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Original Research Article

Screening of Five Herbal Formulations Sold in South-East Nigeria for their Phytochemical Properties, *In Vitro* Antioxidant, Antiplasmodial and Cytotoxic Activities

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ABSTRACT

Due to their rich bioactive secondary metabolites, herbal formulations sold in Nigeria are believed to have a curative effect on ailments including malaria. The study assessed the phytochemical properties, antioxidant properties, antiplasmodial, and cytotoxic effects of five commercially available herbal formulations. Preliminary phytochemical analysis for alkaloids, flavonoids, saponins, steroids, tannins, and terpenoids was carried out following standard procedures. *The in vitro* antiplasmodial assay was evaluated using the DD2 chloroquine resistant *Plasmodium falciparum* strain, while cytotoxicity was assessed using the tetrazolium-based colorimetric (MTT) assay, and the antioxidant properties were evaluated by assessing (2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging activities of these herbal formulations. Results revealed that the five herbal formulations had antiplasmodial potential and also had a cytotoxic effect on jukart cell lines used for the assay, and finally, there was variation in phytochemical constituents, total phenolic content, and antioxidant activity among the five herbal formulations screened. It can be concluded that these herbal formulations possess antimalarial potential and are not toxic to the Red blood cells.

***Keywords*:** Herbal formulations, Phenolic, Antioxidant, Terpenoids, Antiplasmodial, Plasmodium.

Introduction

Malaria infection remains a major source of concern in the developing countries.[1] It is caused by any of the five known *Plasmodium* species namely: *vivax*, *ovale*, *malariae*, *knowlesi* and *falciparum*. However, the main cause of infection in human is the *Plasmodium falciparum* which is transmitted to human when the female Anopheles mosquito has a blood meal on a human host. The parasites thrive in the humid, swampy area.

In Nigeria, malaria infection accounts for about 25% of infant mortality and 30% childhood mortality.[1] Cases of antimalarial (artemisinin) drug resistance has been reported in some part of Asia (Cambodia and Thailand).[2,3] About 2.5 million species of plants have not been investigated for their pharmacological activities. [4,5] it is a known fact that Africa has a high incidence rate of malaria deaths with over half of the cases linked to Nigeria.[6]

Oxidative stress is the main causative factor giving rise to many chronic and degenerative diseases, including diabetes mellitus, cancer, heart diseases and aging.[7]

Antioxidants help to mop up free radicals which cause oxidative stress via cell damage in the body. Scientists have shown interest in medicinal plants for evaluation of antioxidants and phytochemicals such as phenols, flavonoids and tannins which have gained more recognition for their role in the prevention of human diseases.[8]

The evaluated herbal formulations are claimed by the manufacturers to have so much medicinal importance and can be used to treat several ailments, including malaria, typhoid, and serve as antioxidants, antibacterial, and antifungal remedies in traditional medicine practice in Nigeria. However, there has not been any scientific validation to back up these claims. The present study assessed the qualitative phytochemical properties, *in vitro* antioxidant, antiplasmodial and cytotoxicity effect of these herbal formulations sold in Nigeria.

Materials and Methods

Parasites strain

The (DD2) chloroquine resistant strain of the plasmodium parasite was acquired from the Department of Clinical Pathology, Noguchi Memorial Institute for Medical Research, Legon, in Accra, Ghana.

Cell culture

Leukemia (Jurkat) cells were obtained from RIKEN BioResource Centre Cell Bank (Japan). The cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Urbana, IL, USA) and 1% of penicillin streptomycin L-glutamine (Sigma-Aldrich, Urbana, IL, USA). Cells were grown in a humidified incubator (Panasonic Healthcare Company Limited, Japan) at 37°C, supplied with 5% CO_2 and passaged on reaching about 90% confluency.[9]

Herbal formulation

The herbal formulations were purchased from drug stores in the south-east region of Nigeria between the months of March and April, 2021. Twenty millilitres (20 mL) from each herbal formulation were measured and transferred into a Labconco fast-freeze flask and stored

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in the freezer at -20°C. The herbal formulations were removed and placed in a freeze dryer (Labconco FreeZone 6, USA) with reduced pressure of 12 mbar at -40°C to remove all moisture for complete dryness. The dried extracts were weighed using an electronic weighing balance, transferred into glass tubes, and stored for further analysis.

Phytochemical analysis
Phytochemical tests for the presence of saponins, tannins, terpenoids, flavonoids, steroids and alkaloids were carried out as previously described, [10] with slight modifications.

Determination of total phenolic content
Total phenolic content of the herbal products was determined using the Folin-Ciocalteau method as previously described, [10,11] with some modifications. In brief, two-fold dilutions were prepared from a stock solution of 10 mg/mL of each herbal product. Then, 790 µL of distilled water was added to 10µL of each sample dilution followed by 50 µL of Folin-Ciocalteau reagent. The mixture was thoroughly mixed and incubated for 8 minutes in the dark. Subsequently, 150 µL of 7% Na_2CO_3 (Sodium carbonate) was added and the mixture was incubated in the dark for 2 hours at room temperature. The absorbance of the mixture was measured using a microplate reader (Tecan Infinite M200 PRO, Switzerland) at a wave length of 750 nm. A standard Gallic acid calibration curve was plotted and used to estimate the concentration of total phenolic content of each herbal product. The total phenolic content of each sample was expressed in grams of Gallic acid equivalents (GAE).

Determination of antioxidant activity (DPPH Assay)
The scavenging activities of the herbal mixtures on the stable free-radical DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) were assayed according to a method previously described, with slight modification.[12,11] Serial dilutions of each sample were prepared from a stock of 20mg/ml to obtain the various concentrations (10, 5, 2.5, 1.25, 0.625, 0.3125, 0.156 and 0 mg/ml. Each reaction mixture comprised 100µl of 0.5mM DPPH solution (Methanolic DPPH) and 100µl of the sample in 96-well plates. The plates were incubated for 20minutes at room temperature in the dark, and the absorbance was read using a microplate reader (Tecan Infinite M200, Austria) at 517nm. Triplicate experiments were performed. Ascorbic acid was used as positive control and the diluents (water or methanol) were used as blanks.

$$\text{Radical scavenging (\%)} = \frac{(A0 - A1)100}{A0}$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample extracts. Control is the test solution without a sample. Ascorbic acid was used as standard. A similar procedure was followed with the ascorbic acid solution. The antioxidant activity of each sample was expressed in terms of IC_{50} (concentration required to inhibit DPPH radical formation by 50%). Where 50% of the free-radical activity of DPPH is quenched, was extrapolated from graph of percent antioxidant activity versus sample concentration.[11,12]

Preparation of herbal formulation
Five different concentration of each herbal formulation was assayed from a stock solution of 50 mg/mL and filtered using a 0.22 µm membrane (Millipore) filter. Two-fold serial dilutions were employed giving a final concentration value ranging from 6.25 – 100 µg/mL for the herbal formulations and 0.19 – 6.6 nM for artesunate. Artesunate served as the positive control.

Assessment of antiplasmodial activity
The screening of the antiplasmodial activities of the five herbal formulations was performed using the SYBR green I-based fluorescence assay as previously described.[14,15] The infected Red blood cell were incubated with herbal formulation and artesunate under culture conditions using 1 % parasitaemia and 2 % hematocrit. The negative controls were not treated with any drug. The plates were covered and mixed gently to ensure even distribution. Using the candle jar method, the microplates were incubated at 37 °C for 72 hours in a modular chamber under low oxygen and carbon dioxide level. After 72 hours, 100 µL of SYBR Green lysis buffer was added to the well, the content was mixed slowly and incubated in the Dark for 3 hours. The Tecan fluorescence (Tecan Infinite M200, Austria) multi-plate reader was used to assess the fluorescence in each well at excitation and emission wavelength of 485 and 530 nm, respectively. The intensity of the fluorescence signals was plotted against the drug concentrations to obtain a dose-response curve. The curve was analyzed to determine 50 % inhibitory concentrations (IC_{50}) of the drugs using GraphPad Prism 6.01 (GraphPad Software Inc, San Diego, CA).

Screening of herbal formulations for red blood cell cytotoxicity
The MTT 3-(4, 5-dimethylthiazol-2-yl)-5-diphenyltetrazolium bromide-based assay as described in earlier studies with slight modification was used for this study.[14,15] Briefly, for erythrocyte survival assay, a 100 µL of washed Red blood cell at 2% hematocrit was added 100 µL volumes of serially diluted herbal formulations (For each herbal formulation, five duplicate dilutions were prepared with concentrations ranging from 6.25 µg/mL to 100 µg/mL. The plates were incubated at 37 °C for 72 hours in a modular chamber under low oxygen and carbon dioxide. After 72 hours, the MTT-based colorimetric assay was initiated by adding 20 µL of 7.5 mg/mL MTT and re-incubated for another 2hours. The formazan formation was stopped by adding 150 µL of 10 % Triton X-100 in acidified isopropanol and incubated in the dark at room temperature overnight. The optical densities were then read at 570 nm using a Tecan fluorescence (Tecan Infinite M200, Austria) multi-plate reader. A survival curve of OD against concentration was plotted to determine the percent survival of the RBCs. The 50 % cytotoxic concentrations (CC_{50}) were determined by regression analysis. The selectivity indices (SI) of the herbal formulation CC_{50}/IC_{50} values were determined.

Determination of cytotoxicity effect of the herbal formulations on Jukart cell lines
The ability of the herbal formulations to inhibit proliferation of cancer cells was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method previously described Appiah-opong et al.[16] Briefly, cells were seeded in 96-well plates at a cell density of 1×10^5 cells per well. The cells were treated with different concentrations of reconstituted herbal formulations (0 – 1000 µg/mL), curcumin served as positive control, or media alone (negative control) and incubated at 37 °C, 5% CO_2 for 72 hours. After incubation, 20 µL of MTT solution (2.5 mg/mL) was added to each well, and the plates were incubated at 37 °C for 4 hours. A volume of 150 µL of acidified isopropanol was added to each well to dissolve any formazan crystals formed and absorbance of each well determined at a wavelength of 570 nm using a microplate reader (Tecan Infinite M200, Austria). All experiments were performed in triplicates, and percentage viability of cells at each concentration of extract was calculated as follows:

$$\%\ Cell\ viability = \frac{(Absorbance\ of\ control - Absorbance\ of\ sample)100}{(Absorbance\ of\ control)}$$

Statistical analysis
The results were presented as the Mean ± SD (standard error mean) of 3 replicates. All data were analysed at a 95% confidence interval using GraphPad Prism 6.01 (GraphPad Software, Inc, San Diego, CA). *P* values less than 0.05 were considered statistically significant. The $IC5_{50}$ and CC_{50} values were obtained from the log-linear regression analysis of log-dose response curves using the GraphPad Prism 6.01.

Results and Discussion

Phytochemical analysis
Table 1 showed that saponins were detected in all herbal formulations followed by flavonoids present in 4 of the herbal formulation except in African IBA. Alkaloids were found to be present in Deep Root, Blood Purifier and Ruzu bitters. Tannins were present in 4 except African Iba

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and the same applies for terpenoids, finally, steroids were absent in all herbal formulations.

Total Phenolic acid Content of Herbal Formulation
It was observed that phenolic acid was found to be present in varying amounts in 4 of the herbal formulations except for African iba (Table 2). Among the 5 herbal products, Deep Root had the highest phenolic content (95.96 mg GAE/g) followed by Blood Purifier (69.13 mg GAE/g), Ruzu bitter (17.87 mg GAE/g), Yoyo cleanser (8.47 mg GAE/g) while phenolic was absent in African Iba.

Antioxidant activity of herbal formulation
The Deep Root herbal formulation has the highest antioxidant value of IC_{50} 0.1957 ± 0.010 mg/mL but African Iba has no detected antioxidant effect (Table 2).

Antiplasmodial assay
African iba has the best antiplasmodial activity with an IC_{50} of 9.61 µg/mL followed by Blood purifier, Yoyo bitters, Deep root and Ruzu bitters with IC_{50} of 14.68, 30.57, 35.34 and 39.39 µg/mL respectively while the positive control (Artesunate) had an IC_{50} of 0.93 µg/mL (Figure 1).

C*ytotoxicity (Jukart cell line and Red Blood Cells)*
Table 3 showed that the 5 herbal formulations had cytotoxic effect on the jukart cell line. The Deep root herbal formulation had relatively stronger toxicity against Jukart cell line followed by Blood purifier, Yoyo bitters, Ruzu bitters and African iba with CC_{50} of 206.50 µg/mL, 570.58 µg/mL, 685.61 µg/mL, 725.16 µg/mL and 1000 µg/mL. Curcumin served as the positive control with CC_{50} value of 2.93 µg/mL. Also, it showed that these five herbal formulations had no negative effect on the Red Blood Cells. These shows that the five herbal formulations had good selectivity towards DD2 since all selectivity indices were greater than 2.
This study assessed the antimalarial activity and also evaluated the cytotoxic effect of the five herbal formulations sold in South-east, Nigeria.

Table 1: Phytochemical composition of the herbal formulations

Phytochemical Compound	The Herbal Formulations				
	Deep Root	Blood Purifier	African Iba	Ruzu bitters	Yoyo cleanser
Saponin	+	+	+	+	+
Flavonoid	+	+	-	+	+
Alkaloids	+	+	-	+	-
Tannins	+	+	-	+	+
Terpenoids	+	+	-	+	+
Steroids	-	-	-	-	-

Key: +: present; –: absent

Table 2: Total Phenolic Acid Content and DPPH Scavenging activity of the Herbal Formulations

The herbal formulations	Total Phenols content (mg/GAE)	DPPH Scavenging activity IC_{50} (mg/mL)
Deep root	95.96 ± 2.56	0.190 ± 0.01
Blood purifier	69.91 ± 2.94	0,886 ± 0.002
African iba	None	None
Ruzu bitters	17.87 ± 0.28	3.39 ± 0.05
Yoyo cleanser	8.47 ± 0.21	6.89 ± 0.33

Data are expressed as Mean ± SEM

Phytochemical screening of various extracts revealed the presence of secondary metabolites.[17,18] Herbal formulations have occupied a unique position as the treatment therapy for a wide variety of diseases. The presence of biologically active compounds in plants has proven to have medicinal values in several experimental disease models.[19] Such compounds include flavonoids, tannins, steroids, Terpenoids, saponins. These compounds are believed to exhibit activity against pathogens and also aid the antimicrobial activities of medicinal plants.[20,21]
In this study Saponin and Flavonoid was present in deep Root, Blood Purifier, Ruzu bitters and Yoyo cleanser but not present in African iba. Alkaloid was absent in African iba and Yoyo cleanser but present in the 3 other herbal formulations. Tannin was present in 4 herbal extracts (Deep Root, Ruzu bitters, Blood Purifier, Yoyo cleanser) but not in African iba. Steroids were absent in all herbal formulations. The presence of alkaloids in this herbal formulation indicates that they can be used in medicine as anesthetic agents.[22] The presence of Saponin in plants has been suggested to be responsible for the tonic and stimulating activities observed in Chinese and Japanese medicinal herbs.[22]
Flavonoids is integral phytochemical constituents of higher plants believed to possess antioxidant potentials hence could offer protection against heart diseases and cancer, [22] probably by enhancing the body's defence against pathology-induced free radicals. Tannins can evoke an antidiarrheal effect and these substances may precipitate proteins on enterocytes reducing peristaltic movement and intestinal secretion.[24]
Phenolic compounds are considered secondary metabolites and are synthesized by plants during normal development, and in response to infections, wounds, UV radiation and insects**.** They have been identified to be present in both plants fit to be eaten and non-edible plants and have been linked to have multiple biological effects, including antioxidant activity.[24]
Herbs are known to produce secondary metabolites of which these metabolites play a vital role in their growth and development including protection from prey and environmental stress.[24] From our study, it was observed that among the 5 herbal formulations screened for total phenolic acid content Deep Root herbal mixture had the highest with a value of 95.96 mg GAE/g while Yoyo cleanser had the lowest value of 8.47 mg GAE/mg. The difference in the total phenolic content of these herbal products screened for this study may be due to differences in parts of the plants used for the preparation of each herbal.[25] Studies on phytochemical and antioxidant screening of these 5 herbal formulations have not been carried out before, indicating that this study is the first report of this screening on these formulations.
The DPPH radical is mostly used in the assessment of free radical scavenging activity because of the ease of the reaction.[26] In DPPH assay, the ability of these herbal formulations to donate hydrogen atoms or electron n transformation of DPPH radical into a reduced form DPPH was investigated. From our result, it was observed that Deep Root herbal formulation is higher and showed $1C_{50}$ value of 0.195 ± 0.01 mg/mL, followed by Blood Purifier with $1C_{50}$ of 0.89 ± 0.002 mg/mL, Ruzu bitters with 3.39 ± 0.05 mg/mL, Yoyo cleanser with $1C_{50}$ value of 6.89 ± 0.35 mg/mL while ascorbic acid which served as the positive control with an IC_{50} value of 0.0089 ± 0.0002 mg/mL. Infected cells including erythrocytes are always under oxidative stress; therefore in some disease conditions it is helpful for

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some of the agents used in treatment to possess antioxidant potential/activities.[19]

The result showed that African iba herbal formulation has a very promising *in vitro* antiplasmodial activity with IC_{50} value of 9.61 µg/mL against chloroquine resistant *plasmodium falciparum* (DD2). This result is in contrast to the earlier result obtained by Ikem *et al*., (2020) which stated that IC_{50} of African iba against chloroquine sensitive *plasmodium falciparum* (3D7) had a poor activity over 100 µg/mL.[14] This could be attributed to the difference in genetic makeup between both parasites.[27] From the manufacturers claim it was observed that African Iba herbal formulation was prepared using *Kigelia africana* and *Nauclea latifolia*. *Nauclea latifolia* and *Kigelia africana* has been reported in previous literatures to possess antiplasmodial effect.[28] *Kigelia africana* is believed to also possess anti-inflammatory activities.[28] The herbal formulation African iba showed a moderately active IC_{50} of 9.61 µg/mL, it has been established that extracts that showed an IC_{50} of antiplasmodial activity with values less than 5 µg/mL were considered active, while IC_{50} between 5 – 10 µg/mL were considered moderately active and values greater than 10 µg/mL were listed as weakly active.[28,29] Blood purifier, Yoyo bitters, Deep root and Ruzu bitters had a very weak activity with IC_{50} of 14.68 µg/mL, 30.57 µg/mL, 35.34 µg/mL and 39.39 µg/mL respectively. These values showed a weak activity when compared to Artesunate which served as the positive control with IC_{50} value of 0.93 ng/mL.

Some of the plant extracts contained in these formulations including *Magnifera indica*, *Uvaria chamae*, *Cymbopogon citratus*) have been reported to have antimalarial potential.[14,30]

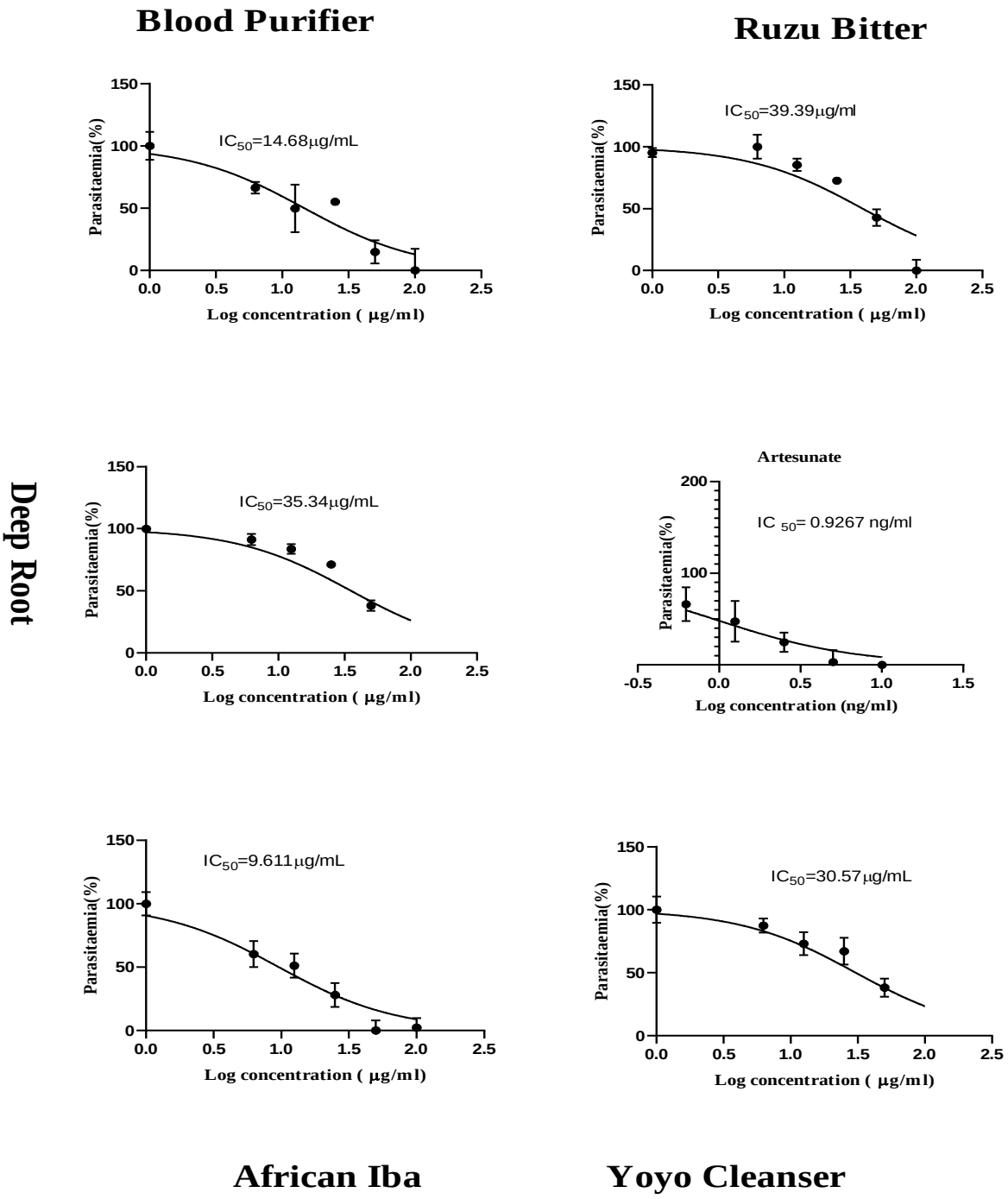

Figure 1: IC_{50} of the anti-malarial Herbal formulations and artesunate (Antiplasmodial assay)

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Table 3: Cytotoxicity effect and Selectivity index of the herbal formulations on Jukart cell line and Red Blood Cells

Herbal formulations	CC_{50} values (µg/ml)		Selectivity index on	
	Jukart Cell line	Red Blood Cells	Jukart Cell line	Red Blood Cells
African iba	1000	108.77	104.06	11.32
Deep root	206.50	107.79	5.84	3.05
Blood purifier	570.58	106.27	38.87	7.24
Yoyo bitters	685.61	102.00	22.43	3.34
Ruzu	725.16	102.07	18.41	2.59
Artesunate	ND	ND	ND	ND
Curcumin	2.93	ND	ND	ND

ND: Not determined; CC_{50}: Concentration of herbal extracts/Drugs which causes 50% cytotoxic effect.

Table 4: The five herbal formulations with their respective NAFDAC numbers

The herbal formulations	Nafdac number	Production Date	Expiration Date
Deep root	A7-0912L	12/19	12/21
Blood Purifier	A7-1390L	02/21	02/24
African iba	A7-0476L	06/20	06/23
Ruzu bitters	A7-1102L	01/20	01/22
Yoyo cleanser	A7-1055L	08/20	08/22

The jukart cell lines were exposed to these five herbal formulations with increment on concentrations. Curcumin served as the positive. Deep root herbal formulation out of the five herbal formulations showed the strongest cytotoxic effect on jukart cell line with an IC_{50} of 206.50 µg/mL while African iba had the least cytotoxic effect with an IC_{50} of 1000 µg/mL.

There has been claim that most herbal formulations marketed in Nigeria can act as antimalarial and anticancer agents, but this has not been validated. From the results obtained the herbal formulation screened had varying cytotoxic effects on the jukart cell line when compared to the positive control (curcumin) which had a high cytotoxic effect on the cell line with IC_{50} of 2.93 µg/mL. These five herbal formulations showed satisfactory selectivity indices ranging from 102.00 to 108.77 µg/mL.

It was observed that the selectivity of the five herbal formulations is greater than 2 (Table 2). This indicate that the herbal formulations have good selectivity for malaria parasite not harmful to the Red blood cell and making them good drug candidate due to the selectivity values been greater than 2.[19,31]

Conclusion

In conclusion, it can be stated that the five herbal formulations possess potential anti-malarial, good selectivity index, antioxidant activities, and possess active secondary metabolites. The five herbal formulations are not harmful to the erythrocytes.

Further studies are suggested to screen the herbal formulations for comprehensive *in vivo* studies, assessment of the liver enzyme activities and mode of action

Conflict of Interest

Authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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