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Evaluation of the Phytochemical Constituents and Antiprotozoal Effects of *Vernonia Amydalina* on *Entamoeba Histolytica*

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Abstract

The aim of this study was to determine the phytochemical constituent and the antiprotozoal activity of *V. amygdalina* on *Entamoeba histolytica*. Fresh leaves of *V. amygdalina* were collected from Amagu Street, Sakamories quarters, Abakaliki, Ebonyi State, Nigeria. The plant was washed, soaked, filtered, and air-dried to get the aqueous extract. Semi-quantitative phytochemical identification, as well as antibacterial assays of the extracts against *Entamoeba histolytica* isolated from faeces by agar well diffusion method, were carried out. The clinical isolate was obtained from the Department of Medical Microbiology, Alex Ekwueme Federal Teaching Hospital Abakaliki, Ebonyi State, Nigeria. Metronidazole and Tinidazole as standard medications were used as controls. Phytochemical analysis of the crude extracts showed the presence of saponin, tannins, resin, carbohydrates, glycosides, steroids, fats and oil, acidic compounds, and reducing sugar. The aqueous extract shows a potent antiamoebic effect against *E. histolytica* at different incubation periods. The longer the incubation period the higher the inhibitory effects and also the higher the concentration of the extract the higher the inhibitory effect. In conclusion, this study revealed that the aqueous extract of *V. amygdalina* contains a high amount of phytoconstituents, especially tannins and it may be responsible for its good antiamoebic activity. Further studies are necessary to isolate and identify the active compounds in the crude extract fraction of *V. amygdalina* responsible for the antiamoebic activities.

Keywords: *Vernonia Amygdalina*, *Entamoeba Histolytica*, Phytochemical Constituent, Antiprotozoal, Metronidazole, Tinidazole

1. Introduction

With the recent trend of high resistance of antimicrobial drugs and multiple drug-resistant microbial strains, efforts are being intensified by researchers to search for and develop possible alternatives for the treatment of microbial diseases [1]. Medicinal

plants and traditional preparation with antimicrobial activities have been used extensively in the West African regions. These plants of medicinal importance have been proven to be very effective even where treatments with antibiotics failed [2]. *Vernonia amygdalina* commonly called bitter leaf (English), Shuwaka (Hausa), Ewuro (Yoruba), Oriwo (Edo), and Olubu (Igbo) is a perennial shrub belonging to the family Asteraceae [2]. *V. amygdalina* is a shrub that can grows to 10 m tall with petiole leaf of about 6 mm in diameter and elliptic in shape and grows throughout tropical Africa and has been domesticated in various parts of West Africa including Nigeria, where it is locally used as vegetable in soups [3]. The leaves are green and have characteristic odour and bitter taste [4]. The presence of bitter principles also protects *V. amygdalina* from most of the animals, insects and microbes but it suffers from the attack by *Coleoptera curculionidae*, weevil *Lixus camerunus* and *Zonocerus variegates* (which utilized it as a source of protein) [5]. The total area of leaf that is susceptible to insect attack ranges from 0.2 to 12%. Thus, this plant is suitable to be planted in either small or large scale as a source of income to farmers. The processed *V. amygdalina* is being exported to Europe and North America restaurants for preparation of African dishes [5] *V. amygdalina* with just a little amount of processing can be classified as healthy food because it promotes the healthy development of the body. It contains not only active drug molecules but also other substances that are necessary for maintaining health and physiological functions of the body without manifestation of toxicity [6]. As a result, *V. amygdalina* serves well as a low-cost and readily available source of important nutrients for humans [7]. Besides, this plant has also been widely used as fuelwood, stakes, fodder, construction poles, fencing of the agroforestry buffer zone, and as an ingredient for compost. Due to its bitterness, it also can be used as a bittering agent, a hop substitute, and for the control of microbial contamination in beer brewing without affecting the quality of malt. In Ethiopia, it is used to make honey wine called Tei [8].

In some African countries including Nigeria, this plant species is traditionally used to treat many ailments including diabetes, malaria, helminth infections, fever, wound healing and to treat microbial infections [6]. Also, the Hausa tribe of the northern part of Nigeria used the root and twig of *V. amygdalina* to treat stomach-ache and gastrointestinal troubles [3]. It is also prescribed to nursing mother as it improves lactation [9]. The usage of *V. amygdalina* as medicinal herb started when zoopharmacologists found that sick chimpanzees with empty stomach sucked pith and juice from the unsavoury Vernonia plant stalk (which was not their common diet) for self-deparasitization, enhanced body fitness, increased strength or appetite and reduced constipation or diarrhoea especially during rainy season [10]. The bitter taste of *V. amygdalina* was suspected as a guide for them to choose for the appropriate plant, plant part and amount of intake [10]. Other than animals, some of the citizens in Africa especially patients who were less educated with low or middle income also liked to use this plant, due to cultural and economic reasons [11].

Phytochemicals are chemicals which are obtained from plants. They have been shown to have medicinal values since they produce physiological actions on the human body. Some phytochemicals that have medicinal values include alkaloids, tannins, saponins and flavonoids. The reported biologically active phytoconstituents from *V. amygdalina* are alkaloids, flavonoids, terpenes, saponins, coumarins, xanthones, phenolic acids, lignans, steroids, anthraquinones [2]. Despite the vast traditional used of *V. amygdalina*, it is still considered among the under exploited crops of economic significance. The broad aim of the study was to determine the phytochemical constituents and the antiprotozoal activity of *V. amygdalina* on *Entamoeba histolytica*.

2. Materials and Methods

2.1 Study Area

The study area is Abakaliki local government, capital of Ebonyi State, south east Nigeria. Ebonyi State occupies a land area of 5,533km^2 with a population size of 2,176,947 as at 2006 census, and is located on the coordinates 6015'N and 8005'E, generally populated by the Igbo's. The capital city is Abakaliki. There are several Igbo dialects spoken in Ebonyi State which includes Edda, Ehugbo (Afikpo), Izzi-Ezza-Mgbo-Ikwo dialect cluster, Oshiri, Unwara, Akpoha, Okposi and Onicha. Most of the inhabitants are farmers.

2.2 Collections and Preparation of Samples

The leaves of *Vernonia amydalina* were collected from 11 Amagu Street, Sakamories quarters Abakaliki, Ebonyi State, Nigeria. The plants were examined and authenticated by Nwankwo O.F. in the Herbarium Section in the Department of Biological Science, Applied Biology Unit, Ebonyi State University Abakiliki, Nigeria. The identified plant samples were transferred to the Microbiology Unit of Genbuk Diagnostic Laboratory for processing.

2.3 Aqueous Extraction/ Preparation of Extract

At arrival, the plants were washed with distilled water to reduce the bacterial load. The leaves were spread under a moist temperature to dry. This was done for about 3 to 4 days. The leaves were grounded with a mortal and pistol into a powder form. The powdered form were poured into a sterile container and a measurable amount of water was added into it. The mixture were stirred intermittently to ensure; total submerging of the leaves as well as actualizing homogenecity. The soap was left for 4hours for it to macerate and mixed well. Filtration was done using sterile filter cloth (sieve) and the filtrate was collected in a sterile stainless plate while the residue was discarded. The filtrate was transferred into the stainless plate and was left for about 5 days under air current to dry. The dried leaves were removed with a sterile sharp object. The leaves were scrapped out and were added into another sterile container.

2.4 Extract Dilution

1. Three concentration of the extract: *Vernonia amygdalina*, were measured using a measuring equipment.
   - First, 0.6g of the extract was first measured and added in to a clean test tube.
   - Secondly 0.3g of the extract was measured and added in to the second test tube.
   - Thirdly, 0.2g of the extract were also measured and added into the third test tube.

2. To each of the tubes well arranged on the testing rack, 2ml of distilled water were added into each of them to produce the three concentrations (300mg/ml, 150mg/ml and 100mg/ml respectively).

3. The tubes were left on the rack for 5minutes to allow the extract to dissolve well.

2.4.1 Calculation
For 300mg/ml

0.6g→2ml

Divide both side by two

0.3g→1ml
Convert g to mg

0.3g×1000 →1ml

=300mg/ml

Further dilutions (150mg/ml and 100mg/ml) are gotten through similar calculation

2.5 Organism Source
The organisms used were clinical isolates of *Entamoeba histolytica*. They were obtained from the Department of Medical Microbiology, Alex Ekwueme Federal Teaching Hospital Abakaliki, Ebonyi State, Nigeria. All the *E. histolytica* positive faecal samples were confirmed by Sct. Nkiru, using conventional cultural, morphological and biochemical methods and maintained at 4°C in nutrient agar slants.

2.6 Phytochemical Analysis
The preliminary phytochemical screening of the aqueous extract of *Vernonia amydalina* was carried out in order to ascertain the presence of various constituents viz. steroids, alkaloids, flavonoids, tannins, sugars and glycosides by utilizing standard conventional protocols [12].

2.6.1 Test for Tannins
About 2 ml of the aqueous extract was stirred with 2 ml of distilled water and few drops of FeCl3 solution were added. The formation of a green precipitate was an indication for the presence of tannins [12].

2.6.2 Test for Saponins
About 5 ml of aqueous extract was shaken vigorously with 5 ml of distilled water in a test tube and warmed. The formation of stable foam was taken as an indication for the presence of saponins [12].

2.6.3 Test for Flavonoids
To 1 ml of aqueous extract was added 1 ml of 10% lead acetate solution. The formation of a yellow precipitate was taken as a positive test for flavonoids [12].

2.6.4 Tests for Steroids
A red colour produced in the lower chloroform layer when 2 ml of organic extract was dissolved in 2 ml of chloroform and 2 ml concentrated sulphuric acid added indicates the presence of steroids [12].

2.6.5 Test for Alkaloids
About 3 ml of aqueous extract was stirred with 3 ml of 1% HCl on a steam bath. Mayer's and Wagner's reagents were then added to the mixture. Turbidity of the resulting precipitate was taken as evidence for the presence of alkaloids [12].

2.6.6 Tests for Carbohydrates
About 3 ml of the aqueous extract was added to 2 ml of Molisch's reagent and the resulting mixture shaken properly. About 2 ml of concentrated H2SO4 was then poured carefully down the side of the test tube. A violet ring at the interphase indicates the presence of carbohydrate [12].

2.6.7 Tests for Glycosides
About 2 ml of the organic extract was dissolved in 2 ml of chloroform and 2 ml of acetic acid was added and the solution cooled well in ice. Sulphuric acid was then added carefully. A colour change from violet to blue to green indicates the presence of a steroidal nucleus (that is, a glycone portion of glycoside) [12].

2.7 Preparation of Modified Pancreas Extract of Homemade Serum Media (PEHPS)
- Freshly obtained liver was washed with distilled water and pounded to form a suspension.
- Finally the medium was autoclaved at 121c for 15mins at 15rpi. After that 100ul chloramphenicol was added into it.
- It was mixed well by shaking to ensure homogenecity.

2.8 Culturing of the Sample on the Modified PEHPS Media
A confirmed *Entamoeba histolytica* faecal sample was collected and a representative portion of it were inoculated into the media. The cultivated media were incubated for 48hours. At the end of incubation, a smear was made with the broth to confirm the growth of the trophozoites. After which it was counted using the improved Neubauer Counting Chamber to evaluate the exact number of cells present in 1ul of the sample.

2.9 Evaluating the Effect of *Vernonia Amydalina* on *Entamoeba Histolytica*
2.9.1 Study Design
6 pairs of laboratory test tubes were placed on a test tube rack and were labelled as follows.
- The first pair of test tube was labelled *Vernonia amydalina* 0.6g (Vn 100ul and Vn 150ul).
- The second pair of test tube was labelled *Vernonia amydalina* 0.3g (Vn 100ul and Vn 150ul).
- The third pair of test tube was labelled *Vernonia amydalina* 0.2g (Vn 100ul and 150ul).
- The fourth pair of test tube was labelled water (W50 and W 100) which served as a negative control.
- The fifth pair of test tube was labelled Metronizadole (Met 50ul and Met 100ul).
- The sixth pair of test tube was labelled Tinidazole (Tin 25ul and Tin 50ul).

2.9.2 Procedure
- Five (5ml) of freshly prepared PHEPS medium was added into the 6 pairs of test tubes that were labeled respectively.
- A Hundred (100ul) and 150ul of the extract (*Vernonia amydalina*) were added into the pair of the test tubes labeled (0.2g, 0.3g and 0.6g) respectively

- A Hundred (100ul) of the samples was added into each of the test tube.
- Each of the test tubes containing the mixture was thoroughly mixed together, to attain homogeneity.
- The mixture were incubated at room temperature.
- Microscopic examinations were made using Neubauer Counting Chamber every 24hours to evaluate the response of the parasite to the extract using 1:10 saline dilution for 72hours.

2.10 Statistical Analysis
The mean of the data collected were calculated using Statistics software (Genstat version 4).

3. Result
The results revealed that the tested extract of *V. amygdalina* possessed significant antiamoebic activity against *E. histolytica* (Table 1).

Table 1 shows that *V. amygdalina* at the concentration of 100mg/ml gave a lesser cell count of 20.6×10^5 (24hrs), 15.5×10^5 (48hrs) and 12.4×10^5 (72hrs) when compared to the negative control which cell count was 50×10^5 (24hrs), 61.2×10^5 (48hrs) and 71.3×10^5 (72hrs) and a higher cell count when compared to the metronidazole which cell count was 13.3×10^5 (24hrs), 12.5×10^5 (48hrs) and 6.0×10^5 (72hrs) and tinidazole which cell count was 15.0×10^5 (24hrs), 13.5×10^5 (48hrs) and 12.3×10^5 (72hrs). The result also shows that *V. amygdalina* at the concentration of 150mg/ml gave a lower cell count of 36.6×10^5 (24hrs), 20.3×10^5 (48hrs) and 4.6×10^5 (72hrs) when compared to the negative control which cell count was 62.2×10^5 (24hrs), 72.2×10^5 (48hrs) and 75.3×10^5 (72hrs) and a higher cell count when compared to metronidazole which cell count was 12.5×10^5 (24hrs), 8.0×10^5 (48hrs) and 4.1×10^5 (72hrs) and tinidazole which cell count was 11.0×10^5 (24hrs), 9.4×10^5 (48hrs) and 5.2×10^5 (72hrs). The result shows that *V. amygdalina* at the concentration of 300mg/ml gave a lower cell count of 15.4×10^5 (24hrs), 5.0×10^5 (48hrs) and 2.0×10^5 (72hrs) when compared to the negative control which cell count was 67.5×10^5 (24hrs), 73.3×10^5 (48hrs) and 75.6×10^5 (72hrs) and a higher cell count when compared to metronidazole which cell count was 6.0×10^5 (24hrs), 4.3×10^5 (48hrs) and 0.1×10^5 (72hrs) and tinidazole which cell count was 11.0×10^5 (24hrs), 7.1×10^5 (48hrs) and 3.0×10^5 (72hrs). Fig 1 shows that *V. amygdalina* at the concentration of 100mg/ml and 150mg/ml gave the highest cell count after 24hrs of incubation of *E. histolytica* and the lowest cell count after 72hrs of incubation. Hence, the longer the time of incubation the higher the antiamoebic activity of *V. amygdalina*. The result also shows that *V. amygdalina* at the concentration of 300mg/ml had more inhibitory effects than *V. amygdalina* at the concentration of 150 mg/ml and 100mg/ml of incubation. Hence, the higher the concentration the higher the inhibitory effect.

Table 1: Cell Count of *E. histolytica* after Inoculation with *V. amygdalina* Extract

	100mg/ml (10^5)				**150mg/ml (10^5)**				**300mg/ml (10^5)**			
	Time after inoculation				Time after inoculation				Time of inoculation			
Product used	0hr	24hrs	48hrs	72hrs	0hr	24hrs	48hrs	72hrs	0hr	24hrs	48hrs	72hrs
V. amygdalina extract	50	20.6	15.5	12.4	50	36.6	20.3	4.6	50	15.4	5.0	2.0
Negative Control	45	50	61.2	71.3	55.0	62.2	72.2	75.3	37.5	67.5	73.3	75.6
Positive control I (Metronidazole)	55	13.3	12.5	6.0	54	12.5	8.0	4.1	55	6.0	4.3	0.1
Positive control II (Tinidazole)	60	15.0	13.5	12.3	53	11.0	9.4	5.2	55	11.0	7.1	3.0

Fig 2 shows the cell count of *E. histolytica* when inoculated with the negative control and positive controls at the concentration of 100mg/ml. The negative control gave more cell count than the positive controls. The negative control gave the lowest cell count after 24hrs of incubation and the highest cell count after 72hrs of incubations. Hence the longer the time of incubation the higher the growth or cell count when inoculated with the negative control. Fig 2 also shows that positive controls had an antiamoebic effect. Metronidazole in Fig 2 gave more antiamoebic effect than tinidazole. Metronidazole and tinidazole gave the highest cell count after 24hrs of incubation and the lowest cell count after 72hrs of incubations. Hence the longer the time of incubation the lower the growth or cell count when inoculated with the positive control.

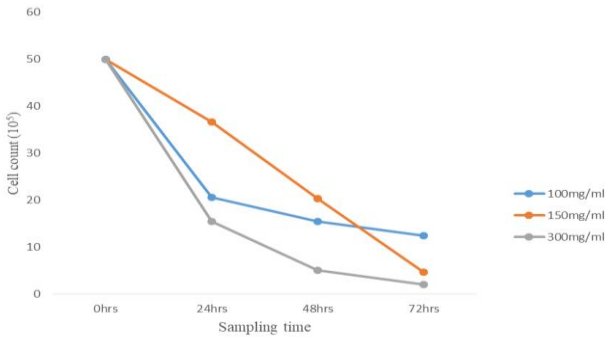

Fig 1: Cell count of V. amygdalina against sampling days

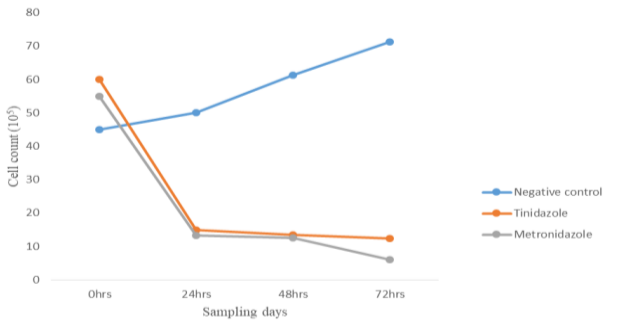

Fig 2: Cell count of E. histolytica after inoculation with control at 100mg/ml against sampling days

Fig 3 and 4 shows the cell count of *E. histolytica* when inoculated with the negative control and positive control at the concentration of 150mg/ml and 300mg/ml respectively. The negative control also gave more cell count than the positive controls. Both positive and negative controls at the concentration of 150mg/ml and 300mg/ml gave a similar antiamoebic effects with that of 100mg/ml. The positive controls gave the highest cell count after 24hrs of incubation and the lowest cell count after 72hrs of incubations while the negative control gave the highest cell count after 72hrs of incubation and the lowest cell count after 24hrs of incubations.

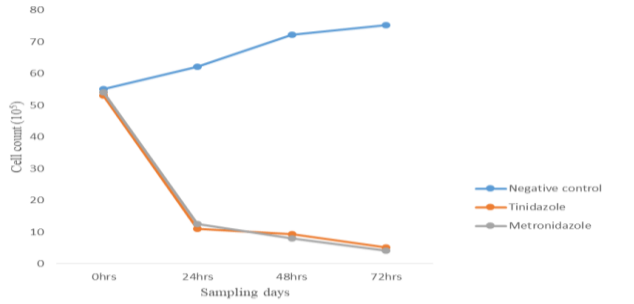

Fig 3: Cell count of E. histolytica after inoculation with control at 150 mg/ml against sampling days

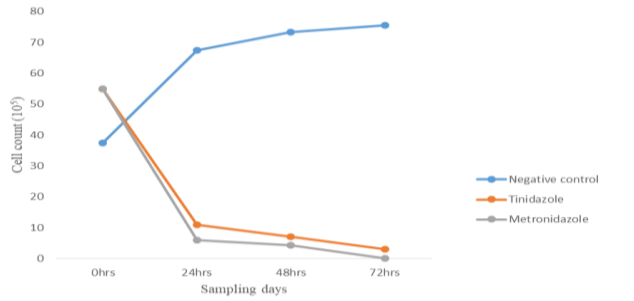

Fig 4: Cell count of E. histolytica after inoculation with control at 300mg/ml against sampling days

Phytochemical screening results of the aqueous crude extracts of *V. amygdalina* are shown in Table 2. The presence of saponin, tannins, resin, carbohydrate, glycosides, steroids, fats and oil, acidic compouds and reducing sugar were noted in the aqueous extracts.

Table 2: Phyochemicals Identified in *V. amygdalina* aqueous crude extracts

Extracts	Saponin	Tannin	Reducing Sugar	Carbohydrate	Glucoside	Resin	Flavonoid	Steriods
V. amygdalina	++	++	++	++	++	-	-	++

Keys: indicates absence, + indicate positive

4. Discussion

Antimicrobial compounds from plants represent a potentially novel source of antimicrobial substances since they act against pathogens via mechanisms that are different from those of currently used antibiotics and may thus have a clinical value in the treatment of antibiotic resistant antimicrobial strain [13].

Plants contain numerous chemical constituents, many of which are known to be bioactive and are responsible for exhibiting diverse pharmacological activities [2, 14-16]. It is therefore desirable to have knowledge of the chemical constituents of plants to discover new therapeutic agents and lead compounds that may lead to the synthesis of more potent analogs of great economic value. Phytochemical analysis revealed the presence of saponin, tannins, resin, carbohydrate, glycosides, steroids, fats and oil, acidic compounds and reducing sugar. Secondary compounds, which include tannis, saponins, cardiac glycosides and alkaloids were reported by Kaufman *et al.* [17], to be present in higher plants. The compounds were reported by Kaufman *et al.* [17] to be indicative of the potential medicinal value of the plants in which they appear. This contradict the findings of Ezeonu *et al.* [18] that resin is not found in the aqueous extract of *V. amygdalina*. The presence of saponin is of particular interest in this study. Saponins are complex glycosides made up of sugar (glycone) and non-sugar (aglycone) groups. The glycone can consist of a single group of sugars (monosaccharides) or several groups of sugars (oligosaccharides) (LASCU, 2008). The foaming characteristic usually observed with saponins is caused by the combination of the non-polar aglycone and the water soluble side chain (glycone) and on contact with water, the sugar group dissolves releasing the non-sugar group [19]. Saponins have been reported to have biological activities including anti-inflammatory, anticancer, immune stimulating, antimicrobial and anti-plasmodial properties [19]. In addition, saponins are believed to be non-systemic and able to escape digestion in the upper gut to arrive in the colon [19]. This latter property is an important property of all prebiotics. The phytochemical constituents of a plant often determine the physiological action on the human body. Antioxidants are agents that protect cells against damage caused by molecules known as free radicals. The antioxidant activities of extracts are mainly due to the presence of phenolic compounds such as flavonoids, phenolic acids, tannins, and phenolic diterpenes [20]. Hence, the constituents of the extracts, such as tannins, play a major role in the wound healing by preventing and protecting oxidative damage from free radicals [21].

The findings of this study revealed that the aqueous extract of *V. amygdalina* possess good antiamoebic activity (Table 1 and Fig 1). The findings of this study that the extract of *V. amygdalina* has antiamoebic effects is consistent with the report of Bracha and Mirelman [22], which suggested that *V. amygdalina* may be used as the alternative source for treating several infectious diseases caused by *E. histolytica*. The study shows that *V. amygdalina* aqueous extract has more inhibitory effects on the clinical isolates as compared to water (negative control) (Table 1). This study also shows that metronidazole has more inhibitory effects than the extract and tinidazole.

5. Conclusion

From the entire work, it was deduced that *V. amygdalina* had antiamoebic effects. Also, the presence of saponin, tannins, resin, carbohydrate, glycosides, steroids, fats and oil, acidic compouds and reducing sugar were confirmed in aqueous extract of *V. amygdalina*. Moreover, the plant was confirmed to be rich in tannin, which are suspected to be responsible for the antiamoebic as well as antioxidant potential of the plant.

6. Compliance with Ethical Standards

Acknowledgments

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Disclosure of Conflict of Interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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