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Research Article

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Phytochimic, cytotoxic, anti-hemolytic and antioxidant activities of leaf and bark of extracts of *Azadirachta indica* (Meliaceae)

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Abstract For decades, plants have been a source of inspiration for new drugs. Almost all ancient civilisations and cultures depended entirely or partially on phytotherapy because of its effectiveness, accessibility, availability and dose-related low toxicity. Thus, the know-how of traditional healers is a starting point for phytochemical and pharmacological investigation of these natural medicines. Therefore, the present study was aimed at studying phytochemical, cytotoxic, anti-haemolytic and antioxidant activities of leaf and bark extracts of *Azadirachta indica.*
The leaves and bark of *A. indica* were harvested, dried and reduced to powder. Then the powder was extracted in different solvents (distilled water, ethanol and ethanol/water 70% (v / v)). The resulting extracts were subjected to phytochemical analysis by colorimetric reactions followed by the cytotoxicity test on the Vero cell line using colorimetric method at MTT. The antihemolytic activity of the promising extracts was performed on the red blood cells and the antioxidant properties of the extracts were evaluated by the DPPH radical scavenging method and Ferric ion reduction**.**
The results show that the extraction yields ranged from 5.02 to 20.7%. The phytochemical screening revealed the presence of alkaloids, polyphenols, saponosides and steroids. All the extracts were non-cytotoxic with CC_{50} values ranging from 169.73 to greater than 1000 µg/ml and two of these exhibited antihemolytic activities with 62% and 57% protection percentages. The extracts had DPPH IC_{50} values ranging from 14.933 to 95.360 µg/mL. The ferric ions reducing power (OD) of the different extracts ranged from 0.15805 to 3.40545.
The activities of *Azadirachta indica* extracts indicate that this plant is a potential source of active compounds.

Keywords *Azadirachta indica*, cytotoxicity, anti-hémolytic, antioxidant

Introduction

For decades, plants have always been used as food or medicine worldwide. Herbal medicines are considered as inoffensive and less toxic compared to pharmaceutical drugs. This may explain the increasing interest of pharmaceutical industries in the ethnobotanical studies of plants. Africa has a significant diversity of medicinal plants. Medicinal plants represent valuable resources for the vast majority of African rural populations where more than 80% use these plants for their primary health care [1]. However, today, Africa is increasingly challenged with the emerging of new oxidative stresses-related diseases. In the biological system, oxidative stress is the consequence

of an imbalance between the antioxidant defense system and the production of reactive oxygen species (ROS), leading to biochemical lesions in the body cells; at the molecular level, this may be associated with alterations in protein and lipid molecules, or breaks in the DNA molecules, or impairment of the integrity of the cell membrane [2]. This may be due to oxidative attack of ROS in the erythrocytes targeting hemoglobin and the cell membrane. However, under high stress conditions, despite the establishment of an effective defense system, the iron (II) contained in oxyhemoglobin can transfer an electron to oxygen by generating a superoxide radical, which will lead to the transformation of hemoglobin into methemoglobin, hence hemolysis [3]. Most pathologies, whether of genetic origin or not, have their origin in the structural and / or functional alteration of proteins of interest. Aggregation, glycation and oxidation are the main types of protein modifications commonly seen in many diseases such as diabetes, arteriosclerosis and neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease [4]. The use of synthetic antioxidants in food technology, cosmetics and pharmaceuticals, known to address the health problem associated with oxidative stress, is currently being compromised by its potential toxicological risks. In fact, a long-term use of synthetic antioxidants is believed to be teratogenic, mutagenic and carcinogenic [5].

Conscious of these shortcomings, the World Health Organization (WHO) has urged African countries to develop regional strategies including undertaking more in-depth research on medicinal plants and traditional medicines to promote their optimal uses in health care delivery systems [6]. Though, many medicinal plants are still unexploited, others such as *Azadirachta indica* (Meliaceae), also called Neem in the regions of North Cameroon have been the object of many research investigations. It is an herbaceous that grows in the tropical and sometimes temperate regions of the globe. From ethno-pharmacological point of view, Neem has been used as antifungal and in the prevention and treatment of malaria [7]. Traditionally it is a powerful disinfectant used in Ayurvedic medicine. In addition, studies conducted by Ekanen in 1976 demonstrated also the schizonticidal activity of *Azadirachta indica* bark extract [8].

The aforementioned claims about *Azadirachta indica* trigger our interest in the evaluation of the cytotoxic, anti-hemolytic and antioxidant activity of its extracts in order to contribute to the treatment or the prevention of oxidative stress-based diseases.

Methodology

Drying and grinding of leaves and bark

The plant material (leaves and bark) was dried in a sun and light free environment for six days and then ground into fine powder in the laboratory before undertaking different extraction techniques.

Preparation of plant extracts

Water extraction was performed by macerating 500 g of the powdered leaves and barks of *Azadirachta indica* with 2000 ml of distilled water every 24 hours for three days. The macerate obtained after filtration was dried in an oven at 45 ° C for 3 days and 5 days for the leaf and bark extracts, respectively.

Hydroalcoholic extraction was achieved by macerating 500 g of the powdered leaves and barks with 600 ml of distilled water and 1400 ml of alcohol at 95 °C every 24 hours for three days. The macerate obtained was dried in the oven at 45 °C for 2 days.

Alcohol extraction was done by macerating 500 g of the powdered leaves and barks with 2000 ml of 95 °C alcohol every 24 hours for three days. Then the macerate was left to evaporate at room temperature for three days. The resulting crude extracts were weighed and their extraction yields were calculated according to the formula below:

$$\text{Yield\%} = (\text{mass of the dry extract}) / (\text{mass of the initial powder}) \times 100$$

Phytochemical Screening

Phytochemical screening of the different extract concentrates was carried out to assess the presence of different groups of secondary metabolites (polyphenols, flavonoids, alkaloids, tannins, saponins, steroids, polyterpenes, coumarins and anthocyans) following the modified protocols described by Harborne (1976), Odebeyi and Sofowara (1978), Trease and Evans (1989), Sofowora, (1993) [9-12].

Presence of Alkaloid by Mayer Test

First, 5 mL of 10% H_2SO_4 were added to 1 g of powder (plant extract). After 24 hours, the macerate was filtered and washed with water. Five drops of Mayer reagent were added to1mL of the filtrate. The mixture was left on for 15 minutes. The development of a creamy white or yellow-white precipitate indicated the presence of alkaloids.

Polyphenols identification test

Five milligram of extract were dissolved in a test tube containing 1 mL of ethanol, then 3 drops of 10% Iron Chloride III were added to the mixture. The appearance of a blue-violet or greenish coloration indicated the presence of polyphenols.

Presence of flavonoid by Shinoda test

In a test tube containing 1 mL methanol, 5 mg of extract were dissolved. To this solution, a few magnesium chips and 3 drops of concentrated H_2SO_4 were added. The presence of flavonoids was indicated by the appearance of the following colorations: yellow for flavones, red for flavonols and pink for flavonones.

Tannin assay using Stiasny reagent

To 0.5 mg of the extract, 2 ml of distilled water were added and then 15 ml of the Stiasny reagent, after 30 minutes of standing in a water bath at 80 °C. A precipitate indicated the presence of catechin tannins. The previous solution was filtered and the filtrate was saturated with sodium acetate. After adding 3 drops of iron chloride, the appearance of a blue-black coloration indicated the presence of gallic tannins.

Presence of saponoside by foam test

In a test tube containing 5 ml of distilled water, 5 mg of dry extract were added and the whole mixture was allowed to boil for 5 minutes. After cooling, the content of the tube is stirred in the vertical direction for 15 seconds, then left to rest. The appearance of a persistent foam of a height of more than one centimeter attested the presence of saponosides.

Presence of sterols and triterpenes using Liebermann Burchard test

In a test tube containing 1 mL of methanol, dissolve 5 mg of extract and add 0.2 mL of each of the following reagents: chloroform, glacial acetic acid, concentrated H_2SO_4. The appearance of a violet or greenish coloration indicated the presence of triterpenes and sterols.

Presence of coumarins

In a test tube containing 2 ml of distilled water and 2 ml of sodium hydroxide, 0.1 g of extract was dissolved. The observation of an intense blue fluorescence after addition of ammonia indicated the presence of coumarins.

Evaluation of cytotoxic activity [13]

The cytotoxicity of the compounds was carried out on the Vero cell line according to the colorimetric method with MTT (3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide) as described by Mosmann et *al*., 1983.

Principle

Step 1: Cell culture

Vero cells were maintained in culture in 75 cm^2 T-Flask containing DMEM supplemented with 10% FBS (Fetal Bovine Serum) and 1% streptomycin-penicillin antibiotic, standard condition with 5% CO_2 at 37 °C. The medium was renewed every 3 days after cultivation. Concerning the preparation of the cell suspension, the cells were detached by introducing 5 ml of 0.25% trypsin-EDTA into the T-Flask after triple rinsing with PBS (Phosphate Buffer Saline). After 5 minutes of incubation at 37 °C., the detached cells were centrifuged at1800 rpm for 3

minutes and the resulting pellets were suspended in 1 ml of medium. The suspension (20 µl) was added to 20 µl of trypan blue solution in order to evaluate the cell viability and the calibrated cell load via the Neubauer hematimeter.
Step 2: Cytotoxicity Screening at 1000 µg / mL
In a 96-well microplate, 100 µl of cell suspension loaded with 5×10^4 cells / wells were introduced into all the wells. At the end of a 24h incubation period, the medium contained in the wells was removed and 90 µl of a new medium were introduced, then 10 µl of our extracts at a concentration of 1000 µg / ml were also added. The plates were incubated for 48 h under a humidified atmosphere at 37 ° C. and 5% CO_2. The positive control consisted of 90 µL of cell solution plus 10 µL of 10% DMSO while the negative control consisted of 100 µL of the cell solution. At the end of 48 hours of incubation, 20 µl of MTT solution (Sigma) were introduced into each well and the whole mixture was homogenised and incubated for 4 hours at 37 °C. At the end of this incubation period, the content of the wells was carefully drained, replaced with 100 µL of DMSO in order to dissolve the formed formazan. The latter was quantified by reading the optical density at 570 nm using the TECAN plate reader (Infinite M200).
Step 3: Determination of the cytotoxic concentration 50
The cytotoxic extracts at 1000 µg / mL were diluted to obtain the following final concentrations of extracts: 500; 250; 125; 62.5; 31.25; 15.625µg / mL.
In a 96-well microplate, 100 µl of culture medium were introduced into all the wells except for line 1. Then, 180 µL of culture medium plus 20 µL of our extracts were introduced into the wells of line 1; remove then 100 µL of the content of line 1 and make second-order dilutions of concentrations ranging from (500 to 15.625µg / mL) from line 2 to 7; then discard the contents of the tip and all this will be done in triplicate.
From the obtained optical densities, the percentages of viable cells were calculated using the formula below:
Percentage inhibition (%) = (DO positive control-DO test) / (positive DO control) × 100
Cytotoxic concentrations (CC_{50}) were determined using the Statgraphics software. Following the defined by the American National Cancer Institute (NCI) criteria, an extract is considered cytotoxic if CC_{50} on the host cells is less than 30 µg / ml [14].

Anti-haemolytic activity
Principle
Step 1: preparation of erythrocytes
Before the rats were sacrificed, they were first exposed to ether for a few seconds for stunning purposes. The rats were now sacrificed and blood was collected with a syringe in the ventricle. The collected blood was distributed into sterile heparinised tubes and centrifuged at 1500 rpm for 5 min. After centrifugation, the supernatant was discarded and the erythrocytes were washed three times with 0.9% NaCl solution. A suspension of red blood cells was made from the obtained erythrocytes in order to adjust the hematocrit to 10%. The suspension was kept cool at 4 °C.
Step 2: Anti-haemolysis experiment
Before carrying out the test, we first performed a series of dilutions of our extracts. It was a second order dilution to achieve the following concentrations: 1000; 500; 250; 125; 62.5 µg / ml. Thus, at 0.5 ml of each of the extracts and at different concentrations, we added 0.25 ml of the erythrocyte suspension (10% hematocrit) and incubated for 5 min at room temperature. After incubation, we added 0.25ml of the 0.3% H_2O_2 solution and then incubated again for 4 hours at 37 °C. After incubation, the mixture was centrifuged at 2500 rpm for 10 min and then 100 µl of the supernatant were transferred to a microplate and the absorbance corresponding to the release of hemoglobin was measured at 540 nm. The negative control consisted of 0.9% sodium chloride and erythrocyte while the positive control consisted of 0.9% sodium chloride, erythrocyte and hydrogen peroxide.
The percentage of hemolysis and stabilisation or protection of the membrane was calculated using the following formula: Percentage of Hemolysis [%] = (Absorbance Test / Absorbance of Negative Control) * 100
Results were expressed as: protection [%] = 100 - [hemolysis]

Antioxidant activity

The evaluation of the antioxidant activity of the ethanol, hydro-ethanolic and water extracts of the leaf and bark powder of *Azadirachta indica* bark was carried out by appropriate tests as described below.

DPPH method

Determination of anti-radical activity on DPPH

The DPPH radical was used as a reference free radical to determine the ability of a plant extract to yield protons to stabilise a free radical. The protocol used for this method was the modified method described by Bassene (2012).

The extracts were diluted to obtain the following final concentrations: 250; 125; 62.5; 31.25; 15.625; 7.8125; 3.90625; 1.953125 and 0.9765625µg / mL.

In a 96-well microplate, introduce 100µl of ethanol into all the wells except for line 1. Then introduce 200µl of our extracts into the wells of line 1 and remove 100µl of each extract from line 1 and make second-order dilutions of concentrations ranging from (250 to 0.97µg / mL) from line 2 to 10 and then discard the contents of the tip. Line 11 was the negative control consisting of DPPH (without extract) and line 12 was the positive control (ascorbic acid) at the same concentrations as our extracts. The dilution (25µl of each extract) was added to a new microplate and 75µl of the 0.01% DPPH solution were added. Reading optical densities at 517 nm was performed after 30 minutes of incubation in the dark and at room temperature. The tests were performed in duplicates. The inhibition percentages were calculated from the optical densities according to the following formula: PI (%) = [(Ac-Ae) / Ac] * 100

Where: PI = percentage of inhibition

Ab =absorbance of the negative control

Ae = absorbance of the extract after 30 min of incubation

50% inhibition (CI_{50}), CE_{50} and PA were deduced from the variation of the anti-radical activity as a function of the concentration of the extracts.

Where: CI_{50} (SC_{50}) = anti radical concentration allowing to capture 50% of free radicals.

The effective concentration 50 (EC_{50}) is the concentration of extract required to capture ½ mole of DPPH. It was calculated by the following formula: CE_{50} SC50 = / ([DPPH])

[DPPH]: DPPH concentration

The anti-free radical power (PA) is the opposite of the effective concentration; the higher it is, the better the anti-radical power of the extract is; it is given by the following formula:

PA = 1 / (CE_{50}) with: PA: anti radical power

Reducing activity of Fe^{3+} ions

The Fe^{3+} reduction test was performed according to the modified protocol described by Path Canada (1994).

Principle

In a 96-well microplate, 100 µL of solvent (ethanol) were introduced into all the wells except those of the first line 1. Then 200 µL of our extracts were introduced into the wells of line 1; a dilution series of order 2 was carried out up to line 10. Dilution (25µl of each) was introduced into a new microplate and 25µl of Fe^{3+} solution to 1.2mg / ml were added, the mixture was incubated for 15min. After this incubation, 50 µl of the 0.2% orthoPhenantroline solution were added and the plate was re-incubated for 20 minutes still at room temperature. At the end of this incubation, the optical density of the content of the wells was read at 505 nm with a plate reader (TECAN Infinite). The test was performed in duplicates. The negative control which corresponded to 0% reduction consisted of solvent, Fe^{3+} and orthophenantroline and the positive control which corresponded to 100% reduction consisted of the hydroxylamine treated as the extracts. The results were expressed as reducing power (OD) as a function of the concentration of extract. It was calculated based on the equation of the straight line in hydroxylamine.

Results

Extraction efficiency

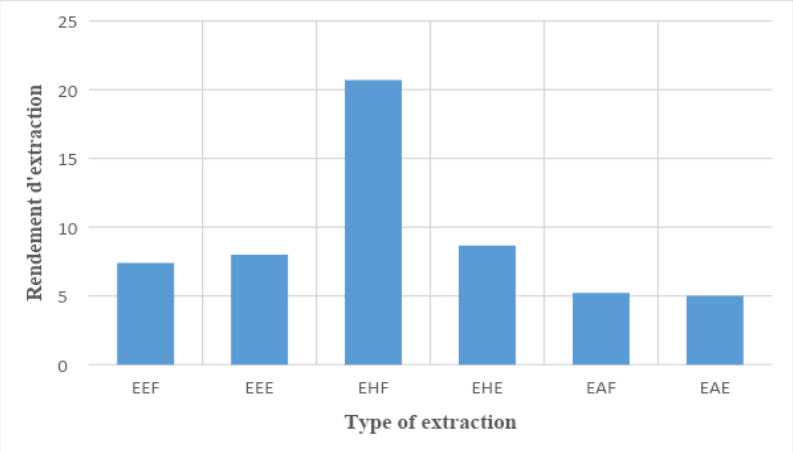

Figure 1: Yield of different leaves and barkextracts of of Azadirachta indica; EEF: ethanolic leaf extract; EEE: ethanol bark extract; EHF: hydro-ethanolic extract of the leaves; EHE: Hydro-ethanolic extract of bark; EAF: aqueous extract of the leaves; EAE: Aqueous extract of bark.

Phytochemical Screening of Extracts of interest

Phytochemical screening showed that alkaloids, polyphenols and flavonoids were present in all the extracts, while no extract contained terpenoid and coumarin. Moreover, all bark extracts and the aqueous leaf extract of *Azadirachta indica* do not have steroids. However, hydroethanolic leaf extract, aqueous leaf extract, and both ethanol and water bark extracts commonly possess tannins, and saponosides.

Table 1: The families of secondary metabolites present in the extracts

Code of Extract	EEF	EHF	EAF	EEE	EHE	EAE
Alkaloids	+	+	+	+	+	+
Polyphenol	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+
Tannins	-	+	+	+	+	+
Saponosides	-	+	+	+	-	+
Terpenoids	-	-	-	-	-	-
Coumarins	-	-	-	-	-	-
Steroids	+	+	-	-	-	-

Cytotoxic activity

The cytotoxic profile of plant extracts was determined by evaluating the percentage inhibition of normal cell growth (Vero cell). Results indicated that both aqueous bark and leaf extracts, ethanol bark and hydro-ethanol bark were not toxic. On the other hand, ethanol and hydro-ethanol leaves extracts were toxic. A series of dilutions of the toxic extracts was used to determine the concentration capable of lysing 50% of the cells

Table 2: Cytotoxic concentration 50 of the different extracts

Plant part	Type of Extract	CC_{50} (µg/ml)	Interpretation
Bark	Water extract	> 1000	Not toxic
	Ethanol extract	> 1000	Not toxic
	hydro-ethanolic extract	> 1000	Not toxic
Leaves	Water extract	> 1000	Not toxic
	Ethanol extract	170.9 ± 1.27	Not toxic
	hydro-ethanolic extract	366.3 ± 49.64	Not toxic

Anti-haemolytic activity of extracts

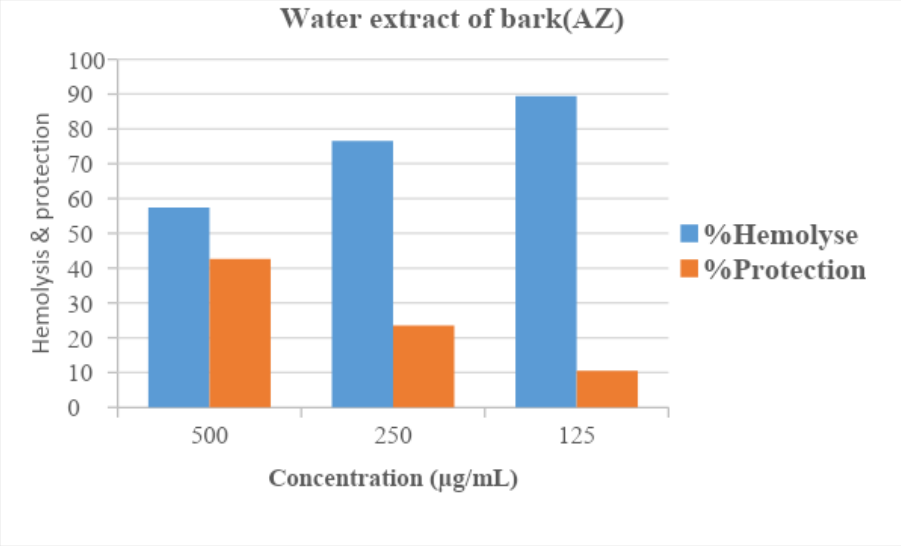

Figure 2: Percentage of hemolysis and protection of the water bark extract

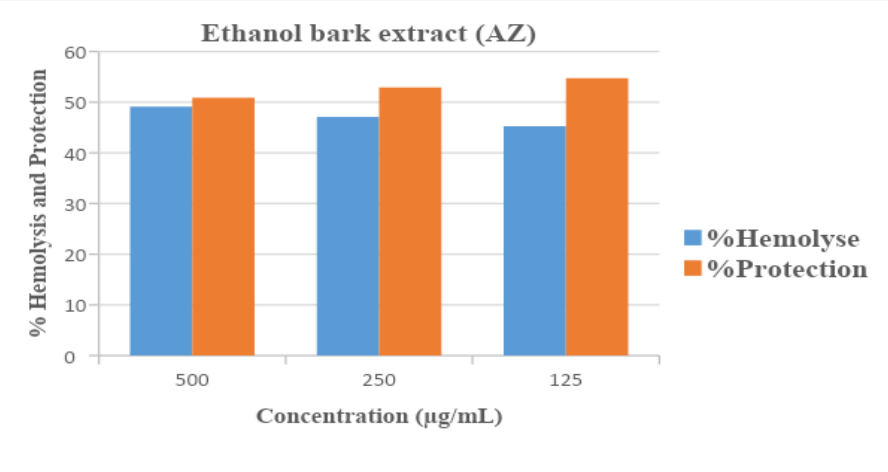

Figure 3: Percentage of hemolysis and protection of ethanol bark extract

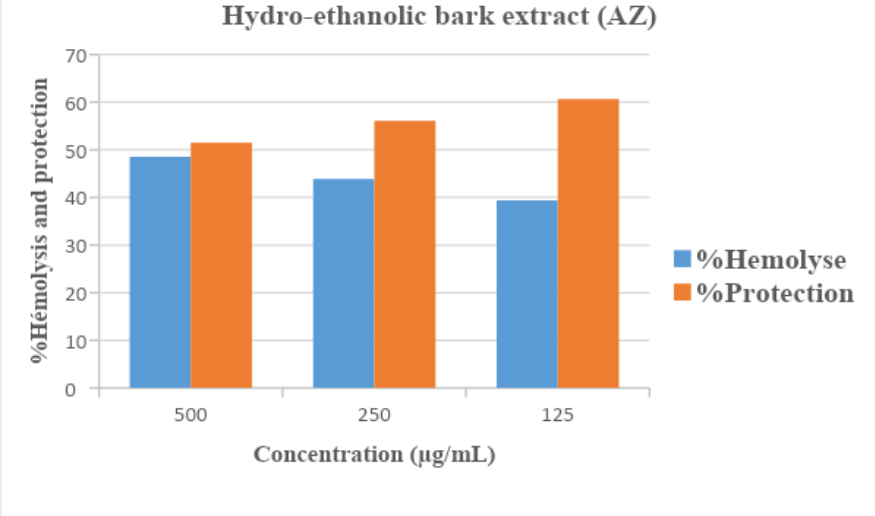

Figure 4: Percentage of hemolysis and protection of hydro-ethanolic bark extract

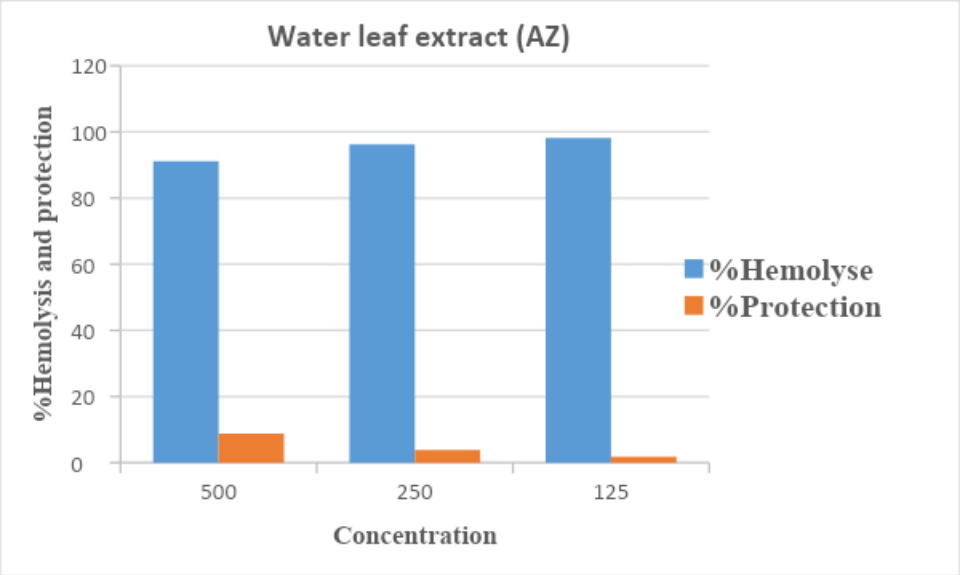

Figure 5: Percentage of hemolysis and protection of aqueous leaf extract

Results in figures 2 to 5 described the variation of the percentages of hemolysis and protection of plants extracts. In general, this variation was concentration-dependent; the higher the concentration of extract decreased, the higher the percentage of protection and hemolysis decreased and increased respectively for both aqueous leaf and bark extracts. On the other hand, the more the concentration of ethanol and hydroethanolic bark extracts decreased, the more the percentage of hemolysis decreased and the greater the percentage of protection increased. At the concentration 125 µg / ml, the hydroethanolic bark extract showed both the highest percentage of protection and the lowest percentage of hemolysis (about 38%), , while the aqueous leaf extract registered the lowest percentage of protection (about 5%) and the highest percentage of hemolysis (about 95%).

Antioxidant activity

Activity on the radical DPPH

The results of Table 3 show that the ethanol leaf extract had the highest anti-radical power (106.6 ± 93.333ab), while the hydro-ethanolic bark extract of the bark recorded the weakest anti-radical power (10.7 ± 1.991).

Table 3: Parameters of anti-radical activity of different extracts and vitamin C

Code of extract	IC_{50} (µg/ml)	CE_{50} x 10^3	PA x 10^{-5}
EAE	21.470 ± 1.830^{bc}	2.147 ± 0.183^{bc}	46.8 ± 4.001^{bc}
EAF	33.180 ± 1.280^{c}	3.318 ± 0.128^{cd}	30.2 ± 1.164^{cd}
EEE	47.360 ± 0.110^{d}	4.736 ± 0.011^{d}	21.1 ± 0.051^{d}
EEF	14.933 ± 0.245^{ab}	1.493 ± 1.025^{ab}	106.6 ± 93.333^{ab}
EHE	95.360 ± 17.400^{e}	9.536 ± 1.740^{e}	10.7 ± 1.991^{e}
EHF	27.730 ± 0.160^{bc}	2.773 ± 0.284^{bc}	36.3 ± 3.741^{bc}
VitC	2.710 ± 0.080^{a}	0.271 ± 0.008^{a}	368.9 ± 11.089^{a}

Reducing activity of Fe^{3+}

The reducing activity of Fe^{3+} was determined by the increase in the absorbance of the Fe^{2+} -ortho-phenanthroline complex formed as a function of the concentration of the extracts and the results are described in the Figures 6 and 7.

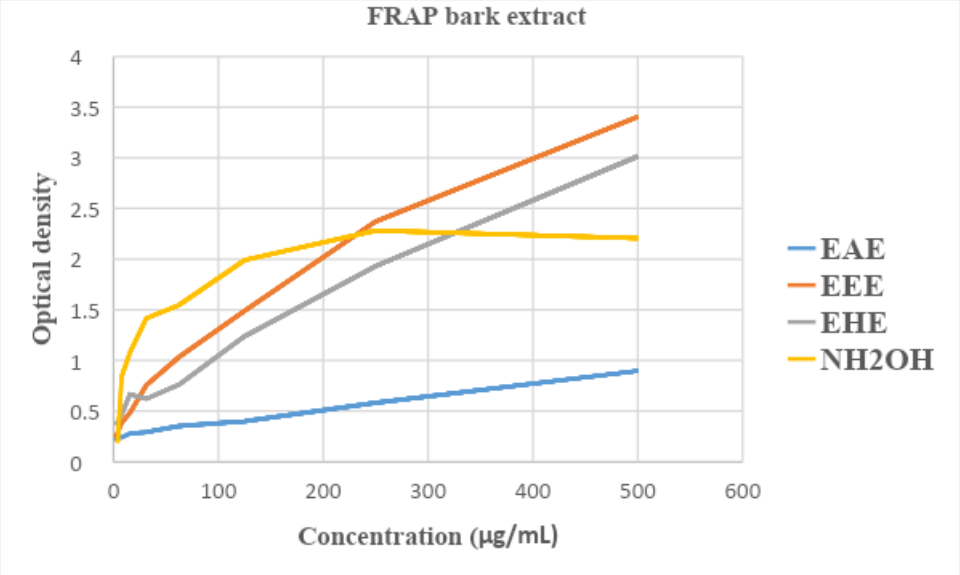

Figure 6: Absorbance of the Fe^{2+} -ortho-phenanthroline complex as a function of the concentration of bark extracts

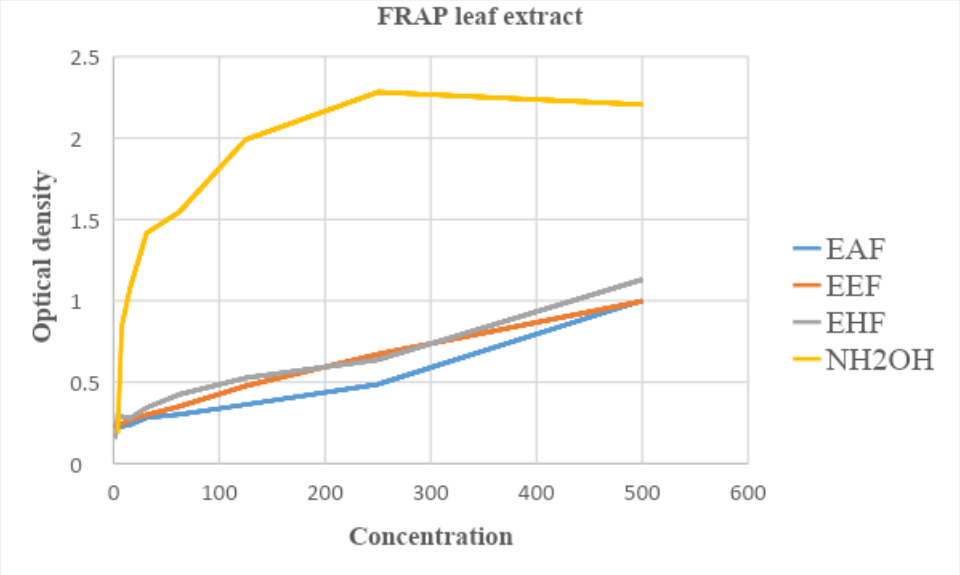

Figure 7: Absorbance of the Fe^{2+} -ortho-phenanthroline complex as a function of the concentration of leaf extracts

Figures 6-7 indicate that the absorbance of the Fe^{2+} -ortho-phenanthroline complex increased proportionally with the concentration of extract. Consequently, a high absorbance reflects the presence of a large amount of Fe^{2+} -ortho-phenanthroline complex thereby a strong reducing activity of the extracts.

Discussions

The present work was aimed at determining the chemical potential and the cytotoxic, anti-hemolytic and antioxidant properties of leaf and bark extracts of *Azadirachta indica*. The extraction yields of the *Azadirachta indica* ranged from 5.02 to 20.7% and with the leaves having the best yield. This result can be explained by the fact that the leaves are the site of photosynthesis in the plant and therefore the privileged place of synthesis of plant metabolites [15].

Phytochemical screening results show that all the extracts contained alkaloids, polyphenols and flavonoids though terpenoid and coumarin were absent. Tannins, saponosides and steroids were unevenly distributed. These results are in agreement with the work carried out by Effiong and others [16], who similarly confirmed the presence of alkaloids, steroids, flavonoids, polyphenols and absence of saponosides and tannins in the ethanol leaf extract of

Azadirachta indica. In addition, they also noted the presence of alkaloids and the absence of terpenoids and steroids in the ethanol bark extract [16]. Moreover, in 2015, Emran and colleagues also reported the presence of tannins, flavonoids, saponosides, alkaloids and the absence of terpenoids in the ethanol bark extract, as described in this study [17]. Nevertheless, our results differ from those obtained by Susmitha and others in 2013, who revealed the presence of alkaloids, steroids, flavonoids, saponosides and tannins in the ethanol leaf extract of this same plant [18]. Phytochemical tests on the aqueous leaf extract of *Azadirachta indica* revealed the presence of tannins, saponosides, flavonoids and alkaloids. Similarly, Dash and colleagues in 2017 obtained the same result with the aqueous extract [19]. The differences between our results and those of other researchers could be explained by the fact that the synthesis and the accumulation of secondary metabolites vary according to the biotic factors (intrinsic factors) and abiotic factors (related to the environment where the plant develops) [20]. Biotic factors include the parts of the plant used, the age of the plant at harvest or the developmental stage of the plant while the abiotic factors include climate, season, time and place of harvest, application of fertilisers, the density of the crop [21,22].

Some plants used for their therapeutic purpose can in high doses, pose a threat to the health of the consumer. The toxicity of any plant of interest should be considered; thus the reason for assessing the cytotoxic activity of the ethanol, hydro-ethanolic and aqueous extracts of *Azadirachta indica* on the Vero cell line. Results indicated that the CC_{50} of plant extracts ranged from > 1000 to 170.9 µg/ml thereby demonstrating that all the extracts were non-cytotoxic. Similarly, Jumba and his teammates concluded that the ethanol leaf extract of *Azadirachta indica* which had an IC_{50} of 149 µg/ml was not toxic as well [23]. On the contrary, the present results showed that the aqueous leaf extract of this plant was toxic since its IC50 was greater than 1000 µg/ml, far above 30 µg/ml, the minimum concentration required to induce cytotoxicity. These results corroborate those of Renugadevi and co-workers in 2012, who obtained with the aqueous leaf extract an IC_{50} of 72 µg/ml on the previous cell line [24].

In addition to the cytotoxic activity, the anti-haemolytic properties of the leaf and bark extracts of *Azadirachta indica* was performed on red blood cells. Results of the present study reveal that the percentage of anti-haemolytic effect varied based on the concentration of the extract. Maximum haemolysis (> 98%) was recorded with the aqueous leaf extract at a concentration, 125 µg / ml. The aqueous leaf extract at this concentration was therefore the most toxic towards red blood cells achieving cell lysis of > 98%. However, the aqueous bark extract at this same concentration registered slightly lesser cell lysis (> 89%) than the aqueous leaf extract. The pronounced anti-hemolytic effect of ethanol leaf and bark extracts at three test concentrations can be attributed to the secondary metabolites such as flavonoids and polyphenols present in the latter. Indeed, several studies have shown that some phenolic compounds, particularly flavonoids possess anti-radical properties thereby neutralizing or trapping free radicals [25]. In addition, polyphenols are known as transition metal chelators such as Fe^{2+}, thus reducing the rate of Fenton reaction. They can then prevent oxidations caused by the hydroxyl radicals [26] and the passage of H_2O_2 through the erythrocyte membrane and the generation of free radicals [27]. On the other hand, the pronounced haemolytic and protective effects of the aqueous leaf and bark extracts irrespective of the concentration can be attributed to their richness in saponosides [28]. In fact, saponosides have the ability to induce the formation of pores across cell membranes, leading to hemolysis and release of hemoglobin in plasma. These results do not corroborate with the findings of Somsak and teammates in 2015 who worked on mice and found that the extracts possessed anti-haemolytic activity with hematocrit percentages ranging from 35% to 70% [29].

The antioxidant potency of our *Azadirachta indica* extracts (Table 3 and Figures 6 & 7) show that the IC_{50} varied from 14.933 to 95.360 µg / ml depending on the extract. The best IC_{50} (14.933 µg / ml) was obtained with the ethanol leaf extract. In addition, the aqueous leaf extract and the ethanol bark extract, each showed a CI_{50} of 33.180 µg / ml and 47.360 µg / ml, respectively, meaning that 50% of these extracts might have trapped the radical DDPH at a closer concentration (33 µg/ml and 47 µg/ml). This result is superior to that of Sithisarn and other who obtained a CI_{50} of 26.48 µg / mL and 30.55 µg / mL with the leaf aqueous leaf extract and ethanol bark extract of *Azadirachta indica*. This difference could be attributed to the ecological and geographical factors [30]. The percentages of DPPH radical inhibiting activity of the aqueous bark extract ranged from 69.20% to 40.66% at concentration ranging between 250 and 15.625 µg / ml confirming its antioxidant property. Our results are superior to those reported by Nahak and others who obtained inhibition percentage range between 30.50 and 18.64% with aqueous bark extracts

of *Azadirachta indica* at concentrations ranging from 200 to 40 µg / ml [33]. Moreover, the percentages of DPPH radical inhibiting power of the ethanol bark extract ranged from 68.53 to 33.95% at concentrations varying between 250 and15.625 µg / ml. Once more, our results agree with those of Nahak and teammates who obtained the percentages of DPPH radical inhibiting of 66.94 to 53.33% at concentrations between 200 and 40 µg / ml with the same extracts [31]. The antioxidant power of the aqueous leaf extracts, hydroethanolic and ethanol bark extracts was demonstrated with their respective DPPH inhibition percentages of 62.13%; 70.03% and 53.27% at the concentration, 125 µg / ml. Again, our results are superior to those of Ghimeray and others who obtained a percentage inhibition of 12.56% at a concentration of 100 µg / ml with the methanol leaf extract [32]. Moreover, results of the anti-free radical powers (PA) in Table 3 indicate that the ethanol leaf extract had the highest anti-radical power (PA=106.6 $x10^{-5}$), followed by the aqueous bark extract (PA= 46.8 $x10^{-5}$). The higher the PA value, the more the extract has a strong proton-donating capacity in order to stabilise the radical [33]. The hydro-ethanolic bark extract which obtained an optical density of 1.2395 (Figure 6) had the highest reducing power at the concentration, 125 µg / ML. Indeed, the absorbance of the Fe^{2+} -orthophenantroline complex increases with the concentration of extract, which in return is proportional to the amount of reduced Fe^{3+} ions. Therefore, a high absorbance reflects the presence of a large amount of Fe^{2+} -orthophenantroline complex thereby indicating a strong reducing activity of the extracts. The aqueous leaf extract exhibited reducing power with an absorbance ranging from 0.2 to 1. The reducing power exhibited by the aqueous leaf extract in the present study is superior to that of the aqueous leaf extract described by Cheung and colleagues, which showed antioxidant activity at optical density ranging between 0 and 0.2 [34]. Moreover, the present research work reveal that the antioxidant activity of the ethanolic bark extract was greater than that of the leaves on the basis of optical density (absorbance ranging 0.2 to 3, 3 for bark extract against 0.2 to 1 for the leaf extract). Our results are similar to those of Abdulkadirb and teammates who realised that the ethanol bark extract of this plant had higher antioxidant activity to that of the leaves and found that the FRAP value in the ethanol bark extract (1668.44 ± 11.10 (mM Fe (II) /g) was significantly higher than that found in leaves (1131.78 ± 34.65 (mM Fe (II) / g) [35]. The observed antioxidant activity may be due to the presence of polyphenols and the difference in the chelating capacity may be attributed probably to the variation in the polyphenol content as well as to the physicochemical properties of the different compounds present in our plant extracts [36]. For example, the presence of flavonoids in plant extracts gives them a good antioxidant potential and metal chelating capacity for transition metals such as Fe^{2+} through their -OH groups [37].

Conclusion

The present study focused on the phytochemical study, the evaluation of the cytotoxic, anti-hemolytic and antioxidant activities of *Azadirachta indica* extracts. The extraction yields, which depended on plant parts and the extraction solvent ranged from 5.02 to 20.7%. The best yield (20.7%) was obtained with the hydro-ethanolic leaf extracts while the lowest yield (5.02%) was recorded by the aqueous bark extracts of *Azadirachta indica.* The phytochemical analysis of *Azadirachta indica* revealed the presence of polyphenols, flavonoids, tannins, alkaloids, steroids and saponoside. Generally, extracts were safe to Vero cells with CC_{50} values ranging from greater than >1000 to 170.9 ± 1.27 µg / mL. Moreover, while the aqueous leaf and bark extracts exhibited anti-haemolytic activity, the ethanol bark extract and the hydroethanolic bark extract did not show any anti-hemolytic activity. In addition, extracts from *Azadirachta indica* were associated with a high antioxidant power that varied with the type of extraction solvent used and the method of analysis. The ethanol leaf extract recorded the best DPPH radical inhibiting activity with IC_{50} value of 14.933 ± 17.400 µg / mL whereas the hydroethanolic extract had the best iron chelating ability with an OD of 1.2395.

The presence of phenolic compounds such as flavonoids, polyphenols and tannins in the extracts of *Azadirachta indica* provides this plant with pharmacological properties thereby, supporting its traditional use for the relief of various pathologies.

Finally, given the results obtained, as future perspective, we intend to evaluate some biological activities such as anti-cancer, anti-inflammatory, antimicrobial activities as well as quantify the phytochemicals present in the extracts including their toxicological profile beyond the present study site.

Conflict of interest statement

We declare that we have no conflict of interest.

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