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Screening of the phytochemicals, antimicrobial, proximate and heavy metals compositions of *Pueraria phaseoloides* leave extracts

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Abstract
The preliminary investigation of different phytochemicals, antimicrobial, proximate and heavy metal analyses of three extracts of *Pueraria phaseoloides* leaves is reported. The extraction was done using three different solvents namely; distilled water, ethanol, and n-hexane respectively. Ten different phytochemicals were screened namely; alkaloids, flavonoids, phlobatannins, saponins, steroids, tannins, terpeniods, carbohydrates, phytosterols and phenolic compounds. The ethanol extract revealed the presence of only five out of the ten phytochemicals that were screened namely; alkaloids, flavoniods, saponins, tannins and carbohydrates in varying amount while steroids, phobatannins, Phytosterols, terpenoids and phenolic compounds respectively were absent. The aqueous (water) extract revealed the presence of all the phytochemicals screened while the n-hexane extract showed the presence of only three out of the ten phytochemicals namely; saponins, tannins and carbohydrates. The antimicrobial screenings of the extracts were carried out via the agar well diffusion method against one gram positive bacterium (*staphylococcus aureus*) and two gram negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). All the extracts showed remarkable activity against the microorganisms. Proximate analysis of the leaves showed the following results; ash content (3.5%), crude fiber (16.9%), moisture content (4.40%), protein (0.34%) and carbohydrate content (74.86%). Heavy metals determination of the leaves revealed Zinc (Zn) with highest value of (16.249 ppm) followed by others Lead (Pb) (1.025 ppm), Copper, (Cu) (0.281ppm), Cadmium (Cd) (0.112 ppm), Mercury (Hg) (0.083 ppm) respectively while Arsenic (As) was absent. However, the values of all the metals were below the W.H.O acceptable limit of 0.05ppm apart from Mercury (Hg) which had a value slightly higher. The above results obtained from this study support the claim that *Pueraria phaseoloides* leaves have medicinal value as speculated by traditional medicine practitioners and that they are also safe for consumption.

Keywords: phytochemicals, *Pueraria phaseoloides*, heavy metals, proximate analysis, antimicrobial

1. Introduction
The use of plants as medicine predates human history. Many of the herbs and spices used by humans to season food also yield useful medicinal compounds. The use of herbs and spices in cuisines as a response to the threat caused by food borne pathogens has become a welcome development [1]. Studies have shown that in tropical climates where pathogens are the most abundant, recipes are mostly spiced and those spices with the most potent antimicrobial activity are carefully selected and used traditionally. The World Health Organization (W.H.O) estimated that about 80% of the people in developing countries of the world rely on traditional medicine for their primary healthcare, and about 85% of the traditional medicines involve the use of plant extracts [1, 2, 3].

Interestingly, in most cultures, vegetables are eaten more often than meat, presumably because they are always readily available. Many of the common herbs that are found around human settlements, such as nettle, dandelion, chickweed, to mention but a few have medicinal properties. Hence the use of herbs to treat diseases is becoming a welcome universal development among developing societies. Many of the pharmaceuticals currently available today are products of herbal remedies, such opium, aspirin, digitalis, and quinine. One of the advantages of herbal remedies is that they are cheaper than modern medicine and possess fewer side effects [3, 4]. However caution should be taken in terms of dosage with regards to heavy metals toxicity when administering the plants as medicine to minimized poisoning [4].

Pueraria phaseoloides is a plant species that belongs to the pea family (*Fabaceae*) and its subfamily is *Faboideae*. It is a promising forage and cover crop found in the tropics with very many uses such as a cover crop, a vegetable and an herbal medicinal material [5]. It grows very wild and it is sometimes referred as a weed. It is called *puero* in Australia and *Tropical kudzu* in most tropical regions [6]. It is closely related to other species in the genus *Pueraria* and it is crossable with the other species of *Pueraria* family like *Pueraria montana* and *Pueraria edulis*.

P. phaseoloides is used as a cover crop due its comparable high nitrogen composition which helps in the improvement of the soil structure owing to its deep rooting system [7]. It can be used as a grazed forage crop for live stocks and as a source of green manure in crop rotations system of faming. Furthermore, it helps to prevent soil erosion on sloppy soils. [8, 9, 10].

Due to limited scientific reports on this plant at the moment we have decided to investigate on it in order to extract and present useful scientific information with regards to the presence of the phytochemicals, antimicrobial activity, proximate and heavy metal compositions of *Pueraria*

phaseoloides leaves.

2. Materials and Methods

The reagents used for this research were of analytical grade and were obtained from Zayo-Sigma in sealed containers and were used without further purification. The preparations of the reagents were done according to the specified standard.

2.1 Preparation of plant materials

Fresh leaves of *Pueraria phaseoloides* were collected from Ndiabor in Aniri local Government Area of Enugu state, Nigeria and were identified to the leaves of *Pueraria phaseoloides* by Mr. S. I. Ijearu of the Department of Botany, Nnamdi University Awka, confirmed it as *Pueraria phaseoloides.*

The leaves were air dried and pulverized in an electric blender, sterilized with ethanol (70%) and stored in an air tight container for further analysis. The extracts were obtained through soxhlet extraction method.

200g of the powdered material was packed into the extractor and extracted with 500ml of ethanol, methanol, n-hexane, and distilled water respectively. The crude extracts were obtained by concentrating them via a rotary evaporator. They were stored in sterilized bottles and properly labeled for further analyses.

Below is a picture of *Pueraria phaseoloides*.

Fig 1: The *Pueraria phaseoloides* plant.

2.2 *Phytochemical Screening*

The four extracts of *Pueraria phaseoloides* leaves respectively were screened for the presence of the following phytochemicals namely; flavonoids, alkaloids, steroids, saponins, tannins, phlobatanin, phytosterols, terpenoids and phenolic compound according to standard methods [2,11,12, 13].

1g of each of the crude extracts obtained was dissolved in 100ml of each of the solvents used to obtain the stock solution and was subjected to phytochemical screening as described below.

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 2min. 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 shows 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 bacterium (*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 hexane extracts respectively was carried out using agar well diffusion method according to [13, 14] as described below.

20g of Muller Hinton agar was weighed and diluted in 500ml 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 121ºC for 15min and allowed to cool.

Thereafter, 15ml of molten Muller Hinton agar was poured into five 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 121ºC 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 the agar plates of different organisms prepared above, holes were bored using a cork borer with each plate containing five (3) wells for the extracts while the sixth (4^{th}) well in the middle was for the control antibiotics (Ciproflaxine). Three drops of both the antibiotics and the extracts were placed in their respective holes. The extracts and the antibiotics (Ciproflaxine) were allowed to diffuse for 30min and then incubated at 37ºC for 24h. The degree of antimicrobial activity for each of the extracts was determined by 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 weighed in crucible and place in a muffler furnace. It was burn for 1 hour (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 moisture content can be analyzed 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 52ºC 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 respectively. The residue was removed and then transferred to an ashing crucible whose weight has been noted (W_1). The residue was dried for 2h at 130ºC and cooled in a desiccator and reweighed (W_2). The residue was again placed in a crucible and ignited for 30min at 600ºC and finally cooled in desiccators and reweighed again (W_3) [15]. 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 [14]. 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 [15, 16].

i) 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 and 380 ºC. 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 [14, 16, 17].

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

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

ii) Distillation

During the distillation step the ammonium ions (NH4 are converted into ammonia (NH_3**)** by adding 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 [14, 16, 17].

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

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

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

iii) 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 [16, 17].

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

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.

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.

2.4.6 Heavy metals

Some heavy metals were analyzed for the plant, these include: Arsenic, mercury, lead, cadmium, zinc and copper. Using nitric- pechloric acid digestion method as recommended by AOAC [18]. 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 minutes 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, further filtered through whatman filter paper and transferred to a 250ml volumetric flask by adding distilled water to the mark. It was further transferred to a labeled bijou bottle and sent for further analysis of some heavy metals at the spring board laboratory for further analysis of these elements using Atomic absorption spectrophotometer (AAS).

3. Results and Discussion

3.1 Phytochemical Analysis

The presence of secondary metabolites in *Pueraria phaseoloides* is a good indicator that it possesses medicinal value [2, 8]. This is because the phytochemical constituents contribute immensely to the medicinal aspect of a plant [12, 13, 19].

From the result of the phytochemical screening tabulated in table **1** below, it can be observed that saponins, tannins and carbohydrates were present in the n-hexane extract. The ethanol extract revealed the presence of alkaloids, flavonoids, phlobatanins, saponins, phytosterols and phenolic compounds. However, the aqueous extract showed the presence of all the phytochemicals that were screened.

Table 1: Qualitative Report on Phytochemical Analysis

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

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

3.2 Antimicrobial Analysis

Antimicrobial properties of substances are desirable tools employed to control the growth of undesirable microorganisms especially in the treatment of infectious diseases and in food spoilage. The active components of the medicinal plants usually interfere with growth and metabolism of the microorganisms [19, 20]. The sensitivity test of the aqueous, ethanol and hexane extracts of *Pueraria phaseoloides* leaves are given below in table 3, 4 and 5 respectively. The aqueous extract was active only against two out of the three bacteria. However, the ethanol and n-hexane extracts respectively showed remarkable activity against all the microorganisms.

Table 3: Sensitivity of aqueous extract against test organisms.

S/N	Organism	Zone diameter for water extract	Zone diameter for Ciprofloxacin (Standard).
1.	Staphylococcus aureus	0.2mm	40mm
2.	Escherichia coli	15mm	50mm
3.	Pseudomonas aeruginosa	13mm	35mm

Table 4: Sensitivity of ethanol extract against test organisms.

S/N	Organism	Zone diameter for ethanol extract	Zone diameter for Ciprofloxacin (Standard).
1.	Staphylococcus aureus	7mm	35mm
2.	Escherichia coli	17mm	30mm
3.	Pseudomonas aeruginosa	4mm	40mm

Table 5: Sensitivity of n-Hexane extract against test organisms

S/N	Organism	Zone diameter for n-hexane extract	Zone diameter for Ciprofloxacin (Standard).
1.	Staphylococcus aureus	10mm	30mm
2.	Escherichia coli	19mm	45mm
3.	Pseudomonas aeruginosa	2mm	32mm

3.3 Proximate Analysis

Table 6 shows the result of the proximate analysis. From the result obtained, ash content was observed to be 3.5%. The percentage of crude fibre was 16.9%. 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 [20].

Table 6: Result on proximate analysis

Components (%)	Amount present (%)
Ash content	3.50
Crude fibre	16.90
Moisture content	4.40
Proteins	0.34
Carbohydrate content	74.8

3.4 Heavy Metals Analysis

Table **7** shows the result of heavy metal analysis. From the result tabulated it can be observed that lead (Pb), cadmium (Cd), zinc (Zn) and copper (Cu) were all within W.H.O permissible limit. However, the concentration of Mercury was slightly higher than W.H.O permissible limit with the value of 0.955 against the 0.05ppm W.H.O permissible limit [20, 21]. The implication of this result is that the leaves *Pueraria phaseoloides* are safe for consumption since they contain heavy metals below the W.H.O permissible limits. But the slight increase in concentration of Mercury may be attributed to the environment where this plant was harvested [21, 22].

Many metals such as zinc, copper, chromium, iron, and manganese are essential to body provided they are present in the acceptable proportions. But if their presence exceeds the acceptable limits, they can be accumulating in our bodies over time when the plant material is consumed; this could results to serious poisoning with damaging effects [20, 21, 22].

Table 7: Result of Heavy metals Analysis of *Pueraria phaseoloides*

S/N	Element	Concentration in ppm	Permissible limit by W.H.O (ppm)
1.	Zinc	16.249	50
2.	Arsenic	0.00	0.01
3.	Mercury	0.083	0.05
4.	Lead	1.025	10
5.	Cadmium	0.112	1.5
6.	Copper	0.281	2.3

4. Conclusions

The aqueous, ethanol and hexane extracts of *Pueraria phaseoloides* leaves have been discovered to possess promising medicinal potentials. From the antimicrobial analysis result, it is observed that the aqueous, ethanol and n-haxane extracts of the above showed remarkable activity against *S. typhi* and *S. aureus* and *Pseudomonas aeruginosa* respectively**.** The leaves showed the presence of a good number of photochemicals which is an indication that they possess medicinal value and 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 leaves are below the W.H.O permissible limit, it is an indication that they safe for consumption.

Conflicts of Interest

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

5. References

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