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PHYTOCHEMICAL, ANTIMICROBIAL, PROXIMATE AND HEAVY METALS ANALYSIS OF EXTRACTS OF THE BARK OF TETRAPLEURA TETRAPTERA FRUIT

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Abstract: Extraction of the bark of *Tetrapleura tetraptera* fruit was done using methanol, ethanol and distilled water to obtain the crude extracts. These were subjected to antimicrobial, proximate and heavy metal screenings. Ten different phytochemicals were screened namely; alkaloids, flavonoids, phlobatannins, saponins, steroids, tannins, terpeniods, carbohydrates, phytosterols and phenolic compounds. The phytochemical analysis of ethanol extract revealed the presence of almost all the phytochemicals above with exception of saponins and phytosterols, however, methanol and water extracts, revealed the presence of most phytochemicals except flavonoids, phlobatanin and phytosterols respectively. Antimicrobial analysis of the aqueous, ethanol and methanol extracts respectively was done using the agar well diffusion method against clinical isolates of gram positive bacteria (*staphylococcus aureus*) and gram negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). The result of methanol extract showed higher inhibition than that of water and ethanol extracts when compared with the positive control Ciprofloxacin. Proximate analysis of the plant showed the following results; ash content (85%), crude fiber (7.8%), moisture content (38%), protein (0.2%) and carbohydrate content (69.38%). Heavy metals analysis conducted, showed the presence of zinc with the highest value of (31.309ppm) followed by lead (Pb) with (1.574ppm), then Arsenic with (0.955ppm), copper (0.138ppm), cadmium (0.073ppm) and mercury (0.068ppm). Mercury (Hg) is the least metal present in this plant. All were within W.H.O acceptable limit except Arsenic. These results is a confirmation that the bark of *Tetrapleura tetraptera* has medicinal value as speculated by the orthodox medicine, but the presence of some heavy metals in the plant especially lead (Pb) should be taken into consideration to avoid taking contaminated extracts as medicine.

K**eywords:** Phytochemicals; *Tetrapleura tetraptera*; Heavy metals; Proximate analysis, Antimicrobial analysis.

1. Introduction

Over the past years, plants have become an indispensable source of food and medicine. To a larger extent, most people depend greatly on medicinal plants as an important source of remedy and treatment for some casual and life-threatening diseases [1].

As a result of this, there is a growing demand all over the world for these medicinal plants. Aside from tackling diseases, most people are resorting more to these medicinal plants as a means of reducing the use of chemical (orthodox) medicines because of their numerous side effects.

Interestingly, most of these plants are used in our everyday cooking as herbs, spices, seasonings and preservatives. Furthermore, the use of these medicinal plants as food, preservatives, spices [1, 2] and as instrument for preventing and tackling the development of diseases in human bodies has become an area of extensive studies.

One of such valuable medicinal plants is Aridan (*Tetrapleura tetraptera*), locally known as Oshosho. This is a flowering plant that belongs to the mimosaceae or pea family native to western Africa [2]. The fruit hangs on stout stalks at the edges of the branches and are characteristically brownish in colour. The fruit (pod) measures about 15 to 25 cm long and is distinguished by its four (4) longitudinal ridges that are slightly curved. Two of the ridges are woody while the other two contains soft, aromatic and oily pulp. The pod contains tiny, hard seeds that measure approximately 8 mm long. The fruit is distinguished by its fleshy pulp when fresh but this fleshy pulp tends to be very strong when dried. The inner part of the *Tetrapleura tetraptera* fruit is characterized by tiny black-brownish seeds. Aridan is highly sought after due to its high medicinal and aromatic values and as such it is used for several purposes ranging from culinary, healing, and therapeutic to cosmetology. Researchers also reveal that this plant has anti-inflammatory, hypertensive, neuromuscular, cardiovascular, anti-ulcerative, molluscicidal and anti-microbial properties [2, 3]. The tree has many uses. Its sweet fragrance is highly valued; its fruit is used to spice dishes such as banga (Palm fruits) soup, and also for medicinal purposes. Being originally from Western Africa, various countries and tribes from this part of the world have different names for the aridan plant for example; the Igbo part of Nigeria refers it as oshosho or osakirisa, the Yorubas in Nigeria call it aidan, or ubukirihu, Ibibio calls it Uyayak, Hausa calls it dawo, while Boki in Cross River state called Kekpen. Also the Twi in Ghana refers to it prekese [3]. The aim of this work is to determine the phytochemical, antimicrobial, proximate and heavy metal analyses of the aqueous, ethanol and methanol extracts of the bark of *Tetrapleura tetraptera* fruits respectively.

2. Materials and Methods

The regents used for this work were of analytical grade and were obtained from the Biochemistry laboratory of the Chemical Sciences Department, Godfrey Okoye University, Enugu, Nigeria. Their preparation was carried out according to the specified standard.

2.1. Preparation of plant materials

Figure **1** is the picture of *Tetrapleura tetraptera* fruits. The fruits were purchased in Abakpa market in Enugu state, Nigeria and were identified and confirmed by Mr Ijearu, a botanist in the Department of Botany, Nnamdi University Awka, Nigeria.

The fruits were cut opened and the seeds were removed while the barks were crushed in a wooden mortar with a pestle. Thereafter they were pulverized in an electric blender, sterilized with ethanol (70%) and stored in an air tight container for further analysis.

200g each of the pulverized plant material was packed into a soxhlet extractor and extracted with 500ml of methanol, ethanol and distilled water respectively. The extracts were concentrated using a rotary evaporator and semi-solids crude extracts were obtained. They were properly stored in well labeled sterile samples bottles for further analyses.

Figure 1. *Tetrapleura tetraptera fruit.*

2.2. Phytochemical Screening

The extracts obtained above were screened for the presence of flavonoids, alkaloids, steroids, saponins, tannins, phlobatanin, phytosterols, terpenoids and phenolic compound according to standard procedures [4]. 1g of each of the crude extracts obtained was dissolved in 100ml of its solvent and was subjected to the qualitative screening as described below.

2.2.1. *Test for presence of alkaloids*

To 2ml of each of the filtrates of the crude extracts, a drop of Mayer's reagent was added. A creamy white precipitate was formed which indicates the presence of alkaloids.

2.2.2. *Test for presence of carbohydrates*

To 1ml of each of the filtrates of the extracts, 1ml of Benedict's reagent was added and the mixtures were heated on water baths for 2mins. A reddish precipitate was formed indicating the presence of sugar (carbohydrates).

2.2.3. *Test for the presence of saponins*

5ml of each of the extracts was diluted with distilled water and made up to 20ml. The suspension was poured into a graduated cylinder and shaken for about 10min. A layer of foam was obtained which indicates the presence of saponins.

2.2.4. *Test for presence of phytosterols*

2ml of each of the extracts was mixed with 2ml of acetic anhydride respectively. Two drops of concentrated H_2SO_4 were added to each of the mixtures slowly along the sides of the test tubes. An array of colour changes show the presence of phytosterols.

2.2.5. *Test for presence of phenolic compounds*

2ml of each of the extracts was diluted in 5ml of distilled water respectively. Thereafter two drop of 5% ferric chloride solution was added to the mixtures. A dark green coloration was obtained which indicates the presence of phenolic compounds.

2.2.6. *Test for presence of tannins*

0.5g of the dried powdered sample was poured into a test tube and boiled in 20ml of distilled water. This was later filtered and few drops of 0.1% ferric chloride solution were added. A brownish green coloration formed indicates the presence of tannins.

2.2.7. *Test for presence of flavonoids*

5ml of dilute ammonia solution were added to 5ml of the aqueous extract, followed by the addition two drops of concentrated H_2SO_4. The appearance of a yellow coloration indicates the presence of flavonoids.

2.2.8. *Test for presence of terpenoids*

5ml of each of the extracts was mixed with 2ml of chloroform and concentrated H_2SO_4. The mixtures separated into two layers. At the interface of the layers a reddish brown coloration was formed which indicates the presence of terpenoids .

2.2.9. *Test for the presence of phlobatannins*

5ml of the aqueous extract of plant samples was boiled with 1% (2ml) aqueous hydrochloric acid. The formation of a red precipitate indicates the presence of phlobotannins.

2.3. Antimicrobial Screening

2.3.1. Test Organism

The microorganisms used for the antimicrobial screening were obtained from the Microbiology laboratory, Department of Biological Sciences, Godfrey okoye university, Enugu, Nigeria. These include one gram-positive bacteria (*Staphylococcus aureus*) and two gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*)

2.3.2. Antimicrobial Sensitivity Test

The antimicrobial screening of the aqueous, ethanol and methanol extracts respectively was carried out using agar well diffusion method [5] as described below.

20.50g of Muller Hinton agar was weighed and diluted in 540ml of distilled water contained in a 1000ml flat bottom flask, following manufacturer's instructions. The flask was shaken and then covered with an aluminum foil and autoclaved at 121^{o}C for 15min and allowed to cool. Thereafter, 15ml of molten Muller Hinton agar was poured into the Petri dishes with flame beside to avoid contamination by aerobic organisms and were left to solidify for 5h. 13g of nutrient broth was also prepared following manufacturer's instructions and was diluted in 30ml of distilled water, autoclaved for 15min at 121^{o}C and allowed to cool. Thereafter, 10ml of nutrient broth was placed in three (3) test tubes respectively to standardize the organisms used. These organisms were smeared using swab sticks on the solidified Petri dishes and incubated for 24 hours. After that, serial dilutions were carried out for both antibiotics that as used as a positive control (Ciproflaxine 500mg) and the crude extracts. The following concentrations were used (250mg, 125mg, 62.5mg, 31.25mg, 15.625mg) in 2ml of distilled water respectively for each of the test tubes. On agar plates of different organisms prepared above, holes were bored using a cork borer with each plate containing five (5) wells for the extracts while the sixth (6th) well in the middle was for the antibiotics (Ciproflaxine). Three drops of both the antibiotics and the extracts were placed in their respective holes. The extracts and the drug were allowed to diffuse for 30 minutes then incubated at 37^{o}C for 24h. The degree of antimicrobial activity of each of the extracts was measured as the inhibition zone diameter in millimeters.

2.4. Proximate analysis

2.4.1. Ash content

An empty platinum crucible was washed, dried and weighed. 1g of the plant sample was poured into the crucible and place in a muffler furnace. It was burn for 1h (until sample turned to white ash) and thereafter cooled in a desiccator and the new weight was noted.

2.4.2. *Moisture content*

The moisture content of the plant was determined using a moisture analyzer. The dry sample was placed in the analyzer and calibrated for 30 min for result output. Using the values obtained from the analyzer the moisture content was determined thus;

$$M_n = \frac{M_w - M_d}{M_w} \times \frac{100}{1}$$

Where; M_n = moisture content of the material

M_w = wet weight of the sample

M_d = weight of the sample after drying.

2.4.3. *Crude fibre*

2g of the plant sample was boiled in petroleum ether at 52^{o}C in order to remove any fat present and was allowed to dry. Thereafter the dried plant material was placed in a 250ml conical flask containing 100ml dilute 1.25% H_2SO_4 and boiled on a hot plate for 30 min. The residue was then filtered and washed with hot distilled water and tested for acidity with the aid of a pH paper. Afterward, the residue was resoaked in 1.25% NaOH (200ml) and boiled for 30min and filtered. The residue was then washed with 25ml of 1.25% H_2SO_4 and with three 50ml portions of distilled water and 25ml of ethanol. The residue was removed and the transferred to an ashing crucible whose weight has been noted (W_1). The residue was dried for 2h at 130^{o}C and cooled in a desiccator and reweighed (W_2). The residue was again placed in a crucible and ignited for 30min at 600^{o}C and finally cooled in desiccators and reweighed again (W_3) [6]. The percentage of the crude fibre in the sample was determined thus:

$$\%\ \text{of Crude fibre} = \frac{\text{Loss in weight on ignition } (W_2 - W_1) - (W_3 - W_1)}{\text{Weight of sample}} \times \frac{100}{1}$$

2.4.4. Crude Protein (Kjeldahl method).

The Kjeldahl method is an official method that has been described in different normatives such as Association of Official Analytical Chemists (AOAC), the United States Environmental Protection Agency (USEPA), and International Organization for Standardization (ISO), Pharmacopeias and different European Directives. Its procedure involves three major steps namely; digestion, distillation and titration. In the digestion step, organic nitrogen is converted into ammonium ions (NH_4^+), in the distillation step; NH_3 is distilled and retained in a receiver vessel while in the titration step nitrogen is determined [5,6].

1. Digestion

The aim of the digestion procedure is to break all nitrogen bonds in the sample and convert all of the organically bonded nitrogen into ammonium ions (NH_4^+). Organic carbon and hydrogen form carbon dioxide and water. In this process the organic material carbonizes which can be visualized by the transformation of the sample into black foam. During the digestion, the foam decomposes and finally a clear liquid indicates the completion of the chemical reaction. For this purpose, the sample is mixed with sulfuric acid at temperatures between 350^{o}C and 380^{o}C. The higher the temperature used, the faster digestion can be obtained. The speed of the digestion can be greatly improved by the addition of salt and catalysts. Potassium sulfate is added in order to increase the boiling point of sulfuric acid and catalysts are added in order to increase the speed and efficiency of the digestion procedure. Oxidizing agents can also be added to improve the speed even further [5].

$$\underset{\text{Protein (-N)}}{\text{Sample}} + H_2SO_4 \xrightarrow{\text{Catalyst}} (NH_4)_2SO_4 + CO_2 + H_2O$$

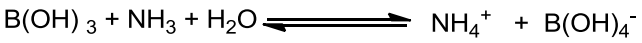
After digestion is completed the sample is allowed to cool to room temperature, then diluted with water and transferred to the distillation unit.

2. Distillation

During the distillation step the ammonium ions (NH_4^+) are converted into ammonia (NH_3**)** by adding an alkali (NaOH). The ammonia (NH_3) is transferred into the receiver vessel by means of steam distillation.

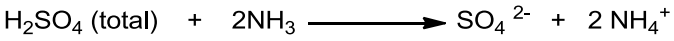
$$(NH_4)_2SO_4 + 2NaOH \rightleftharpoons 2NH_3\,(gas) + Na_2SO_4 + 2H_2O$$

The receiving vessel for the distillate is filled with an absorbing solution in order to capture the dissolved ammonia gas. Common absorbing solutions involve aqueous boric acid [$B(OH)_3$] of 2-4% concentration. The ammonia is quantitatively captured by the boric acid solution forming solvated ammonium ions [5].



$$B(OH)_3 + NH_3 + H_2O \rightleftharpoons NH_4^+ + B(OH)_4^-$$

Also other acids can be used as precisely dosed volume of sulfuric acid or hydrochloric acid that captures the ammonia forming solvated ammonium ions.

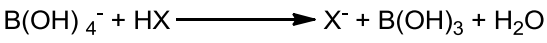


$$H_2SO_4\ (total) + 2NH_3 \longrightarrow SO_4^{2-} + 2\ NH_4^+$$

3. Titration

The concentration of the captured ammonium ions can be determined using two types of titrations:

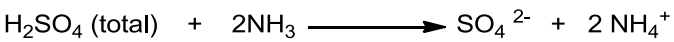
When using the boric acid solution as absorbing solution, an acid-base titration is performed using standard solutions of sulfuric acid or hydrochloric acid and a mixture of indicators. Depending on the amount of ammonium ions present, concentrations in the range of 0.01N to 0.5N are used. Alternatively the end point can be determined potentiometrically with a pH-electrode. This titration is called direct titration.



$$B(OH)_4^- + HX \longrightarrow X^- + B(OH)_3 + H_2O$$

HX= strong acid ($X= Cl^-$, etc.)

When using sulfuric acid standard solution as absorbing solution, the residual sulfuric acid (the excess not reacted with NH_3) is titrated with sodium hydroxide standard solution and by difference the amount of ammonia is calculated. This titration is called back titration [5, 7].



$$H_2SO_4\ (total) + 2NH_3 \longrightarrow SO_4^{2-} + 2\ NH_4^+$$

2.4.4.1 Determination of total nitrogen

2g of powdered sample was digested in a Kjeldahl digestion flask by boiling with 20ml of concentrated H_2SO_4 and a Kjeldahl digestion tablet (catalyst) until the mixture was clear. The digest was filtered into a 250ml volumetric flask and the solution made up to the mark with distilled water and connected to an ammonia distillation unit. Ammonia was steam distilled from the digest to which 50ml of 45% sodium hydroxide was added. Afterward, 150ml of the distillate was collected in a conical flask containing 100ml 0.1M HCl and methyl red indicator. The ammonia that distilled into the receiving conical flask reacted with the acid and the excess acid in the flask was estimated by back titration against 2.0M NaOH and the end point was observed by a colour change [5, 6, 7].

2.4.5. Carbohydrates

The carbohydrate content was determined by summing up the ash, moisture, crude fibre and protein contents and subtracting the value from 100.

2.5.1. Heavy *metals*

Some heavy metals that were analyzed include: Arsenic (As), mercury (Hg), lead (Pb), cadmium (Cd), zinc (Zn) and copper (Cu). Using nitric- pechloric acid digestion method.

1g of sample was placed in 250ml digestion tube and 10 ml of concentrated HNO_3 was added. The mixture was boiled gently for 30-45 min to oxidize all oxidizable matter. After cooling, 5ml of 70% $HClO_4$ was added to the mixture and was boiled gently until dense white fumes appeared. After cooling, 20ml of distilled water was added and the mixture was boiled further to release any fumes. The solution was cooled and filtered and transferred to a 250ml volumetric flask. Distilled water was then added to make up the mark. This was then subjected to analysis using Atomic absorption spectrophotometer (AAS) [7].

3. Results and Discussion

3.1. Phytochemical Analysis

The presence of secondary metabolites in bark of *Tetrapleura tetraptera* fruit is responsible for its medicinal value and use as a local drug [8]. This assertion is in line with the findings of other authors that the presence of phytochemicals in plants is responsible for their medicinal values [9, 10].

The aqueous extract of the bark of *Tetrapleura tetraptera* fruit showed the presence of high saponins and tannins while alkaloids, flavonoids and carbohydrates were absent. Ethanol extract revealed the presence of high tannins and flavonoids while in the methanol extract saponins the highest. The result of phytochemical analysis is summarized in table 1 below.

Table 1*.Qualitative report on Phytochemical Analysis*

S/N	Test	Solvents		
		Ethanol	Methanol	Water
1.	Alkaloids	+	-	-
2.	Flavonoids	+++	-	-
3.	Phlobatanin	-	++	++
4.	Saponins	+	+++	+++
5.	Steroids	+	++	+
6.	Tannins	+++	++	++
7.	Terpenoids	+	+	+
8.	Carbohydrates	-	-	-
9.	Phytosterols	++	+	+
10.	Phenolic compounds	+	+	+

Keys*: + = Insignificantly present, ++= Moderately present, +++ = Abundantly Present, - = Not present.*

3.2 Antimicrobial Analysis

Antimicrobial properties of substances are desirable tools in the control of undesirable microorganisms especially in the treatment of infectious diseases and in food spoilage. The active components of the medicinal plant usually interfere with growth and metabolism of microorganisms [11, 12]. The sensitivity test of the aqueous, methanol and ethanol extracts of the bark of *Tetrapleura tetraptera* fruit are given below in table 3, 4 and 5 respectively. It can be inferred from these tables that methanol extract was active against all the three tested bacteria. Water and ethanol extracts were active against two out of the three tested bacteria. This indicated that methanol extract showed remarkable activity against all the microorganisms used in this work and can be used as antibiotics as speculated.

Table 3: Result of sensitivity test for aqueous extract against test organisms

S/N	Organism	Zone of inhibition for water extract	Zone of inhibition for Ciprofloxacin (Standard).
1.	Staphylococcus aureus	9mm	30mm
2.	Escherichia coli	24mm	40mm
3.	Pseudomonas aeruginosa	0.2mm	40mm

Table 4: Result of sensitivity test for methanol extract against test organisms

S/N	Organism	Zone of inhibition for methanol extract	Zone of inhibition for Ciprofloxacin (Standard).
1.	Staphylococcus aureus	6mm	40mm
2.	Escherichia coli	12mm	40mm
3.	Pseudomonas aeruginosa	12mm	40mm

Table 5: Result of sensitivity test for ethanol extract against test organisms

S/N	Organism	Zone of inhibition for ethanol extract	Zone of inhibition for Ciprofloxacin (Standard).
1.	Staphylococcus aureus	0.2mm	45mm
2.	Escherichia coli	5mm	29mm
3.	Pseudomonas aeruginosa	13mm	40mm

3.3. Proximate Analysis

Table **6** shows the result of the proximate analysis. From the result obtained, ash content was observed to be 4.0%. The percentage of crude fibre was 18.1%. High fibre content in food has been found to have some physiological effect on gastrointestinal tract and low fiber in diet is undesirable and may cause constipation. The protein content was 0.72%. This result shows the bark of *Tetrapleura tetraptera* fruit is not a good source of protein. This is because any source of food that provides less than 12% of the caloric value of protein is not a good source of protein [13, 14].

Table 6: Result on proximate analysis.

Components (%)	**Amount present (%)**
Ash content	4
Crude fibre	18.1
Moisture content	7.8
Proteins	0.72
Carbohydrate content	69.38

3.4. Heavy Metals Analysis

Table **7** shows the result of heavy metal analysis. From the result tabulated it can be observed that mercury (Hg), lead (Pb), cadmium (Cd), zinc (Zn) and copper (Cu) were all within W.H.O permissible limit of medicinal plant. However, the concentration of Arsenic was slightly higher than W.H.O permissible limit with the value of 0.955 against the 0.01W.H.O permissible limit [15, 16]. The implication of this result is that the bark of *Tetrapleura tetraptera* fruit is safe for consumption since they contain heavy metals below the W.H.O permissible limits apart from Arsenic (As). The high concentration of Arsenic may be attributed to the environment where the plant was harvested [17]. Many metals such as zinc, copper, chromium, iron, and manganese are essential to body provided they are present in the acceptable proportions. But if these metals that are present in the plants exceed the acceptable limits, they can be accumulating in our bodies over time as we consume them which could results poisoning with serious damaging effects [18].

Table 7: Result of heavy metals analysis of the bark of *Tetrapleura tetraptera* fruit and their concentrations

S/N	Element	Concentration in ppm	Permissible limit by W.H.O (ppm)
1.	Zinc	31.309	50
2.	Arsenic	0.955	0.01
3.	Mercury	0.068	0.05
4.	Lead	1.574	10
5.	Cadmium	0.073	1.5
6.	Copper	0.138	2.3

4. Conclusions

The aqueous, ethanol and methanol extracts of the bark of *Tetrapleura tetraptera* fruit have been discovered to possess promising medicinal potentials. From the antimicrobial analysis result, it is observed that the methanol, aqueous and ethanol extracts of the above showed significant activity against *S. typhi* and *S. aureus* and *Pseudomonas aeruginosa* respectively**.** The seeds showed the presence of a good number of photochemicals which is an indication that they possess medicinal value hence their ability to exhibit antimicrobial activity on the different microorganisms tested. From the proximate analysis, this plant can be use as a source of energy booster, due its high carbohydrates content. Since the percentage of heavy metals present in the seeds are even below the W.H.O permissible limit, therefore they safe for consumption.

Conflicts of Interest

The author hereby declares that there is no conflict of interest regarding the publication of this article.

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