangolia gradiflora* L. Journal of Pharmacological Science. 1981;10:947-951.
6. Buwa LV, Staden JV. Antibacterial and antifungal activity of traditional medicina plants used against venereal diseases in South Africa. Journal of Ethnopharmacology. 2006;103:139-142.
7. Orwa C, Mutua A, Kindt R, Jamnadass R, Anthony S. *Azanza garckeana* agroforestree database: A tree reference and selection guide version 4.0; 2009. Available:http//www.worldagro-forestry Org/Af/tree
(Data based 2009; 11/12/16)
8. Okafor JC. Selection and improvement of indigenous tropical trees. Journal of Tropical Resources. 1985;2:87-95.
9. Adamu HM, Ushie OA, Lawal DS, Oga IA. Phytochemical screening of fruit of *Azanza garckeana* and root of *Acacia macrothyrsa.* International Journal of Traditional and Natural Medicines. 2013;3(1):19-25.
10. Ochokwu IJ, Dasuki A, Oshoke JO. *Azanza garckeana* (Goron Tula) as an edible indigenous fruit in North Eastern Part of Nigeria. Journal of Biology, Agericulture and Healthcare. 2015;5(15): 26-31.
11. Flayin JM, Goron Tula. An article in new dawn magazine. Published by National Association of Tula Students. 2010;1:16-17.
12. Letcher RM, Shirley IM. *o*-naphthoquinones from the heartwood of *Azanza garckeana*. Phytochemistry. 1992; 31(12):4171-4172.
13. Masila VM, Midiwo JO, Zhang J, Gisacho BM, Munayi R, Omosa LK, Wiggers FT, Jacob MR, Walker LA, Muhammad I. Anti-vancomycin-resistant *Enterococcus faecium* and *E. faecalis* activities of (-)-gossypol and derivatives from *Thespesia garckeana*. Natural Product Communications. 2015;10(4) 613-616.
14. Maroyi A. An ethnobotanical survey of medicinal plants used by the people in Nhema communal area, Zimbabwe. Journal of Ethnopharmacology. 2011;136: 347–354.
DOI: 10.1016/j.jep.2011.05.003
15. Alfred M. Traditional use of medicinal plants in south-central Zimbabwe: Review and perspectives. Journal of Ethnobiology and Ethnomedicine. 2013;9(31):1-18.
Available:http://www.ethnobiomed.com/content /11/11/31
16. Mshelia EH, Watirahyel EM, Maigari AU, Yohanna C, Ismail F. Cytotoxicity and antioxidant activity of stem bark extracts of *Azanza garckeana* (kola of Tula). European Journal of Pure and Applied Chemistry. 2016;3(2).
ISSN: 2398-1385
17. Anyam JN, Tor-anyiin TA, Igoli JO. Studies on *Dacryodes edullis* II: Phytochemical and medicinal principles of boiled seed. International Journal of Current Research in Chemistry and Pharmaceutical Science. 2015;2(4):32-37.
18. Khan ME, Yakubu UD, Kubmarawa D. Determination of mineral composition, anti-microbial activity and anti-nutrient screening of *Cleome gynandra* (Cat's Whiskers). Bristish Journal of Applied Science and Technology. 2015;5(4):396-402.
19. Linus UA, Dikko YJ, Khan ME, John VA, Igoli JO. Phytochemical screening and antimicrobial activity of *Bryophyllum pinnatum* extracts. British Biotechnology Journal. 2016;16(2):1-8.
20. Tor-Anyiin TA, Igoli JO, Anyam, Anyam JN. Isolation and antimicrobial activity of *sarracenin* from root bark of *Strychnos spinosa.* Journal of Chemical Society of Nigeria. 2015;40(1):71-75.
21. Ajayi OO. Antimicrobial effect of flavonoids from the stem of *Annona senegalensis*. Journal of Advances in Biolology and Biotechnology. 2014;2(1): 1081-2394.
22. Enzo AP. Traditional medicine and herbal remedies used in the treatment diarrhoea disease: Mode of action, quality, efficacy and safety considerations. Journal of Pharmacological Science. 2007;2(4):248–260.
23. Michael KG, Onyia LU, Jidauna SB. Evaluation of phytochemicals in *Azanza*

garckeana (Goron Tula) seed. Journal of Agriculture and Veterinary Science. 2015; 8 (5):71-74.

24. Huang S, Cui-ping Z, Kai W, Fu-liang H. Recent advances in the chemical composition of properties. Journal of Molecules. 2014;19:610-63.
25. Iinuma M, Tosa H, Asai F, Kobayashi Y. Antibacterial activity of *Xanthones* from *guttiferaeous* plants against *Mechicillin resistant Staphylococous aureus*. Journal Pharmacology. 1996;48:861-870.
26. Igoli JO, Gray IA. Friedelanone and other triterpenoids from *Hymenocardia acida*. International Journal of Physical Sciences. 2008;3(6):156-158.
27. Nazma P, Mohammad SR, Mohammad SI, Mohammad AR. Chemical and biological investigations of *Dillenia indica* Linn. Journal of the Bangladesh Pharmacological Society. 2009;4:122-125.
28. Ghias U, Bina SS, Muhammad A, Anwar S, Ashfaq A, Ala U. Chemical constituents and phytotoxicity of solvent extracted fractions of stem bark of *Grewia optiva* drummond ex burret. Middle-East Journal of Scientific Research. 2011;8(1):85-91.
29. Shumaia P, Abdul Kader Md, Abdul M, Ekramul H, Ashik M. Triterpenoids and phytosteroids from stem bark of *Crataeva nurvala buch ham*. Journal of Applied Pharmaceutical Science. 2011;1(9):47-50.
30. Fujioka T, Kashiwada Y, Kilkuskie RE, Cosentino LM, Ballas LM, Jiang JB, Janzen WP, Chen IS, Lee KH. Betulinic acid and platanic acid as anti-HIV principles from *Syzygium claviflorum*, and the anti-HIV activity of structurally related triterpenoids. Journal of Natural Products. 1994;57(2):243-247.
31. Chandramu C, Manohar RD, Krupadanam DG, Dashavantha RV. Isolation, characterization and biological activity of betulinic acid and ursolic acid from *Vitex negundo L*. Phytotherapy Research. 2003; 17(2):129-134.
32. Bringmann G, Saeb W, Assi LA, Francois GA, Narayanan SS, Peters K, Peters EM. Betulinic acid: Isolation from *Triphyophyllum peltatum and Ancistrocladus heyneanus,* antimalarial activity and crystal structure of the benzyl ester. Planta Medica. 1997;63(3):255-257.
33. Alakurtti S, Mäkelä T, Koskimies S, Kauha-luoma S. Pharmacological properties of the ubiquitous natural product botulin. European Journal of Pharmaceutical Sciences. 2006;29(1):1-13.
34. Enwerem NM, Okogun JI. Wambebe CO, Okorie DA, Akah PA. Anthelmintic activity of the stem bark extracts of *Berlina grandiflora* and one of its active principles, betulinic acid. Phytomedicine. 2001;8(2): 112-114.
35. Kinoshita K, Akiba M, Saitoh M, Ye Y, Koyama K, Takahashi K, Kondo N, Yuasa H. Antinociceptive effect of triterpenes from *Cacti.* Pharmaceutical Biology. 1998;36(1) :50-57.
36. Zuco V, Supino R, Righetti SC, Cleris L, Marchesi E, Gambacorti-Passerini C, Formelli F. Selective cytoxicity of betulinic acid on tumor cell lines, but not on normal cells. Cancer Letters. 2002;175(1):17-25.

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/17172