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Phytochemical, Antimicrobial and Anti-Epilepsy Characteristics of Root Extract of *Hippocratea welwitschii* (*Celastraceae*) Oliv

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ABSTRACT

The root extract of *Hippocratea welwitschii* Oliv. was investigated for its phytochemical and biological characteristics. Phytochemical analysis showed that it contained glycosides, saponins, triterpenes, phenols and alkaloids. The mineral composition was determined using the atomic absorption spectroscopic methods (AAS). It showed that vital minerals known to play important metabolic and physiologic roles in the living system were present in appreciable concentrations. Antimicrobial tests carried out showed that the crude ethanolic extract was active against Klebb species, *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Candida albicans* with MIC values of 200, 200, 100, 250 and 250 µg/ml, respectively. The anti-oxidant characteristics were investigated using the DPPH method with Vitamin C as reference compound. The brine shrimps lethality test showed that at concentrations as low as 10 ppm all the shrimps were killed by the crude ethanolic extract. The acute oral toxicity test showed that the plant was relatively toxic, but safe for consumption and the LD_{50} value was 3800 mg/kg body weight. The biological and chemical characteristics of the plant support the traditional medicinal uses of the plant, particularly in the management of epilepsy.

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INTRODUCTION

The genus *Hippocratea* belongs to the family of plants known as Celastraceae or staff vine or bittersweet family (website: Celastraceae, 2010). The great majority of the genera are tropical with only *celastrus* (the staff vine), *Euonymus* (the spindles) and *Maytenus* widespread in the temperate climates. Common names of some of its genera include *canotia* (*Crucifixion thorn*), *catcha*, celastrus, *hippocratea* etc (website: Celastraceae, 2010). Most of the representatives of this family are shrubs and some as in *Hippocratea* are like climbers by their branchlets, twisting round their supports.

The species *Hippocratea welwitschii*, is a shrub of closed, primary or mature secondary forest or in thickets of secondary shrub from Guinea to western Cameroon and widespread across Africa to Angola, Uganda and Tanganyika (Tanzania). In Ghana, the root materials have been found to contain a sort of gutta of no recorded usage, but only in small quantity (2.58 % crude and 1.22% pure) (Burkill, 1985). In Ivory Coast, part of the plant is used to ease labour and delivery at childbirth (Burkill, 1985). In Cross-River State, south-south Nigeria the root of the plant is used effectively to manage epilepsy (Oral discussion).

This study aimed to evaluate the chemical and biological characteristics of the root of the plant. In particular, the anti-microbial and anti-oxidant activities, the brine shrimps cytotoxicity, the acute cytotoxicity using albino mice and the phytochemicals of the crude 95% ethanolic extract of the root of this plant were investigated to assess its anti-epilepsy potential.

MATERIALS AND METHODS

The roots of the plant were obtained from a farm in south-south Nigeria, cleaned up to remove the sand particles and dried indoors in an airy corridor. The plant was identified and authenticated by a taxonomist, Mr. Ozioko of Bio-resource Development and Conservation Program, number 114 Aku Road, Nnsuka, Enugu State, Nigeria and a voucher specimen; number BDCP 213 was deposited in the Herbarium. The dried roots were then broken into smaller bits and blended into a fine powder with Waring commercial blender 8011E model 38BL 41, extracted and used for both chemical and biological analysis using standard methods. The mineral composition was determined on aliquots of the solutions of the ash by established atomic absorption/emission spectrophotometer model 200-A, produced by Buck Scientific. The muffle furnace was the Carbolite Eurotherm 2416, produced by Aston Lane

Sheffield, England. Pure test cultures of microorganisms were collected from the Nigerian Institute for Pharmaceutical Research and Development, (NIPRD), Idu Abuja, FCT. They were confirmed and standardized as *Klebsiella species, Staphylococcus aureus, Bacillus subtilis, Escherichia* coli and *Candida albicans*. Reverse phase silica gel (RP-18) and Kieseigel 60 with pore size range of 0.063-0.200 mm, respectively, were used for column chromatographic separations.

Extraction of the root powder of *Hippocratea welwitschii*

Powdered root sample (1300 g) of *Hippocratea welwitschii* was extracted with 95% ethanol for forty eight (48) hours using Soxhlet extractor. The extract was then filtered and evaporated *in-vacuo* using a rotary evaporator to give the crude extract (127 g).

Phytochemical analysis of crude 95% ethanolic root extract of *Hippocratea welwitschii*

The crude ethanol extract of the root was subjected to qualitative and quantitative analyses. The qualitative analysis was carried out using standard methods of analysis (Treatise and Evans, 1989, Sofowora, 1982, Sofowora, 1993). The quantities of the phytochemicals present were determined using the methods of Harborne (1973) and Obadoni and Ochuko (2001). The results are shown in Table 1.

Antimicrobial screening of 95% ethanolic root extract of *Hippocratea welwitschii*

The sensitivity of the ethanolic extract of *Hippocrateae welwitschii* in dimethyl sulphoxide (DMSO) to selected micro-organisms was determined using the disc diffusion method (Bauer *et. al.,* 1966). The disc diameter was 7 mm. Standardized inoculum (1-2 x 107 cfu/ml 0.5 McFarland standards) was each introduced onto the surfaces of sterile agar plates using a sterile glass spreader. Sterile paper discs previously inoculated with known concentrations of extract were carefully placed radially from the center of the inoculated plates in triplicates. Amoxil and dimethyl sulphoxide (each at 30 µg/ml) were used as controls. The plates were then incubated in the oven at a temperature of 37^0C for 24hrs after which they were observed for zones of inhibition. The results are shown in Table 2 and Fig 1.

Mineral elements determination

The mineral elements were determined using standard analytical methods (AOAC, 1990). The results are shown in Table 3.

Free radical scavenging (antioxidant) activity of *Hippocratea welwitschii root extract*

The free radical scavenging activity of the crude ethanol extract of the root of *Hippocratea welwitschii* was evaluated using DPPH with Vitamin C as standard. The method of Ayoola *et al*. (2008), measured at 452 nm using UV spectrophotometer at concentrations ranging from 0.05 -5.0 mg/ml in methanol was used.

A solution of DPPH in methanol (0.1mg/ml) was prepared and 0.5 ml of this solution was added to 3.0 ml of solution of extract in methanol at concentrations 0.05, 0.10, 0.20, 0.5, 1.00, 2.00 and 5.00 mg/ml and Vitamin C (ascorbic acid) was used as the standard antioxidant. The measurements were performed in triplicates. A blank solution was prepared containing the same amounts of methanol and DPPH. The decrease in absorbance was monitored at 452 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity which is the percentage inhibition of free radical by the sample. The radical scavenging activity (RSA) was calculated as the % inhibition of the DPPH de-coloration thus:-

% Inhibition = $\{A_b-A_c / A_b\}$ x 100/1, where $A_{b=}$ absorption of the blank sample and A_c = absorption of the extract

The results are recorded in Table 4 and Fig 2.

Brine shrimps cytotoxicity screening

The method of Fatope*et al* (1993) was used for the screening. The shrimps were hatched in seawater for 48 hours at room temperature. The harvested shrimps were attracted to one side of vessel with a light source. The solutions of the extract were prepared at 10,000, 100 and 10 ppm in triplicates using dimethyl sulphoxide (DMSO) as solvent. The extract (0.5 mg) was introduced into a test tube and seawater (4 ml) was added. Ten (10) shrimps were added to each test tube at each concentration and made up to 5 ml with sea water. Seawater and DMSO in different test tubes were used as controls. The number of dead shrimps was noted after 24 hours and the LC_{50} was calculated (Fatope *et al*, 1993). The results are shown in Table 5

Acute oral toxicity screening

The acute toxicity of the crude ethanolic *Hippocratea welwitschii* root extract was determined using the method of Lorkefor intra-peritoneally (IP) and oral routes in mice (Okoh-Esene*et al.*, 2012). The method consisted of two phases. In the first stage, three groups of three mice each were fed orally with the *Hippocratea welwitschii* root extract at doses of 10, 100 and 1000 mg/kg body weight (*ad labitum*) and observed for signs of toxicity and death within 24 hours. In the second stage, four groups of three each were treated with four more specific doses of the extract based on the result of the first stage carried out using the Swiss albino mice as described below.

Three animals were sorted into each of the cages, weighed and coded for easy identification. The extract (1.0 g) was dissolved in 10.0 ml of water to act as the stock from which other concentrations in milligrams per kilogram body weights of the animals were prepared and the animals dosed as appropriate. The dosing was orally done (*ad libitum*) and the observations were noted.

The LD_{50} value was determined by calculating the geometric means of the lowest dose that caused death and the highest dose for which the animals survived as shown below.

LD_{50} =√maximum tolerated dose x minimum toxic dose, where the maximum tolerated dose was 2900mg/kg and the minimum toxic dose

The results are shown in Table 6.

RESULTS AND DISCUSSION

The crude 95% ethanol extract of the root of *Hippocratea welwitschii* was subjected to qualitative and quantitative phytochemical screening. The results of the phytochemical analysis are shown in Table 1 and revealed that the root extract contains saponins, alkaloids, phenols and glycosides in the following amounts: 1.66 $x10^{-2}$, 3.67 $x10^{-3}$, 2. 64 x 10^{-2} and 2.01 x 10^{-2} µg/g, respectively. Plant saponins generally help humans to fight fungal infections, combat microbes and viruses and knock out some tumor cells, particularly lung and blood cancers (Barakat *et al.*, 1993, Poornima and Ravishankar, 2009). They also bind blood cholesterol, thereby reducing heart problems, but the most exciting and outstanding prospect for saponins is how they inhibit and kill cancer cells (Poornima and Ravishankar, 2009). It

has also been reported that they do so without destroying normal cells in the process, as is the mode of some cancer fighting drugs (Poornima and Ravishankar, 2009, Ryam and Shattuck, 1994). It is also known that trace quantities of phenolic compounds help prevent the death of plants since phenolic compounds from plant extracts act as antimicrobial agents (Ofokansi *et al.*, 2005).

The sedative and anticonvulsant properties of many plants used for treatment of epilepsy in traditional medicines around the world have been attributed to some phytochemicals found in them. Some of these include flavonoids, saponins and isoquinoline alkaloids, particularly berberine (Phytochemicals: websites). The crude 95% ethanol extract was screened for antimicrobial activity. The results (Table 2 and Fig 1) showed that the crude ethanolic extract was active against *Klebb species, S. aureus, B. subtilis, E. coli* and *C. albicans* with MIC values of 200, 200, 100, 250 and 250 µg/ml, respectively. The antibiotic property of the plant observed in Table 2 could be attributed to the presence of saponins, phenols and alkaloids. This is supported by numerous researchers such as the findings of Jacoband Burri(1996). Phenols, saponins and alkaloids in the root of the plant may be responsible for its use in the treatment of cough, dysentery, inflammations and ringworm (Bauer *et al.*, 1966, Fankel *et al.*, 1993, Sterberg, 1999). Their natural tendency to ward off microbes makes them good candidates for treatment of fungal and yeast infections (Eka, 1998).These compounds serve as natural antibiotics, which help the body to fight infections and microbial invasion (Sodipo *et al.*, 2000).

Table: 1 Qualitative and Quantitative Phytochemical Analysis of Root extract of *Hippocratea welwitschii*

Metabolites	Presence	Quantity(µg/g)
Tannins	-	-
Phlobatannins	-	-
Chlorogenic acid	-	-
Anthraquinones	-	-
Saponins	+	1.666×10^{-2}
Alkaloids	+	3.67×10^{-3}
Phenols	+	2.64×10^{-2}
Balsams	-	-
Anthracenes	-	-
Flavonoids	-	-
Resins	-	-
Sterols	-	-
Glycosides	+	2.01×10^{-2}
Terpenoids	+	3.08×10^{-3}

Key: + = Present; - = Absent

The mineral composition of the root of *Hippocratea welwitschii* was determined and the results showed that the elements are present in appreciable concentrations (Table 3). Minerals are known to play important metabolic and physiologic roles in the living system (Oliver-Beber, 1989, Enechi and Odonwodo, 2003).Iron, zinc selenium and manganese strengthen the immune system as antioxidants(Ujowundu *et al*., Talwar *et al*.,1989), while magnesium, zinc selenium are also known to prevent cardiomyopathy, muscle degeneration, growth retardation, alopecia, dermatitis, immunologic dysfunction, gonadal atrophy, impaired spermatogenesis, congenital malformations and bleeding disorders (Enechi and Odonwodo, 2003, Talwar *et al*., 1989). The other elements like copper, molybdenum, chromium and cobalt though in trace amounts are essential for survival of all forms of life, but Nickel has often been associated with allergies Bauer *et al*., 1966). These elements are required in trace amounts (RDA<200mg/day), usually because they play a catalytic role in enzymes (Chaturvedi *et al.*, 2004).Cobalt is required for biosynthesis of vitamin B_{12} family of coenzymes. Animals cannot biosynthesize B_{12}, and must obtain this cobalt-containing vitamin in the diet. Selenium is required for peroxidase (antioxidant proteins) (Nelson and Cox, 2000), while zinc is required for several enzymes such as carboxypeptidase, liver alcohol dehydrogenase, and carbonic anhydrase (Corbridge, 1995).

Table 2: Antimicrobial screening of the crude 95% ethanol extract of the root extract of *Hippocratea welwitschii*

Organism	**Zone of inhibition(mm)**	**MIC(µg/ml)**	**Am**
Klebb spp	12.0	200	18.0
Sa	12.5	*200*	33.5
Bs	*20.0*	*100*	*30.0*
Ca	*10.0*	*250*	*11.0*

Key: *Klepp spp=Klepp species, Sa=Staphylococcus aureus, Bs =Bacillus substilis, Ec=Escherichia coli, Ca=Candida albicans*, Am=Amoxicillin, MIC=Minimum Inhibition Concentration

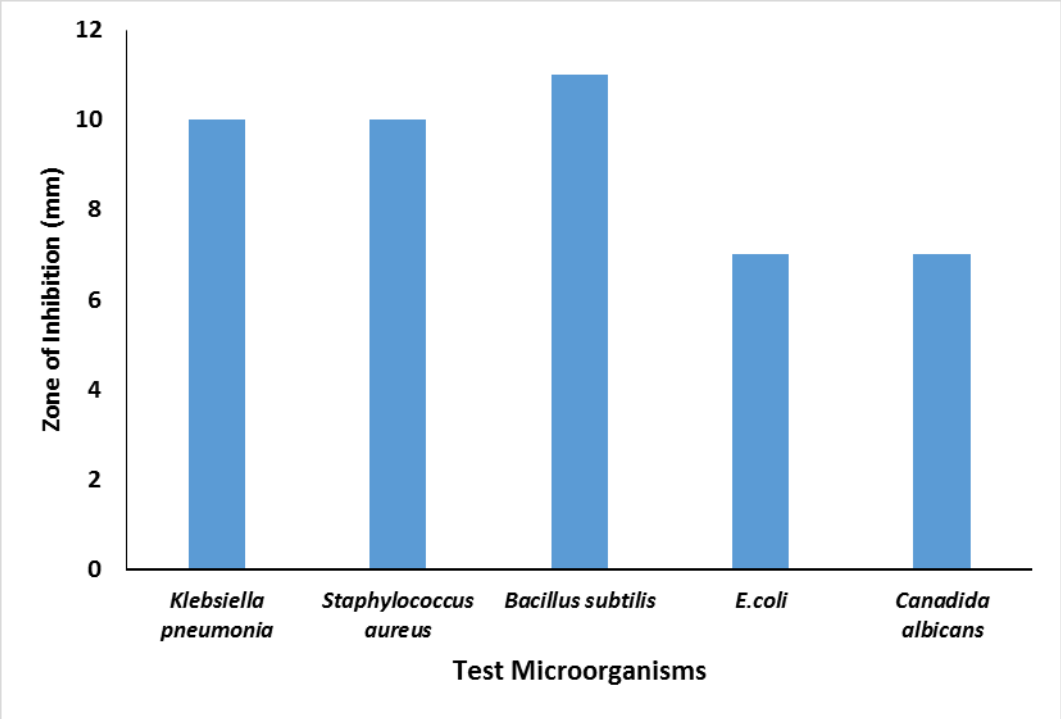

Fig. 1. Antimicrobial screening of crude 95% ethanolic root extract of *Hippocratea welwitschii*

Table 3: Mineral analysis of the root powder of *H. welwitschii*

Element	Quantity (mg/g)
K	1.987
Na	2.324
Ca	0.218
Mg	0.774
Zn	0.041
Pb	0.068
Cu	0.011
Co	0.149
Ni	0.239
Cd	0.197
Mn	0.301
Se	0.162

Potassium, sodium and calcium are common electrolytes needed in the body. Their functions in the muscles and nerves may be responsible for the use of this plant for the treatment of epilepsy (Okoh-Esene*et al.*, 2012).Potassium is essential for heart and nerve health). Calcium is required for nearly all functions in the body including muscle contractions and blood

clotting. The elements are also needed structurally for muscle and digestive system health, bone strength; some neutralize acidity, may help clear toxins, and provide signaling ions for nerve and membrane functions. Magnesium is required for processing ATP and related reactions (builds bone, causes strong peristalsis, increases flexibility and increases alkalinity (Chaturvedi *et al*., 2004).

Table 4: Free-radical scavenging activities of *Hippocratea welwitschii. r*oot extract

Conc. (mg/mL)	Average Absorbance(452 nm)	Blank	%Inhibition
5.0	0.225	-	86.00
3.0	0.235	-	84.48
2.0	0.543	-	66.15
1.0	0.605	1.604	62.31
0.5	0.770	-	52.03
0.1	1.182	-	26.31
0.05	1.273	-	20.64
Vit. C	90.190		90.19

Key: Vit.C = Vitamin C., *H. w =Hippocratea welwitschii*

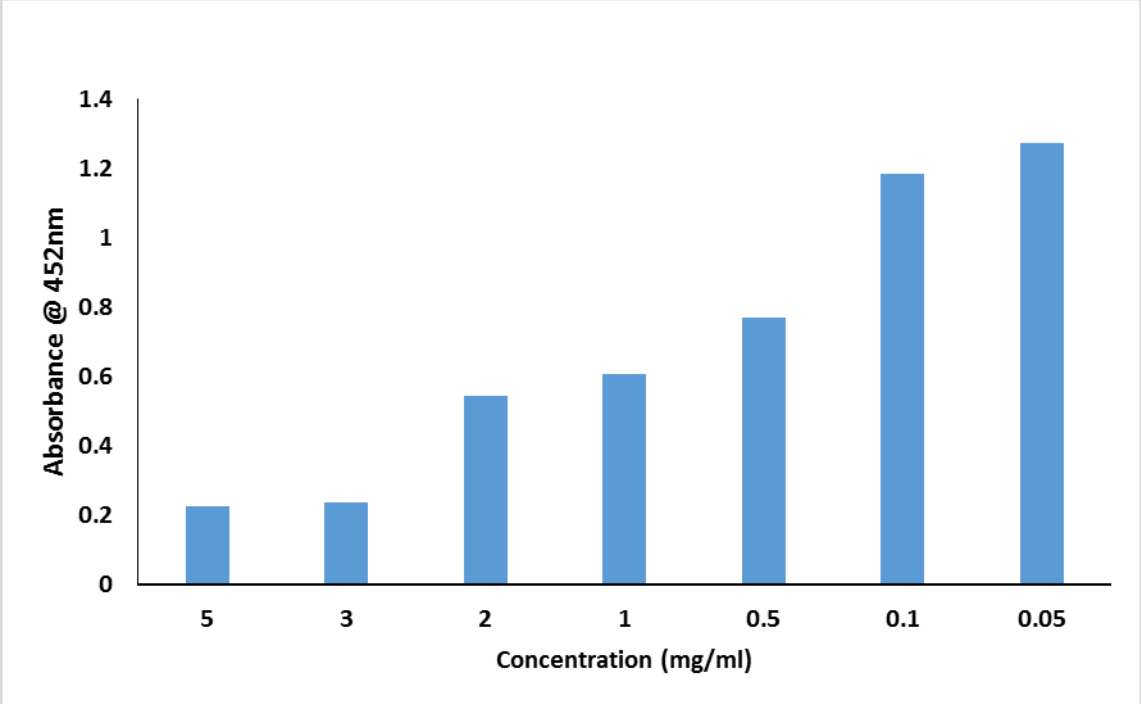

Fig 2.Antioxidant activity of crude 95% ethanolic extract of root of *Hippocratea welwitschii*

From the table above, the free radical scavenging ability of *Hippocratea welwitschii* root extract is very high. This goes to imply that their ability to quench the damaging effects of free radicals which are injurious to health is also very high. Plant saponins, alkaloids and phenols have been reported to generally help humans to knock out some tumor cells, particularly lung and blood cancers (Barakat *et al*., 1993, Poornima and Ravishankar, 2009, Lippard and Berg, 1994). As cellular metabolism or energy production requires oxygen, potentially damaging compounds known as free radicals can form. Most of these are oxidizers (i.e. acceptors of electrons) and some react very strongly. For the continued normal cellular maintenance, growth, and division, these free radicals must be sufficiently neutralized by antioxidant compounds. It has also been found that anti-oxidant therapy has been very successful in improving cognitive function and behavioral deficits in patients with mild to moderate Alzheimer's disease (AD) and epilepsy and natural products have been considered to be the biggest source of anti-oxidants which can be beneficial for AD therapy (Maya and Sarada, 2014, Gutzumann and Hadler, 1998). The high antioxidant activity and the high concentration of phenols (2.64×10^{-2} µg/g) recorded in this study for *Hippocratea welwitschii* root supports its highly successful use in south-south Nigeria in the management of epilepsy and some researchers have proved that phenolic compounds such as phenolic acids and flavonoids are responsible for the antioxidant activity of plant materials (Rice-Evans *et al*., 1996).

Table 5. Brine shrimps lethality test on crude 95% ethanolic extract of root of *Hippocratea welwitschii*

Conc.(ppm)	No. of Shrimps	No. of Deaths	% Death
10,000	10	10	100
1,000	10	10	100
100	10	10	100
10	10	10	100
DMSO	10	01	10
Sea Water	10	02	20

Key: LC_{50} = 0.00ppm (µg/mL) non-toxic; $LC_{50} \leq$ 1000 ppm (µg/mL) = toxic, DMSO=dimethyl sulphoxide

The results of brine shrimp lethality test (Table 5**)** showed high cytotoxicity against shrimps at a concentration as low as 10 ppm. This suggests that the plant extract may also be useful as a possible excellent medication for cancer and as a pesticide since the cytotoxicity screening

using the brine shrimps lethality test(BST) showed very high toxicity (LC_{50} of 0.00µg/ml ≤ 1000µg/ml). Since cancer cells have more cholesterol type compounds on their membranes than normal cells, saponins therefore bind cholesterol and thus interfere with cell growth and division (Poornima and Ravishankar, 2009, Ryam and Shattuck, 1994). Some plant extracts containing some alkaloids (dihydro dioscorine) have been reported to possess a long lasting hypotension and contraction of the smooth muscle fibers in the intestine both *in-vivo* and *in-vitro* when administered to animals (Poornima and Ravishankar, 2009, Sodipo *et al.*, 2000). In the control, sea water and DMSO two and one death occurred, respectively, which could have resulted from starvation as not all of them hatched at the same time. Naturally, those that hatched earlier could have had challenges with food to sustain them.

Table 6. Acute oral toxicity test on crude 95% ethanolic root extract of *Hippocratea welwitschii*

	Doses in mg/Kg Bodyweight Phase 1				Doses in mg/Kg Bodyweight Phase 2				
Conc.(ppm)	0(Control)	10	100	1000	0 (Control)	600	1600	2900	5000
Body weight (g)	20	19	18	17	20	22	19	18	21
		17	17	17		16	17	18	23
		16	15	15		18	17	21	15
Number of Deaths	0/1*	0/3*	0/3*	0/3*	0/1*	0/3*	0/3*	0/3*	3/3*
Clinical Signs	x	+	++	+++	x	++	+++	++++	+++++
		+	++	+++		++	+++	++++	+++++
		+	++	+++		++	+++	++++	+++++

Key: (*)= number of mice which died/number used, (x)= no clinical sign, (+) = mild drowsiness, (++)=sedated, (+++)=highly sedated, (++++)= gravely sedated, (+++++)=sedated and death.

The LD_{50} value was determined by calculating the geometric means of the lowest dose that caused death and the highest dose for which the animals survived as shown below:-

LD_{50} =√maximum tolerated dose x minimum toxic dose, where the maximum tolerated dose was 2900 mg/kg and the minimum toxic dose =5000 mg/kg

=√2900 x 5000 =3800 mg/kg.

The sedative nature of the extract could be attributed to the presence of saponins and since the LD_{50} is 3800 mg/kg, it is relatively safe for consumption. The sedative activity of the

extract also explains the anti-epilepsy property of the extract in traditional medicinal preparations (Dubois *et al.*, 1986).

CONCLUSION

From the results of this study, it has been established that the root of *Hippocratea welwitschii* has antimicrobial, antioxidant and cytotoxic activities which may be attributable to the phytochemicals found in the crude 95 % ethanolic extract. The root also has been shown to contain very important minerals such as potassium, sodium and calcium. These findings provide scientific evidence for the potential of the plant as a source of drugs for the management of cancer, infections and cognitive diseases such as epilepsy. In fact, it supports the claim by the natives of south-south Nigeria where the root has been successfully used to treat epilepsy for over a century. The presence of potassium, sodium and calcium which are electrolytes that have been implicated in heart muscles and nerve contractibility and general good health could be responsible for this. From the result of the cytotoxicity test, it may also be useful in the treatment of some cancers. This is polyvalence because in whole organisms or tissue studies, compounds present may be active against a range of targets.

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