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Phenolic compounds' dosage and antioxidant activities of ethanolic and aqueous extracts of *Cassytha filiformis'* Linne (Lauraceae) lianas saprophyte of *Melaleuca quinquenervia* (Cav.) ST Blake Myrtaceae

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Abstract *Cassytha filiformis* L., plant with several therapeutic virtues (purgative, hemorrhoid, ulcer, gonorrhea ..), is also used in the treatment of liver diseases. Hepatic diseases are diseases related to oxidative stress. The treatment of liver diseases requires therefore the intake of antioxidant substances. The aim of this work is to prove the antioxidant activity's of *Cassytha filiformis'* ethanolic and aqueous extracts. The ethanolic and aqueous extracts were prepared according to the traditional method and use of *Cassytha filiformis* L. The phytochemical screening revealed the presence of alkaloids, polyphenols, flavonoids, tannins, saponosides, anthocyanins, free anthracene derivate, leucoanthocyanins, quinone derivate and reducing compounds. Chemical analysis dosage showed that the ethanolic extract of *Cassytha filiformis* is rich in total polyphenols, flavonoids, tannins and anthocyanins than the aqueous extract. The larval toxicity test has been conducted and allows us to say that both extracts are not cytotoxic. Antioxidant activity's by DPPH and Iron Reduction Test (FRAP), showed that *Cassytha filiformis*' ethanolic extract would be more active than the aqueous one.

Keywords *Cassytha filiformis*, extracts, polyphenols, antioxidant, cytotoxicity

1. Introduction

In recent years, interest in natural antioxidants, in relation to their therapeutic properties, has increased considerably. Scientific research is intensifying for the identification, extraction and quantification of these phytocompounds from medicinal plants and agri-food products [1,2,3]. According to the previous work, the use of natural products (fruits, vegetables) rich in antioxidants could play an important role in the prevention of diseases [4]. Hepatic diseases are linked to oxidative stress [5,6] and constitute a public health problem given the ravages caused by them in the populations. The treatment of these diseases by modern medicine alone cannot meet the expectations of populations given the very high cost of pharmaceutical drugs that do not often satisfy them. The use of traditional medicine is inevitable because it is even a very old practice worshiped and prized by people very attached to the culture and

civilization ancestral for the treatment of diseases through phytotherapy because of the efficacy, the accessibility and availability of medicinal plants [7]. According to WHO, 80% of world's population uses traditional medicine [8]. *Cassytha filiformis* is a leaves-free parasitic plant of the family Lauraceae [9] and well known for its many therapeutic virtues (deliveries, hemorrhoids, ulcers, coughs, dysmenorrhea and post-traumatic haemorrhages ...) [10,11]. It is a widely distributed plant in India, China and South Africa [12]. It is also found in Benin, Sèmè-Podji and Ekpè. Ethnobotanical surveys and bibliographic review revealed that this plant would be hepatoprotective [13]. It is in this context that we have set ourselves the objective in this work, to evaluate the antioxidant activity of the ethanolic and aqueous extracts of this plant in order to provide scientific evidence of its use in the traditional treatment of liver diseases which are oxidative stress diseases [5,6].

2. Material and Methods

2.1. Material

Cassytha filiformis' lianas were harvested at Ekpè in the department of Ouémé. Voucher specimen was identified at the National Herbarium of Abomey-Calavi's University and certified under the number AAC 230/HNB. They are then dried, crushed and stored in glass bottles away from light and moisture for extracts' preparation.

Chemical reagents

The reagents used are: DPPH (2,2-Diphenyl-1-picrylhydrazyl), Folin-Ciocalteu reagent, ascorbic acid ($C_6H_8O_6$), gallic acid ($C_7H_6O_5$), aluminum trichloride ($AlCl_3$), quercetin ($C_{15}H_{10}O_7$). All of these products come from sigma and the solvent used is ethanol 96 °C.

2.2. Methods

2.2.1. Preparation of the ethanolic extract

A test sample of 50 g of *Cassytha filiformis'* lianas powder was macerated in 500 mL of ethanol 96 °C for 72 h under mechanical stirring. The supernatant was then filtered on Whatman paper and the solvent evaporated under reduced pressure to 50 °C using a rotated evaporator (Büchi Rotavapor 200).

2.2.2. Preparation of the aqueous extract

A test sample of 50 g of *Cassytha filiformis* lianas's powder was decocted for 30 minutes. The decoction was filtered on Whatman paper and the filtrate deposited in the oven and then scraped. The extract obtained is kept in the freezer.

2.2.3. Phytochemical Screening

Phytochemical analysis based on differential reactions (coloration and precipitation) of the main groups of chemical compounds contained in the aqueous and ethanolic extracts were carried out according to the method of [14].

2.2.4. Compounds dosage

Determination of total polyphenols

125 µL of the extract (10 mg/mL) are collected and added to 625 µL of Folin-Ciocalteu reagent. After incubation for 5 min at 28 - 30 °C, 500 µL of sodium carbonate (Na_2CO_3) at 75 mg/mL and 4.75 mL of distilled water are added. The vortex mixture is incubated for 1 hour at 28 - 30 °C. Absorbance reading was made at the Biomate spectrophotometer Thermo Spectronic Genesys 6 (Rochester NY USA) at 760 nm. The concentrations of the polyphenols are deduced from calibration ranges established with gallic acid and are expressed in mg of gallic acid equivalent per gram of extract [15].

Determination of Flavonoids

The total flavonoids content of plant extracts can be estimated by the aluminum trichloride ($AlCl_3$) method [16,17]. 500 µL of $AlCl_3$ ethanol solution (2%) are taken and added to 500 µL of the sample (10 mg/mL). To this mixture, 3 mL of distilled water are added. The blank is composed of 500 µL of $AlCl_3$ and 3.5 mL of distilled water. The

absorbances are read spectrophotometrically at 415 nm after 10 min incubation at 28 - 30 °C. The concentrations of flavonoids are deduced from calibration ranges established with quercetin and are expressed in mg of quercetin equivalent per gram of extract.

Dosage of condensed tannins

To 500 µL (10 mg/mL) of the sample or standard, 3 mL of the solution of sulfuric vanillin (4%) is added. The mixture is incubated for 15 minutes at 28 - 30 °C and the absorbance is read at 500 nm. Concentrations of condensed tannins are deduced from calibration ranges established with pyrogallol (0-300 µg/mL) and are expressed in mg of pyrogallol equivalent per gram of extract [18].

The contents of the polyphenols, flavonoids and condensed tannins are determined by the following formula:

$T = (C \times Vr) / (Vp \times Cp)$

T = content of the compounds; C = Concentration obtained from the calibration curve; Vr = Reaction volume; Vp = Volume of extract taken; Cp = Concentration of the extract solution

Dosage of Anthocyanins

The extraction of anthocyanins and flavonic aglycones is carried out according to the technique of [19]. The flavonic aglycones are extracted first with ether and the anthocyanins with butanol from the residual acidic aqueous phase. This solvent causes the red colored anthocyanins from the oxidation of proanthocyanins. 2 mL of this butanolic epiphase are determined by spectrophotometry between 480 and 600 nm [19,20]. The proanthocyanins content expressed in milligrams of procyanidin equivalent per gram of extract is given by the following formula:

T (anthocyanins) = (OD.MVd) / (ε.p)

With T(anthocyanins): anthocyanin content (in% or mg/g);

OD: Optical density at the maximum absorption wavelength;

ε: molar absorption coefficient of cyanidine (34,700) ;

M: molar mass of procyanidin (306);

V: volume of the butanolic solution;

D: dilution factor;

p: dry weight of hydrolyzed plant material

The differential dosage of flavones and flavonols is based on the specific chelation of flavonoids by Al^{3+} ions. For the assay, 500 µL of $AlCl_3$ ethanol solution (1%) is taken and added to 500 µL of the sample. To this mixture, 2 mL of ethanol is added. The blank is composed of 500 µL of $AlCl_3$ and 2.5 mL of ethanol. After 10 minutes of contact, the presence of flavonols is indicated by a peak between 420 and 440 nm and that of flavones by an absorption maximum between 390 nm and 415 nm [21]. Thus, the aglycone content, expressed in milligram equivalent of quercetin (control flavonol) per gram of extract, is calculated according to the following formula:

T (aglycones) = (OD.MVd) / (ε.p)

T(aglycones): Aglycone content (in % or mg / g);

OD: Optical density at the maximum absorption wavelength;

ε: molar absorption coefficient of quercetin (23,000) ;

M: Molar mass of procyanidin (302);

V: Volume of the ethanolic solution of aglycones;

D: dilution factor;

p: Dry weight of hydrolysed extract

2.2.5. Antioxidant activity

Iron reduction test FRAP (Ferric reducing antioxidant power)

The Iron reduction test FRAP is performed according to the method of [22] with some modifications. To 100 µL of extract of concentration 1 mg/mL, 3000 µL of FRAP reagent is added. After incubating the mixture in a

thermostated bath at 37 °C for 30 minutes in the dark, the absorbance is measured at 593 nm [23,24]. Ferrous sulphate was used to draw the calibration's curve. For calibration, it made the mixture of 3000 µL FRAP and 100 µL of each concentration of the standard solutions. Blank was prepared by mixing 3000 µL of FRAP reagent and 100 µL of ethanol. All the measurements are repeated 3 times.

DPPH test: Determination of IC_{50} extracts

For this test, the samples were prepared by dissolution in absolute ethanol [25]. For each extract, a stock solution in ethanol is prepared at a rate of 200 µg/mL. This solution is then diluted in a geometric series of reason 2 to have different concentrations. In dry sterile test tubes, 1 mL of the solution of the extract to be tested is introduced, 1 mL of DPPH solution (0.04 mg/mL) is added. After vortexing, the tubes are placed away from light at laboratory temperature for 30 minutes. The absorbance is measured at 517 nm using a spectrophotometer (Biomate UV/VIS). For each dilution, a blank consisting of 1 mL of the solution to be tested and 1 mL of ethanol is prepared. The positive control is represented by ascorbic acid (200 µg/mL) and is treated under the same conditions as the test sample.

DPPH test: Antioxidant capacity of extracts

The ability of the extracts to trap the free DPPH (2,2-diphenyl-1-picrylhydrazyl) radical was determined using the procedure described in [26]. A stock solution of concentration 1 mg/mL of the extract in ethanol is prepared which is diluted 1/10. Then, the mixture of 1.5 ml of each extract (diluted solution) is made with 3 mL of the ethanolic solution of DPPH (0.04 mg/mL). The mixture is incubated for 15 minutes at room temperature and the absorbance is read at 517 nm. The antioxidant capacity of the extracts and fractions was determined using a calibration curve of ascorbic acid (0-10mg/mL). Each test is realized in triplicatas. The antioxidant capacity is expressed in mmol Equivalent Ascorbic acid per gram of extract (mmol EAA/g).

2.2.6. Larval toxicity test

This is a preliminary test of non-clinical toxicity that has been proposed by [27], and later developed by [28] and [29]. After incubation and hatching of *Artemia salina* shrimp eggs giving birth to the larvae after 48 hours, sixteen (16) larvae are seeded in each of a range of 10 tubes of decreasing concentrations of *Cassytha filiformis* obtained by dilution in geometric series of reason 2. After 24 hours of incubation at the temperature of 28- 30 °C, the live larvae are counted in each of the tubes, which allows us to draw the curve of dead larvae based on the concentrations [30]. The concentration corresponding to 50% larval mortality (LC_{50}) is determined and compared to the scale of [31] and allows us to predict the safety of the extracts.

3. Results and Discussion

3.1. Phytochemical Screening

Phytochemical screening revealed the presence of several groups of chemical compounds in the extracts of *Cassytha filiformis* lianas. These chemical groups are summarized in Table 1.

Table 1: Chemical groups tests of *Cassytha filiformis* lianas' extracts

Chemical groups	C.f. aq	C.f. eth
Alkaloids	+	+
Polyphénols	+	+
Flavonoids	+	+
Tannins	+	+
Mucilages compounds	+	-
Triterpenoids	-	-
Steroids	-	-
Cardenolides	-	-
Anthocyanins	+	+

Leucoanthocyanins	+	+
Saponosides	+	+
Reducing Compounds	+	-
Quinones derivates	+	-
Anthracene derivates	-	-
Cyanogenic derivates	-	-

- Absent + Present

Screening has revealed alkaloids, polyphenols, flavonoids, tannins, anthocyanins, leuco-anthocyanins, saponosides in both extracts. The aqueous extract of *Cassytha filiformis* alone contains mucilages, reducing compounds and quinone derivatives. The presence of these phytocompounds in both extracts would justify the antioxidant activity of *Cassytha filiformis*. Indeed, polyphenols, flavonoids, tannins are endowed with antioxidant properties. Polyphenols are considered to be a major group of compounds that contribute to the antioxidant activities of plants as free radical scavengers because of their hydroxyl groups [32]. Flavonoids are recognized for their biological activities: anti-inflammatory, antioxidant, antiviral, hepatoprotective and anticancer [33,34]. The presence of phenolic compounds (polyphenols, flavonoids, tannins ...) in these extracts could be the basis of the antioxidant and hepatoprotective activities of *Cassytha filiformis*.

3.2. Content of polyphenolic compounds

The calibration's curves of gallic acid, quercetin, pyrogallol are shown by figures 1,2 and 3 respectively.

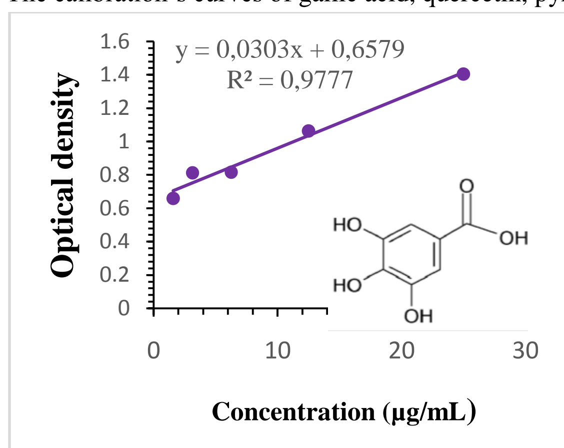

Figure 1: Calibration's curve of gallic acid

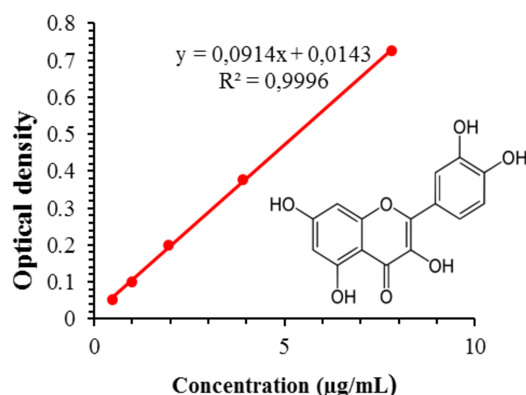

Figure 2: Calibration's curve of quercetin

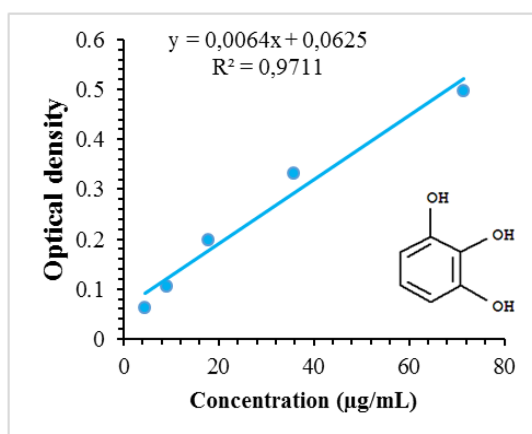

Figure 3: Calibration's curve of pyrogallol

The contents of total polyphenols, flavonoids and tannins are summarized in Table 2, that of aglycones in Table 3.

Table 2: Content in polyphenols

Extracts	T Polyphenols	T Flavonoids	T Tannins
C.f. aqext	024.287 ± 0.000	7.668 ± 0.005	11.200 ± 0.000
C.f. ethext	118.299 ± 0.089	27.072 ± 0.075	356.378 ± 0.713

Table 3: Content in aglycones

Extracts	T Anthocyanins	T Flavonic aglycones	T Flavones	T C-glycosyl-Flavones	T Flavonols
C.f. aq ext	0.052 ± 0.000	0.471 ± 0.000	0.387 ± 0.072	0.339 ± 0.000	0.022 ± 0.000
C.f. eth ext	0.117± 0.000	0.851 ± 0.000	0.771 ± 0.002	0.574 ± 0.002	0.029 ± 0.000

Three colorimetric methods (Folin-Ciocalteu, aluminum trichloride, 4% sulfuric vanillin) were used separately for the determination of total polyphenols, flavonoids and tannins contents in both extracts. The different contents of polyphenols, flavonoids and tannins are respectively reported in mg of gallic acid equivalent (EAG), mg of quercetin equivalent (EQ) and mg of pyrogallol equivalent (EP) per g of extract (Table 2). The results obtained show that the content of the ethanolic extract of *Cassytha filiformis* (118,299 ± 0,089) mg EAG/g is nearly 5 times greater than that of the aqueous extract (24,287 ± 0,000) mg EAG / g for total polyphenols. For the flavonoids content, the ethanolic extract of *Cassytha filiformis* (27.072 ± 0.075 mg EQ/g) would be nearly 4 times rich than the aqueous extract (7.668 ± 0.005 mg EQ/g). The flavonoids would represent 31.57% of the total polyphenols in the aqueous extract. This percentage in flavonoids of the polyphenols would not exceed 22.88% in the ethanolic extract. As for the contents of the tannins, the results give respectively for the ethanolic extract and the aqueous extract, 356.378 ± 0.713 mg EP/g and 11.200 ± 0.000 mg/g. The ethanolic extract would be nearly 32 times rich than the aqueous. Anthocyanins whose content was determined by the method of [19] and expressed in mg of procyanidine equivalent per g of extract, gave as results for the ethanolic extract (0.117 ± 0.000) and the aqueous extract (0.052 ± 0.000). As for the previous chemical groups, the ethanolic extract would be nearly 2.5 times rich than the aqueous extract (Table 3). It can be deduced from this analysis that for the chemical groups (polyphenols, flavonoids, tannins, anthocyanins), the ethanolic extract is 2 to 32 rich than the aqueous extract. This could be explained by the high polarity of the ethanol which concentrates the chemical groups in the ethanolic extract.

3.3. DPPH Antioxidant activity and Iron reduction test FRAP (Ferric reducing antioxidant power)

The antioxidant capacity of the two extracts ethanolic and aqueous of *Cassytha filiformis* was determined by the calibration curve (Figure 4).

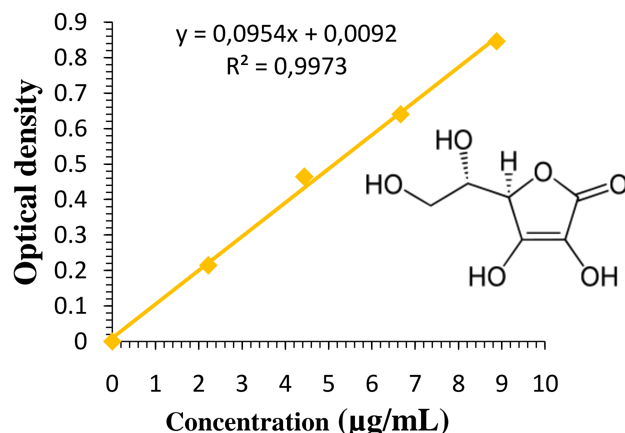

Figure 4 : Calibration's curve of ascorbic acid

Table 4 summarizes the DPPH antioxidant activity and the Iron reduction test (FRAP).

Table 4: DPPH antioxidant activity and the Iron reduction test (FRAP)

Extracts	IC_{50} µg/mL	EC_{50}	APR	I% at 100 µg/mL	CA (mmol EAA/g of extract)	Fe^{2+} (mmol/g of extract)
C.f. aq ext	31.75	793.750	0.001	63.626	31.540 ± 4.153	2.201 ± 0.000
C.f. eth ext	7.07	176.786	0.006	95.015	45.310 ± 8.543	3.285 ± 0.012

The inhibition curves of the free DPPH radical of the ethanolic and aqueous extracts of *Cassytha filiformis* are given in Figures 5 and 6.

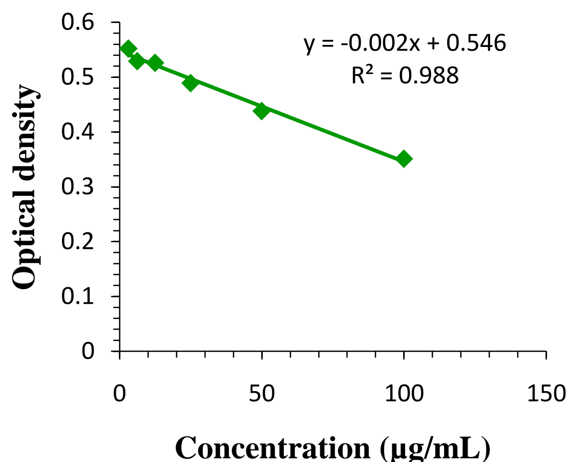

Figure 5: Cassytha filiformis lianas aqueous extract inhibition's of the free DPPH radical

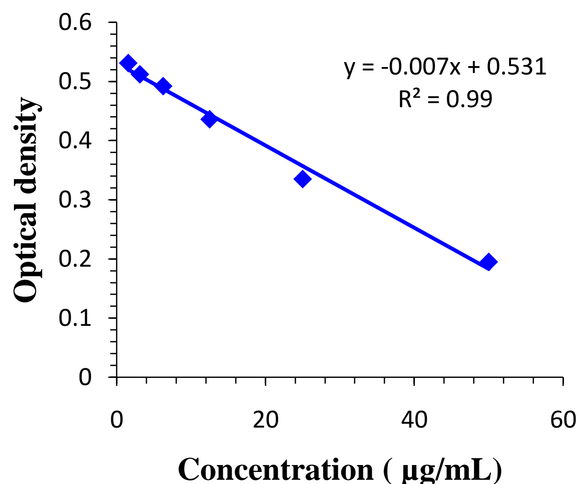

Figure 6: Cassytha filiformis lianas ethanolic extract inhibition's of the free DPPH radical

Free radical scavenging of DPPH (2,2'-diphenyl-1-picrylhydrazyl) and the Iron Reduction Test (FRAP) are used to evaluate the antioxidant activity of the extracts.

DPPH is one of the most commonly used radicals for rapid and direct evaluation of antioxidant activity due to its radical shape stability and simplicity of analysis. The DPPH test is not quantitative, it allows to compare different extracts between them according to their ability to trap DPPH and thus to evaluate the qualitative variations of phenolic compounds [35]. The smaller the IC_{50} value, the greater the antioxidant activity of the tested extract [36]. According to the results recorded (Table 4), the aqueous extract (31,540 ± 4,153 mmol of EAA / g of extract) and ethanol (45,310 ± 8,543 mmol of EAA / g of extract) have a proven antioxidant power and their EC_{50} are respectively 793,750 µg / mL and 176,708 µg / mL. Based on these results, we can deduce that the 50% reduction in DPPH was achieved for all extracts tested and the IC_{50} values were 7.07 µg / mL and 31.75 µg / mL, respectively. Antioxidant chemical groups such as flavonoids and tannins have been shown to reduce and discolor DPPH due to their ability to release hydrogen [37]. The polyphenols contained in extracts of *Cassytha filiformis* are probably responsible for the antioxidant activity of these extracts. The DPPH free radical scavenging test of the ethanolic and aqueous extracts of *Cassytha filiformis* to the DPPH radical was evaluated using a spectrophotometer following the reduction of this radical which is accompanied by its passage from the violet color (DPPH•) to the yellow color (DPPH-H) measurable at 515 nm. This reduction capacity results in a decrease in the absorbance induced by antiradical substances [38]. The results of the antioxidant power of the extracts tested show that at a concentration of 100 µg / mL, the percentage inhibition of the ethanolic extract is greater than 90% while the aqueous extract of *Cassytha filiformis* is greater than 60%. The ethanolic extract of *Cassytha filiformis* would be more active than the aqueous extract. This could be explained by the high content of phenolic compounds, especially flavonoids. The ethanolic extract of *Cassytha filiformis* would be more active than the aqueous extract and would be more active than the chloroformic extract (IC_{50}: 14 µg / mL) obtained by [13].

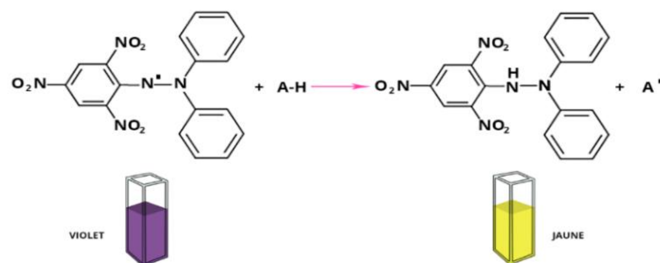

Figure 7: DPPH· DPPH-H

The FRAP method is a colorimetric assay of electron transfer, based on the ability of the products tested to reduce iron (the transition from ferric to ferrous form) [22,23]. It consists to observe after 30 minutes the change in absorbance at 593 nm due to the reduction of the Fe^{3+} - TPTZ complex (Fer 2, 4, 6-tripyridyl-s-triazine) and the appearance of a blue color [23,39]. Originally designed to measure the antioxidant potential of blood plasma, the FRAP method has the advantage of being also adequate to measure the potential of pure compounds [38]. Moreover, it is simple, fast and inexpensive. The reducing power of the ethanolic extract of *Cassytha filiformis* is nearly 1.5 times higher (3,285 ± 0,012 mmol of Fe^{2+}/g extract) than the aqueous extract (2,201 ± 0,016 mmol of Fe^{2+}/g extract). The reducing power of the species *Cassytha filiformis* is probably due to the presence of hydroxyl groups in the phenol compounds that can serve as electron donors. As a result, antioxidants are considered as reducing and inactivating oxidants [40]. Some previous studies have also shown that the reducing power of a compound may be a significant indicator of its potential antioxidant activity [41,42]. The ethanolic extract of *Cassytha filiformis* would have a higher antioxidant activity than that of the aqueous extract.

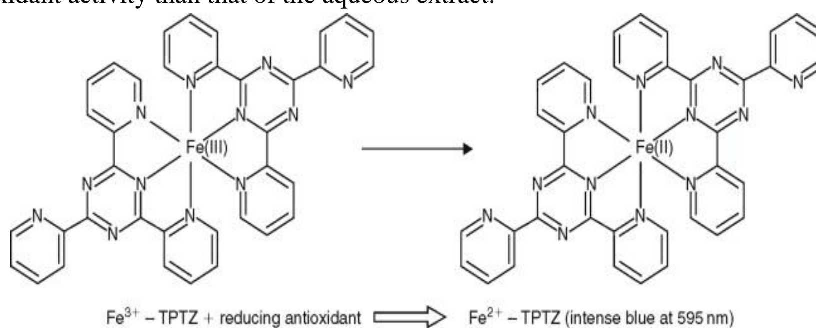

Figure 8: FRAP method

3.4. Larval toxicity

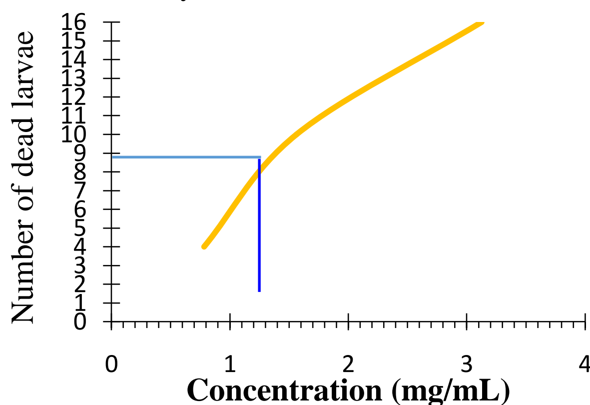

Figure 7: Larval toxicity of aqueous extract of Cassytha filiformis lianas

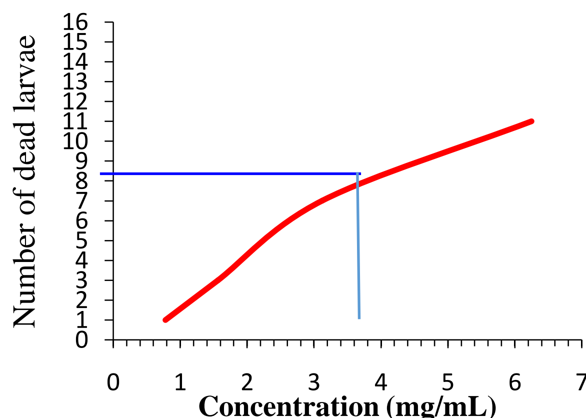

Figure 8: Larval toxicity of ethanolic extract of Cassytha filiformis lianas

The test was carried out on shrimp larvae *Artemia salina* according to the technique proposed by [27]. The lethal concentration (LC_{50}) was determined using the curves given in Figures 9 and 10.
Artemia salina shrimp larvae are microorganisms that are used in larval toxicity research. The larval toxicity test is a quick, inexpensive and highly reliable simple test to predict the safety of a substance intended for consumption. Indeed, there is a correlation between cytotoxicity on *Artemia salina* larvae and on 9PS and 9KB cells (human nasopharyngeal carcinoma on the one hand [43], pulmonary carcinoma A-549 cells and colon carcinoma HT-29 cells on the other hand [44]. Therefore, a toxic substance for the larvae, is also for the above-mentioned human cancer cells. The comparison of LC_{50} (Lethal concentration which causes the death of 50% of the population of these microorganisms) on the scale of [31] provides information on the safety of this substance. In the light of the results obtained, the two extracts ethanolic (LC_{50}: 3,699 mg/mL) and aqueous (LC_{50}: 1,272 mg/mL) of *Cassytha filiformis* would not be toxic on the larvae.

4. Conclusion

At the end of this work, it can be concluded that the ethanolic and aqueous extracts of *Cassytha filiformis* contain different groups of chemical compounds such as polyphenols, flavonoids, tannins, saponosides and anthocyanins. The aqueous extract alone would contain mucilages, quinone derivates and reducing compounds. The determination of the chemical groups common to both extracts revealed that the ethanolic extract of *Cassytha filiformis* would be rich than the aqueous extract in each of the chemical groups concerned. The search for antioxidant activity by the DPPH and the FRAP method showed us that both extracts are endowed with antioxidant activity and that the ethanolic extract of *Cassytha filiformis* would be more active than the aqueous. The search for safety by the larval toxicity test of the extracts allows us to say that the two extracts ethanolic and aqueous of *Cassytha filiformis* would not be cytotoxic on the larvae.

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