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PROXIMATE ANALYSES AND GAS CHROMATOGRAPHY – MASS SPECTROMETRY IDENTIFICATION OF BIOACTIVE CONSTITUENTS OF THE HYDRO-METHANOLIC SEED EXTRACT OF AZANZA GARCKEANA

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ABSTRACT

Fresh fruits of *Azanza garckeana* harvested from Tula in Kaltungo LGA, Gombe State, Nigeria were obtained and subjected to the crude maceration extraction using the combination of water and methanol (20:80 v/v) to constitute the solvent for the extraction. The protocol of the Association of Official Analytical Chemists (AOAC) was adopted for the proximate analysis of the plant sample.The standard procedure for identification of bioactive constituents using the GCMS was carefully used. The result of the proximate analysis revealed that the levels of moisture content, ash content, total fatty acid (TFA), crude fibre (CF), nitrogen, crude protein (CP) and carbohydrate in percentages (%) were found to be 5.53 ±0.03, 1.31 ± 0.00, 6.86 ± 0.01, 1.06 ± 0.00, 2.65 ± 0.00, 16.59 ±0.06 and 65.72 ±0.34 respectively. The GCMS identified bioactive constituents of the extract were up to 24 including 2-Furancarboxaldehyde (or furan-2-carbaldehyde); 1-Hexene; Furfural; Pentadecanoic acid; 2,5-Furandicarboxaldehyde; Benzoic acid; 4-Methylpyridazine; 2H-Pyran-2-one (or pyran-2-one; 3-Methyl-4-nitro-5-(1-pyrazolyl)pyrazole; 1-Pentyne; 2-Propanol; 2H-Oxireno[3,4]cyclopenta[1,2-c]furan-2-one; 2(1H)-Pyridinone (or 1H-pyridin-2-one); Propanoic acid; Butanedioic acid (or Succinic acid); 1-Methylcyclopropene; Heptadecanoic acid; 1-Oxetan-2-one; Isonicotinic acid N-oxide; Benzoyl chloride; Oleanitrile; 2-Hexene; 2-Butenoyl chloride (Crotonyl chloride) and 2,4-Hexadiyne-1,6-diol. There were various beneficial biological properties attributed to the different constituents. In conclusion, present study has revealed that proper profiling of medicinal plant using advance and sensitive analytical technique like the GCMS can helpful in more precisely predicting the therapeutic value of such plant, as with the case of the investigated hydro-methanolic seed extract of *azanza garckeana. Hydromethanolic extract was potentially better at identifying the various chemical constituents 0f the seed of Azanza garckeana.*

KEYWORDS: *Gas Chromatography – Mass Spectrometry; Bioactive Constituents of the Hydro-methanolicextract; Azanza garckeana; Proximate Analyses.*

1. INTRODUCTION

With a broad range of structural diversity and intrinsic biological activity, natural products are essential to the drug discovery process (Newman and Cragg, 2012; Chupakhin et al., 2019). This has been a major rationale for the widespread reliance on herbal or other natural products to meet the therapeutic needs of different populations (Fowler, 2006; Pan et al., 2013). Furthermore, arising from poor economic conditions and scarcity of orthodox medications, the presence of abundant bioactive components in numerous herbs has endeared them to be choice medicaments to different populations (Ekor et al., 2014; Khodadadi, 2015). This has elicited widespread scientific enquiry on their therapeutic utility.

Some of these herbs include *Moringa oleifera, Justicia secunda*, *Citrus lanatus*, *Azanza garckeana*, amongst many others (Ayoka et al., 2008; Ojeka et al., 2018). Considering the wide application of *Azanza garckeana* both as food and in ethnomedicinal applications in tropical Africa (Maroyi 2017), a proper profiling of the plant will be of immense benefits and stimulate in depth investigation. *Azanza garckeana* is a member of the Malvaceae family (Sulieman, 2019); the generic name "*Azanza*" is derived from the word "Azania", a persian word meaning black and surviving in Zanzibar. The specific name "garckeana" is in honour of a German botanist and plant collector, August Garcke (1819-1904) (Maroyi, 2017).

A lot has been said and done on the pulp of the *Azanza garckeana* plant (Jacob et al., 2016; Hlabano et al., 2020; Laz-Okenwa et al., 2023), but the ethnopharmacological value of the seed extract of the plant are still unclear; as much of the information is empirical based and lacking scientific validation (Mojeremane and Tshwenyane, 2004; Yusuf et al., 2020).

In view of the foregoing, the present study attempted an evaluation of the proximate constituents and identification of bioactive compounds in the hydro-methanolic seed extract of *Azanza garckeana* using gas chromatography-mass spectrometric (GC-MS) technique.

2. MATERIALS AND METHODS

All reagents (methanol and others as required by the respective analyses) used in the present study were analytical grade and obtained from authorized dealers.

2.1 Plant Collection and Preparation of extract

Fresh fruits of *Azanza garckeana* cultivated and harvested from Tula in Kaltungo LGA, Gombe state, Nigeria were obtained. Voucher sample of the plant was deposited at the University of Port Harcourt Herbarium (domiciled in the Department of Pharmacognosy and Phytotherapy of the Faculty of Pharmacy) where it was identified by Dr M. Suleiman and authenticated and herbarium number issued—UPHM0596.

The pulps (Sticky bark) of the fruits of the *Azanza garckeana* plant were removed and the seeds air-dried for two weeks and pulverized into fine powder using a motorized blender. The powdered sample obtained was then soaked in hydro-methanol solvent (water: methanol, 20:80 v/v). The mixture was periodically stirred to ensure a uniform mixture. After 72hours of formulating the mixture, it was filtered with Whatman filter paper and then concentrated using a rotary evaporator followed by water bath at 50^{o}C. The recovered gelatinous-like extract was then refrigerated at about 4^{o}C before use.

2.2 Proximate analysis

The protocol of Association of Official Analytical Chemists AOAC (1990) was adopted for the proximate analysis of the plant sample. The procedures for the determination of moisture content, ash content, total fatty acid (TFA), crude fibre (CF), crude protein (CP) and carbohydrate as reported by Sirajo et al., (2022) were carefully followed. Meanwhile, The Kjeldahl method was used to determine the nitrogen content of the plant sample (Rhee ,2001). Here, Copper sulphate (CuSO4) and Titanium oxide (TiO_2) were used as catalysts to digest the sample in sulfuric acid, turning N into NH3, which was then distilled and titrated.

2.3 Gas Chromatography – Mass Spectrometry (GC-MS) Analysis Protocol

The procedure adopted for the GC-MS analysis of the hydro-methanolic seed extract of *Azanza garckeana* for the present study was as earlier described by Laz-Okenwa et al., (2024). The gas chromatography (GC) portion (Agilent technologies, United States of America, Model number 7890(B) was coupled to a mass spectrometer (MS) (Agilent technologies, United States of America, Model number 5975(B).

As was previously reported by Bekinbo et al., (2020), the National Institute of Standards and Technology (NIST) database, with more than 60,000 patterns, was used to analyse the GC-MS outcome of the present study. The obtained spectrum of the unknown constituent was compared to the NIST library of known component spectrums. Further, the names of the compounds, molecular weights, and molecular formulas of all the identified constituents of the study sample were presented.

3. RESULTS

Table 1: Comparison of Proximate analysis of the Hydro-methanolic Seed Extract of *Azanza garckeana* with those of earlier reports.

Nutrients (%)	**Hydro-methanolic Seed Extract of *Azanza garckeana* (%) (Result of present study)**	**Composition of the Seed of *Azanza garckeana* (%) (Result as presented by Sirajo et al., 2022)**	**Composition of the Seed of *Azanza garckeana* (%) (Result as presented by Williams et al.,2020)**
Moisture Content	5.53 ±0.03	6.66 ± 1.66	10.23 ± 0.00
Ash Content	1.31 ± 0.00	5.10 ± 1.33	4.50 ± 0.01
Total Fatty Acid (TFA)	6.86 ± 0.01	6.60 ± 1.21	2.45 ± 0.01
Crude Fibre (CF)	1.06 ± 0.00	29.00 ± 0.45	12.4 ± 0.02
Nitrogen Content	2.65 ± 0.00	—	—
Crude Protein (CP)	16.59 ±0.06	4.85 ± 0.89	9.85 ± 0.01
Carbohydrate	65.72 ±0.34	47.88 ± 1.70	60.57 ± 0.03

Values are presented as mean ± standard error of the mean of 3 replicates (for the first column of the result on the above table).

Table 1 shows the proximate analysis of the hydro-methanolic seed extract of *Azanza garckeana*

The percentage moisture content, ash content, total fatty acid (TFA), crude fibre (CF), nitrogen content, crude protein (CP) and carbohydrate were found to be 5.53 ±0.03, 1.31 ± 0.00, 6.86 ± 0.01, 1.06 ± 0.00, 2.65 ± 0.00, 16.59 ±0.06 and 65.72 ±0.34 respectively.

Comparing these values to same indices of some earlier reports (Williams et al.,2020; Sirajo et al., 2022)on the study plaant, the outcome here is of better yield.

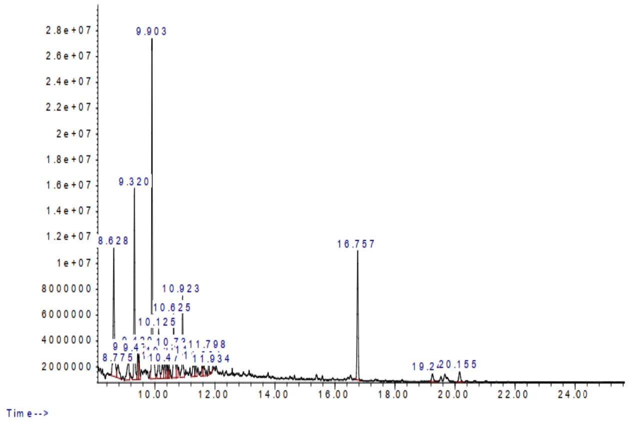

Figure 1: Chromatogram from GC-MS screening of the Hydro-methanolic Seed Extract of *Azanza garckeana.*

Table 2: Identified chemical compounds in the hydro-methanolic Seed extract of *Azanza garckeana.*

S/No	Name of compound	Retention time (RT) (Minutes)	Molecular formula	Molecular weight (g/mol)	Peak Area (%)
1	2-Furancarboxaldehyde (or furan-2-carbaldehyde)	9.903	$C_5H_4O_2$	96.08	22.08
2	1-Hexene	9.320	C_6H_{12}	84.16	13.13
3	Furfural	8.628	C_4H_3OCHO	96.08	12.27
4	Pentadecanoic acid	16.757	$C_{15}H_{30}O_2$	242.4	10.32
5	2,5-Furandicarboxaldehyde	10.625	$C_6H_4O_3$	124.09	5.88
6	Benzoic acid	10.923	$C_7H_6O_2$	122.12	5.43
7	4-Methylpyridazine	10.125	$C_5H_6N_2$	94.11	4.11
8	2H-Pyran-2-one (or pyran-2-one)	9.120	$C_5H_4O_2$	96.08	3.23
9	3-Methyl-4-nitro-5-(1-pyrazolyl)pyrazole	8.775	$C_7H_7N_5O2$	193.16	2.19
10	1-Pentyne	9.429	C_5H_8	68.12	2.06
11	2-Propanol	11.303	C_3H_8O	60.1	1.93
12	2H-Oxireno[3,4]cyclopenta[1,2-c]furan-2-one	10.732	$C_{10}H_{14}O_5$	214.21	1.87
13	2(1H)-Pyridinone (or 1H-pyridin-2-one)	9.471	C_5H_5NO	95.1	1.85
14	Propanoic acid	10.246	$C_3H_6O_2$	74.08	1.79
15	Butanedioic acid (or Succinic acid)	10.326	$C_4H_6O_4$	118.09	1.73
	1-Methylcyclopropene	11.366	C_4H_6	54.09	1.44
16	Heptadecanoic acid,	20.155	$C_{17}H_{34}O_2$	270.5	1.34
17	1-Oxetan-2-one	10.387	$C_5H_6O_2$	98.1	1.34
18	Isonicotinic acid N-oxide	11.798	$C_6H_5NO_3$	139.11	1.22
19	Benzoyl chloride	11.634	C_7H_5ClO	140.56	1.21
20	Oleanitrile	19.247	$C_{18}H_{33}N$	263.5	1.04
21	2-Hexene	10.475	C_6H_{12}	84.16	0.78
22	2-Butenoyl chloride (Crotonyl chloride)	10.431	C_4H_5ClO	104.53	0.77
23	2,4-Hexadiyne-1,6-diol	11.934	$C_6H_6O_2$	110.11	0.15

Table 2 shows the identified chemical compounds in the hydro-methanolic seed extract of *Azanza garckeana.*

In a decreasing order of percentage abundance (peak area in percentage), the following active compounds were identified by the current study the plant extract: 2-Furancarboxaldehyde (or furan-2-carbaldehyde); 1-Hexene; Furfural; Pentadecanoic acid; 2,5-Furandicarboxaldehyde; Benzoic acid; 4-Methylpyridazine; 2H-Pyran-2-one (or pyran-2-one; 3-Methyl-4-nitro-5-(1-pyrazolyl)pyrazole; 1-Pentyne; 2-Propanol; 2H-Oxireno[3,4]cyclopenta[1,2-c]furan-2-one; 2(1H)-Pyridinone (or 1H-pyridin-2-one); Propanoic acid; Butanedioic acid (or Succinic acid); 1-Methylcyclopropene; Heptadecanoic acid; 1-Oxetan-2-one; Isonicotinic acid N-oxide; Benzoyl chloride; Oleanitrile; 2-Hexene; 2-Butenoyl chloride (Crotonyl chloride) and 2,4-Hexadiyne-1,6-diol. The percentage abundance of the aforementioned compounds ranged between 0.15% (for 2,4-Hexadiyne-1,6-diol—the lowest) and 22.08% (for 2-Furancarboxaldehyde— the highest). The retention time, molecular weight, molecular weight are as indicated on table 2 for each identified compound

Table 3: Probable biologic properties of the identified compounds in the hydro- methanolic seed extract of *Azanza garckeana.*

S/No	Name of Compound	Potential Biological Effects
1	2-Furancarboxaldehyde (or furan-2-carbaldehyde)	Exhibits antibacterial and antifungal properties and is useful in pesticides, cosmetics, and pharmaceutical products (Rigal and Gaset, 1983; Oskoueian et al., 2011).
2	1-Hexene	No clear cut biological activity recorded so far but identified to be a possible human metabolite (Chiappe et al., 1998; Onel et al., 2019)
3	Furfural	Used as a flavouring agent at trace levels in a range of food items and alcoholic and non-alcoholic beverages; Occurs naturally in food substances; However exposure of the skin, eyes, nose, and throat to raised levels/concentrated synthetic forms of furfural may result in irritations causing coughing and/or dyspnea (HSFS, 2000; Eseyin, 2015) and an appreciable number of chromosome aberrations (Mehta, 2015; Jilani and Olson, 2023).
4	Pentadecanoic acid	A vital odd-chain saturated fatty acid with a wide range of biological activities: immunological, hepatic, and cardiometabolic health boosting attributes (Venn-Watson and Butterworth 2022). It Known to stimulate adenosine monophosphate -activated protein kinase (AMPK) and promotes basal and insulin-stimulated glucose uptake in C_2C_{12} myotubes (Fu et al., 2021).
5	2,5-Furandicarboxaldehyde	A bio-based crosslinking agent that takes the place of glutaraldehyde in the immobilisation of covalent enzymes (Millan et al., 2021; Danielli et al., 2022).
6	Benzoic acid	Enhances absorption and digestion processes in the gut of animal models: shows antibacterial and antifungal potentials (Mao et al., 2019).
7	4-Methylpyridazine	No clear cut biological activity recorded so far.
8	2H-Pyran-2-one (or pyran-2-one)	Numerous naturally occurring substances, many of which have intriguing pharmacological characteristics, contain the pyran-2-one motif (Parker et al., 1997; Almalki, 2023). Pyran is amongst the commonest structural components of natural products, like benzopyrans, coumarins, flavonoids, and xanthones. It has also shown some neuroprotective properties in mammalian models (Almalki, 2023).
9	3-Methyl-4-nitro-5-(1-pyrazolyl)pyrazole	Numerous biological activities, including anti-microbial, anti-fungal, anti-tubercular, anti-inflammatory, anti-convulsant, anticancer, antiviral, angiotensin converting enzyme (ACE) inhibitor, neuroprotective, cholecystokinin-1 receptor antagonistic, and estrogenic properties, have been documented for pyrazoles (Naim et al., 2016)
10	1-Pentyne	Known to be a portion of a compound (5-Bromo-1-pentyne (5BP)) that serves as a radical chain that make up an enzymatic antioxidant system; which catalyzes the breakdown hydrogen peroxide to water and oxygen (Biosynth, 2024).
11	2-Propanol	Known bioreagent in molecular biology and used to precipitate DNA and RNA from mammalian tissues (Bhat et al., 2015). As a typical ethanol, it is believed to exert inhibitory effect on excitatory neurotransmission and could potentiate or lengthen the duration of CNS conditions (Abramson and Singh, 2000; Punja, 2014).
12	2H-Oxireno[3,4]cyclopenta[1,2-	No clear cut biological activity recorded so far.

	c]furan-2-one	
13	2(1H)-Pyridinone (or 1H-pyridin-2-one)	Possess numerous biological activities, including analgesic, antifungal, antimalarial, anti-inflammatory, antibacterial, anti-HIV, phytotoxic, antitumoral, and antiviral effects (Jacinto et al., 2009).
14	Propanoic acid (or propionic acid)	Compound and derivatives are known to possess numerous biological activities, including antioxidant (Dracheva et al., 2009), antitumor, analgesic, antimicrobial (Avetisyan et al., 2010), and antidiabetic (Berzosa et al., 2011).
15	Butanedioic acid (or Succinic acid)	Exists as the anion succinate in living organisms; it thus serves a variety of biological purposes. It is a metabolic intermediate that the enzyme succinate dehydrogenase transforms into fumarate in complex 2 of the electron transport chain, which is involved in the synthesis of ATP, and it is a signalling molecule (Ackrell et al., 2019).
16	1-Methylcyclopropene	No clear cut biological activity recorded so far.
17	Heptadecanoic acid,	Functions as a mammalian metabolite, a Daphnia magna metabolite and an algal metabolite (Jenkinsetal., 2015; Windisch and Fink, 2018)
18	1-Oxetan-2-one	No clear cut biological activity recorded so far.
19	Isonicotinic acid N-oxide	No clear cut biological activity recorded so far.
20	Benzoyl chloride	No clear cut biological activity recorded so far.
21	Oleanitrile	No clear cut biological activity recorded so far.
22	2-Hexene	No clear cut biological activity recorded so far.
23	2-Butenoyl chloride (Crotonyl chloride)	No clear cut biological activity recorded so far.
24	2,4-Hexadiyne-1,6-diol	No clear cut biological activity recorded so far.

Table 3 shows the probable biologic properties of the identified compounds in the hydro-methanolic seed extract of *Azanza garckeana* as reported by previous studies. These biological attributes vary for the different active constituents and include antibacterial, antifungal and antitumour properties, and immunological, hepatic, and cardiometabolic health boosting attributes, etc (Rigal and Gaset, 1983; Oskoueian et al., 2011; Venn-Watson and Butterworth 202).

4. DISCUSSIONS

New bioactive compounds have been discovered as a result of the advancement and increased sensitivity of contemporary analytical techniques for the identification and quantification of bioactive ingredients in natural products including plants/herbs. These techniques have also made it possible to gather a wealth of information about the therapeutic potential of medicinal plants (Momodu et al., 2022). A typical example of such technique is the GC-MS. Consequently, the present study evaluated the proximate and GCMS screening of the hydro-methanolic seed extract of *azanza garckeana* and made some findings which are presented here.

Comparing the finding of the present study on proximate analysis to reports from previous studies by Williams et al., (2020) and Sirajo et al., (2022), the moisture, ash, and crude contents of the hydro-methanolic seed extract of *Azanza garckeana* investigated by the present study had lower levels. Whereas, the levels of total fatty acids, and carbohydrate were respectively higher in the present study with respect to the earlier stated studies. This variation may be due to the longer air-drying period and multiple blending of the seed to finer powder employed in this present study as against the shorter time of air-drying and the use of pestle and mortar for pulverizing the seed of the plant by Sirajo et al., (2022). The reduced moisture level observed by the present study would less likelihood of bacterial growth in the extract, thus, ensuring longer shelf –life and use. The less fiber content also indicated availability of wider surface area for maximum extraction of the bioactive molecules. In addition, the higher level of total fatty acid and carbohydrate is an indication that the method of processing the extract better conserved the nutrients/active ingredients than recorded in the works of others.

The GCMS identified bioactive constituents of the hydro-methanolic seed extract of *azanza garckeana* in the present study were much more abundant (up to 24) than some earlier report (by Momodu et al., 2022 with less than 20). The outcome of the present study is suggestive that the combination of water and methanol (20:80 v/v) to constitute the solvent for the extraction could be better at harvesting both that using either of polar or non-polar solvent only.

Considering the outcome on the probable biologic properties of the identified compounds in the hydro-methanolic seed extract of *Azanza garckeana* as previously reported by other scholars, some constituents (like 4-Methylpyridazine and 2H-Oxireno[3,4] cyclopenta[1,2-c]furan-2-one) are yet to be clearly explored for their actual biological effects. So, this finding has revealed the need for more studies on such constituents of the extract.

5. CONCLUSION

The present study has shown that proper profiling of a medicinal plant using advanced and sensitive analytical techniques like the GCMS can be helpful at precisely predicting the therapeutic value of such plant, as with the case of the investigated hydro-methanolic seed extract of *Azanza garckeana. Our study also suggest that* longer duration of air drying the seed of *Azanza garckeana* and multiple blending may result in reduced moisture, crude fibre and ash contents and increased nutrient value (carbohydrate and fatty acid) in the extract. Furthermore our study suggest that hydromethanolic extract of the seed of *Azanza garckeana* may produce much abundant polar and non-polar bioactive constituents of the extract than when used individually.

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