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Phytochemical analysis, toxicity and larvicidal activity of extracts from *Launaea taraxacifolia* (Asteraceae) on *Anopheles gambiae*, a malaria vector

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Abstract
In the search for alternative methods to chemical control, the use of bio-insecticides is increasingly developed against mosquitoes, vectors of disease. The extracts of native plants are very promising in this new perspective. This work aims to determine the efficacy of extracts *Launaea taraxacifolia* larvicidal agent as well as its possible use in the control of malaria vectors. To do this, extracts of *L. taraxacifolia* were tested on the larvae (third stage) of *Anopheles gambiae* Kisumu and wild strains, as well as the larvae of shrimp *Artemia salina*. It shows that the hydro-methanol extract was the most active with lethal concentrations LC_{50} of 469.7 ppm and 12.2 ppm in 24 and 48 h of exposure for strain Kisumu; 270.7 ppm and 166.7 ppm in 24 h and 48 h, respectively, for the wild population. Strong lethal doses (LC_{50} > 2668 ppm) extracts on larvae of *Artemia salina*, denote their innocuousness on shrimp. Thus, *L. taraxacifolia* is a good candidate for the development of a bio-larvicide agent for the integrated fight against malaria vectors and the differential activity of its extracts on larvae is due to secondary metabolisms they contain and that it will specifically identify later.

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1. Introduction

1.1. Background

Vector-borne diseases are responsible for over 17% of infectious diseases, and cause over one million deaths each year. Among them, figure malaria which causes over 600 000 deaths per year world-wide, mostly children under five years (OMS, 2016). The mosquitoes responsible for transmitting the Plasmodium (pathogens) to humans belong to the Anopheles genus. This type is one of the most important in Public Health with 484 species of which only sixty induce with more or less efficiency, the transmission of human malaria (Harbach, 2004). Of these, thirty are good vectors while others have a localized or relatively secondary role. The fight against malaria transmission relies both on the declining number of gametocyte carriers by chemical preventive and curative measures, decreasing transmission. Insecticides are used as well as in the campaigns

against malaria vector land to, mainly through the use of are impregnated mosquito nets and in-house spraying. Although these interventions can have a significant impact, they do succeed in total control because they target only adult mosquitoes. Without the vaccine, vector control now appears as one of the main mass prevention methods applicable against malaria. Indeed, it remains the insecticides used to prevent the disease generally belong to the class of organophosphates, pyrethroids and carbamates synthesis. The misuse of these insecticides has caused the emergence of resistance in target vectors including *Anopheles gambiae*. Thus, sensitivity tests in Ivory Coast, Benin and Cameroon, were used to determine the tolerance to insecticides of Anopheles populations (Akogbeto and Yakoubou, 1999; Edi et al., 2012; Nwane et al., 2013). Moreover, these synthetic insecticides are not very selective and cause environmental pollution. Using an integrated control by larvicidal measures is needed in the management of resistance. Indeed, the added value of larvicides along with the use of insecticide-treated nets is that they can induce a further reduction infective bite of up to 73% of the rate of entomological inoculation (TIE) (Fillinger et al., 2009).
Note that the collectors of standing wastewater represent the main suppliers of mosquito *Anopheles gambiae*, a mains malaria vectors in Benin (Larivière et al., 1987), an effective fight against these vectors priori implies good management houses larval through a larva control in a respectful of environmental protection.

One of the strategies is the use of microbial organisms such as *Bacillus sphaericus* (Bs) and *Bacillus thuringiensis var. israelensis* (Bti). These bacilli are seen as effective biological control agents (Becker, 1998). They act on mosquito larvae, black flies and Diptera in general and are used today in a wide range of breeding sites, due to their efficiency and specificity that respect wildlife companion. Although the development of resistance in mosquitoes has not yet been detected with these bio-insecticides, it is important to continue studies to develop new biochemical insecticides to be ready to meet all the eventualities and to have an effective control system of these microorganisms. Thus, another method is the use of active extracts plant-based and environmentally profitable (Chougourou et al., 2012). *Launaea taraxacifolia* shows a characteristic for requirement. From the family of Asteracae, *Launaea taraxacifolia* is a leguminous crop among the 10 plants, is significant by underutilized and is most important in Benin and a high priority for research (Dansi et al., 2012). Furthermore, it is known and domesticated in all West African countries (Adebisi, 2004). Their fresh leaves eaten in salads or cooked as a sauce (Koukoui et al., 2015), are nutritionally rich in vitamins, proteins, minerals, essential fatty acids, fiber , and flavonoids (Adinortey et al., 2012; Dickson et al., 2012). Apart from their use as food, leaves *Launaea taraxacifolia* are widely used as infusion for the treatment of several diseases such as regulation of blood pressure, dyslipidemia, decreased cholesterol, antioxidant properties as well as in viral diseases (Arawande et al., 2013; Dansi et al., 2008; Owoeye et al., 2015). In addition, the work of Gbadamosi et al., (2012) and Adimonyemma et al., (2016) found a proven activity of *L. taraxacifolia* against microorganisms such as *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniea*, *Candida albicans* and *Proteus spp*. However, no study has been published on a probable inhibitory effect of this plant on mosquito larvae.

1.2. Objectives of Research

The present work aims to evaluate the larvicidal activity of *Launaea taraxacifolia* extracts on *Anopheles gambiae* and toxicity on *Artemia salina* shrimp larvae and to characterize by phytochemical screening the different chemical groups of the plant.

1.3. Justification of Research

Launaea taraxacifolia is an edible plant used increasingly in therapeutic formulations in Benin. The extract from its crushing in water is poisonous to insects. Despite its proven geographic availability, *L. taraxacifolia* is classified as insignificant and underutilized plants. Its value therefore requires a detailed study of its pest control activities as well as the identification of compounds responsible for its properties.

2. Materials and Methods

2.1. Collection of plants and preparations of crude extracts

The aerial parts of *Launaea taraxacifolia* were harvested in maize crop fields in Comé in the southern part of Benin and then dried in a cold room at 16 °C in the laboratory before being crushed and powdered. Crude extracts were prepared with two different polarity solvents. For the first extraction, they are stirred 50 g of powder obtained in 500 mL of methanol-water (70: 30; v / v) and 0.5% formic acid. Homogenized for one hour sonicator (ultrasonic bath) then filtered and evaporated to dryness using a rota-vapor (Heidolph efficient Laborota 4000). The second extract was prepared using essentially the previous method except that dichloromethane (99.9%) was used instead of water-methanol mixture. The operations were repeated three times and the obtained dry residue is weighed after each operation to determine the average extraction yield.

2.2. Collection of Anopheles gambiae larvae

Bioassays were carried out on two larvae strains: wild larvae collected from breeding in Cotonou followed by the morphological and behavioral criteria of larvae using taxonomic identification keys (Gillies and Coetzee, 1987) and larvae Kisumu of Kenyan origin obtained Entomological Research Center of Cotonou (CREC). Larvae are held in Kisumu breeding in the laboratory of CREC for several years and their sensitivity is checked regularly.

2.3. Phytochemical analysis

The different chemical groups have been identified in *L. taraxacifolia* exploiting the conventional method of (Houghton and Roman, 1998), used routinely and very recently by (Fagbohoun et *al*., 2015; Fagbohoun et *al*., 2014), when they highlighted the combination of chemical groups sources of natural dyes from dye plants and the variability in polyphenols in the tolerance of species of palm oil towards the larvae *Coelaenomenodera lameensis*. Thus Mayer and Dragendorff tests are used for the alkaloids, the Fehling test for reducing compounds and glycosides, the test Liebermann-Burchard for triterpenoids and steroids, Frothy test for saponins, Shinoda tests and sodium hydroxide to flavonoids, ferric chloride test for tannins, Guignard test for free cyanogenic derivatives and Bornträger test for anthraquinones.

2.4. Sensitivity tests on the larvae of Anopheles gambiae

The standard WHO protocol for testing sensitivity towards the larvae insecticides used in country side, was operated with a slight modification in accordance to our working conditions (W.H.O., 2005). The Kisumu larvae were treated with solutions extracts at concentrations 10, 50, 100, 250, 500 and 1000 ppm, prepared from each type of extract and 1% ethanol; while wild larvae were subject to extract solutions at concentrations 100, 500, 1000, 2000, 3000 and 5000 ppm. The tests were carried out in transparent cup (5 cm in diameter each containing 100 mL of solution and 20 larvae of *Anopheles gambiae* 3rd instar the same category). The same number of larvae was placed in another beaker control containing only 100 mL of 1% ethanol. For each of the concentrations of the extracts as well as for the control, two replicas were made. Larval behavior, by counting the number of survivors was followed for 48 hours and the lethal concentrations (LC_{50}) were determined every 24 hours. In fact, are considered dead, the larvae remain still even in contact with a needle and those that are dying. Data analysis is done using Microsoft Office Excel 2010 version to determine the average mortality rate of Anopheles larvae following the doses and extract the lethal concentrations (LC_{50}).

2.5. Toxicity Test on larvae Artemia salina "Brine Shrimp"

Encysted eggs *Artemia salina* (10 mg) were incubated in 100 ml of seawater from the Atlantic Ocean. After 48 hours of incubation, the larvae are collected using a pasteur pipette are dissolved with 2% DMSO (dimethyl sulfoxide). We then prepare a series of 10 solutions dupliqua two, hydro-methanolic and dichloromethane extracts in varying concentrations and progressive (12.5; 6.25; 3.12; 1.56; 0.78; 0.39; 0 , 19; 0.098, 0.049 and 0.024 mg / mL). A definite number of 16 larvae were introduced into each solution. All solutions including the control-free extract are left with gentle stirring continuously at room temperature and the reading of the results is made after 24 hours, by counting under microscope the number of surviving larvae in each solution (Houngbèmè et al., 2014; Vanhaecke et al., 1981). If the control contains dead larvae, the percentage mortality is corrected using Abbott's formula: % death = [(test-control) / Witness] × 100 (Carballo et al., 2002). Using the Microsoft Office Excel 2010 version software, the dose-response data were log-transformed and lethal concentration of 50% of the larval population introduced into each determined by linear regression extract, are recorded.

3. Results

3.1. Phytochemical analysis

Characterization results of the tests of large chemical groups *L. taraxacifolia* are shown in Table 1. In fact, the qualitative analysis of 17 chemical groups tested in this plant showed that it contains tannins, flavonoids, anthocyanins, leuco anthocyanin, and triterpenes.

Table 1: Phytochemical screening of *Launaea taraxacifolia*

Compounds	***Launaea taraxacifolia***
Tannins	++
Catechin tannins	++
Galic tannins	+
Flavonoids	++
Anthocyanins	+
Leuco-anthocyanins	++
quinone derivatives	-
Saponosides	-
Triterpenes	+
Steroïds	-
cyanogenic compounds	-
Mucilages	-
reducing compounds	-
Coumarins	-
free anthracene	-
O-glycosides	-
C-glycosides	-
cardiac glycosides	-
Alkaloïdes	-

++ : strong presence; + : moderate presence ; - : Absent

3.2. Larvicidal activity of the crude extracts of Anopheles

Two solvents used for the extractions, the aqueous-methanolic mixture is the solvent which produced the greatest amount of mass removed with a yield of 12.28 ± 0.13%, compared to extraction with dichloromethane (2.87 ± 0.19%). This result suggests a wealth of polar compounds *L. taraxacifolia*. Indeed, apart from triterpenes, chemical substances characterized in that kind include polar compounds. Their activity *via* crude extracts tested on larvae of *Anopheles gambiae* strain Kisumu, resulting in Figure 1. This figure shows that the blank of 1% ethanol has no inhibitory effect on larvae of Anopheles strain Kisumu, who continue to live in the experience 48h. On the contrary, in 24 hours, the water-methanol crude extract induces a strong larval mortality beyond 50% at 500 ppm and 100% mortality at 1000 ppm compared with dichloromethane crude extract which caused only 5% of a high concentration of 24 deaths. After 48 hours, larval mortality varies very little according to the dichloromethane extract dose applied. Indeed, mortality was low indicates 10% of larvae to a maximum dose of 1000 ppm. Low concentration of 10 ppm, hydro-methanol extract causes over 48% of deaths of ethnic larvae Kisumu; this mortality reaches 100% at 500 ppm. Moreover, the application of water-methanol crude extract of the *Anopheles gambiae* larvae of wild strain, is comparable with previous results, the only difference in the 24h dose inducing 100% mortality of wild larvae (5000 ppm) is 5 times that of Kisumu strain (Figure2). At this dose (5000 ppm) in addition, the dichloromethane extract produced beyond 50% mortality of wild strains of larvae. Lethal concentrations responsible for 50% mortality of larvae and Kisumu strains in wild 24h and 48h are summarized in Table 2. In 24 hours, the LC_{50} (469.7 ppm) related to the hydro-methanol extract of larvae Kisumu strain is greater than that of wild strain (270.7 ppm). In contrast, after 48 hours, the dose inducing 50% mortality of larvae Kisumu strain is 13 times lower than that of wild strain. Note that the doses of dichloromethane extract, hardly induce mortality of larvae strain Kisumu, the LC_{50} associated with this extract on the wild type larvae are 15 times greater than those of the extract hydro-methanolic. We can deduce a priori that the larval mortality varies among tested strains, doses and duration of the applied dose.

Figure 1: Comparative graph of larvicidal activity of extracts Dichloromethane and Methanol / Water on the larvae of *Anopheles gambiae* of Kisumu strain

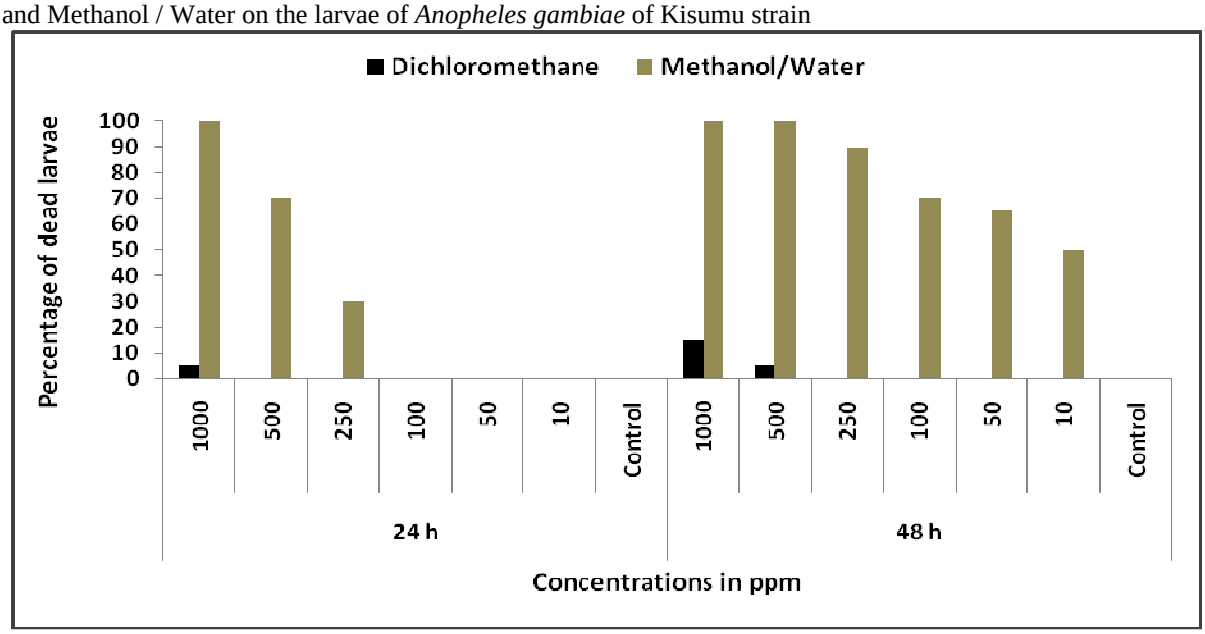

Figure 2: Comparative graph of larvicidal activity of Dichloromethane and Methanol / Water extracts on the larvae of *Anopheles gambiae* of wild strain

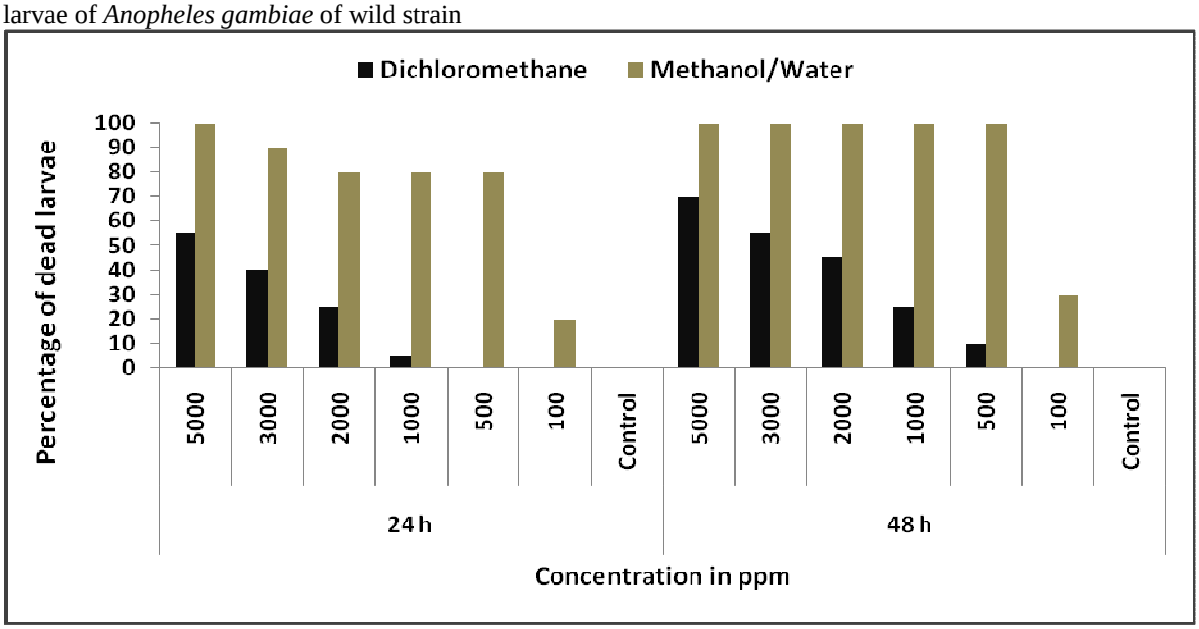

Table 2: LC_{50} values in ppm extracts tested on larvae of *Anopheles gambiae* Kisumu and wild

Extracts	Strain Kisumu		Wild strain	
	24h	48h	24h	48h
Methanol-Water (LC_{50} en ppm)	469,7	12,2	270,7	166,7
Dichloromethane (LC_{50} en ppm)	nd	nd	4247,5	2487,6

nd : not determined

3.3. Cytotoxicity of extracts opposite the larvae of Artemia salina

The evaluation of the cytotoxic activity of extracts of *Artemia salina* (Figure 3), aquatic species, is a base of reference and a screening of the selective toxicity of the extracts studied the Anopheles larvae. It falls within this figure, a sensitivity shrimp larvae extracts at high doses (> 2500 ppm). Table 3 summarizes the half lethal concentration (LC_{50}) extracted graph reflecting the number of larval mortality based on extracts of doses applied with a correlation coefficient R2 greater than 0.85; witnessed a good correlation between the applied concentrations and responses. Indeed, these lethal concentrations are slightly higher than 2660 ppm and vary little according to the extracts tested. However, they are largely high compared to those found on the larvae of *Anopheles gambiae*.

Figure 3: Cytotoxicity of dichloromethane extracts and hydro-methanolic towards the larvae of *Artemia salina*

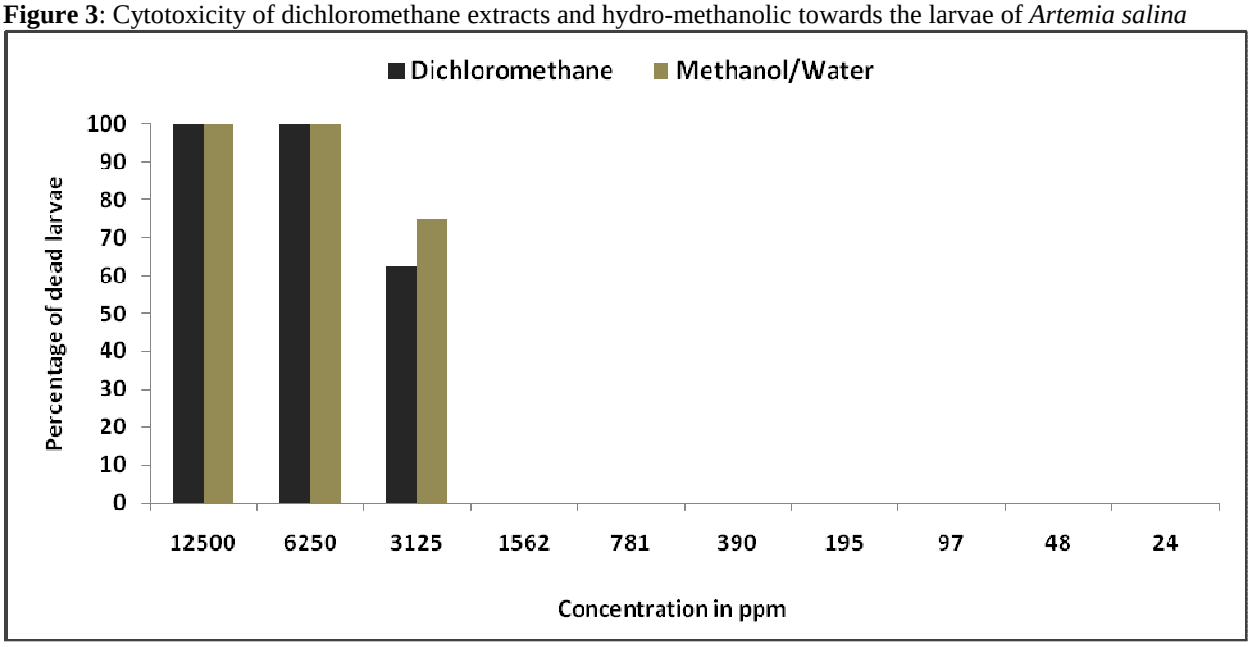

Table 3: Lethal concentrations (LC_{50} in ppm) to 50% of two extracts tested on larvae of *Artemia salina*

Extracts	LC_{50} (ppm)
Methanol/Water	2668,1
Dichloromethane	2873,4

To interpret the results, correlation grids associating the degree of toxicity LC_{50} have been proposed (Moshi et al., 2004).

$LC_{50} \geq 100$ ppm : no toxic
100 ppm > $LC_{50} \geq 50$ ppm : low
50 ppm > LC50 ≥ 10 ppm : average
LC50 < 10 ppm : strong
Given the results, we find that the two extracts from *L. taraxacifolia* are not toxic to the larvae of *Artemia salina.*

4. Discussion

The phytochemical screening of *L. taraxacifolia* harvested in Comé-Benin, revealed the presence of tannins, flavonoids, leuco-anthocyanins which are exclusively polyphenols and triterpenes incidentally. These results are comparable to the recent works of (Adinortey et al., 2012) and (Koukoui et al., 2015). Indeed, apart from secondary metabolites in the species studied, other varieties of *L. taraxacifolia* of Ghanaian origin contain Steroid and saponins (Adimonyemma et al., 2016; Adinortey et al., 2012) on the one hand and mucilage (Koukoui et al., 2015) for the other harvests within the Sakété region of South Benin. It appears from the study compared to chemotypes encountered in the literature; this crop encloses mainly polar chemical groups including polyphenols. This explains, in effect, the relatively high yield of the extraction pipe to the mixture of polar solvent methanol-water (70: 30 v / v) which largely retrieves these polyphenolic substances compared to the extraction conducted with dichloromethane. Evaluation of the larvicidal activity of *L. taraxacifolia* showed that its hydro-methanolic extract had an inhibitory effect on the two categories of *Anopheles gambiae* larvae studied. In fact, 500 ppm of this extract, all larvae (Kisumu and wild) die after 48 hours of exposure. By cons, it is necessary to 5000 ppm of the extract to obtain this result in 24 hours with wild larvae against 1000 ppm for larvae Kisumu. However, it should be mentioned that the lethal concentration LC_{50} of the same extract (water-methanol) which is 270.7 ppm in 24 hours for the wild larvae is significantly less than 469.7 ppm raised in larvae Kisumu; This is contrary to the statements made after 48 hours of exposure (LC_{50} = 12.2 ppm) for larvae and Kisumu (166.7 ppm) for wild larvae). This difference in sensitivity associated with exposure times fall biochemical mechanisms of toxicity inherent strains tested. It could also result in the differential resistance to chemical insecticides increasingly observed among *Anopheles gambiae* mosquitoes in the city of Cotonou and around (Akogbeto and Yakoubou, 1999). Indeed, the LC_{50} values obtained on the *Anopheles gambiae* larvae from the wild population are similar to those obtained on larvae of *Anopheles stephensi* through studies on the leaves of *Ajuga remota*: 0.033% (330 ppm) in 24 hours and 0.029% (290 ppm) in 48 hours (Preeti et al., 2004). The same work on larvae of *Culex quinquefaciatus* (Culicinae) vector of lymphatic filariasis with *Hyptis suaveolens* by (Murugesan et *al*., 2015) in India gave LC_{50} = 485.61 mg/L (ppm) and 344.03 mg/L (ppm) in 24 hours and 48 hours of treatment, respectively.

The results obtained with the dichloromethane extract give lethal concentrations (4247.5 ppm in 24 hours and 2487.6 ppm in 48 hours) highly superior to those obtained by extraction with methanol-water, on wild larvae identical concentrations. Note that the doses tested of dichloromethane extract, hardly induce mortality of larvae strain Kisumu. Therefore, we can conclude that the compounds responsible for the larvicidal activity of *L. taraxacifolia* polyphenols are concentrated in hydro-methanolic extract and without which there is some tolerance of dichloromethane extract towards the larvae. Indeed, Dichloromethane solvent could not extract the active ingredient of the leaf of *L. taraxacifolia*. The molecules responsible for the larvicidal activity would be polar and would be in phase with the methanol-water solvent. Reflecting the strong inhibition of larvae observed in this extract. In addition, the extraction method using methanol-water solvent is that commonly used to extract the polar substances of plants (Aguinaldo et al., 1993; Bhuyan and Saikia, 2005; Purushotham et al., 2010). Note that the active molecules involved in the larvicidal activity belong to the polyphenol family; it is essential to continue work to identify the / molecule (s) responsible for this activity. Lethal concentrations obtained at the larvae of *Artemia salina*: 2668.1 ppm and 2873.4 ppm respectively for hydro-methanolic and dichloromethane extracts are well above the toxicity threshold (100 ppm) and LC_{50} result of their inhibitory effect the Anopheles larvae. These

results show selective toxicity of the extracts tested towards the aquatic species and demonstrate their safety on shrimp larvae *Artemia salina*. Considering the correlation between cytotoxicity shrimp larvae and cell-9 PS 9 and KB (human nasopharyngeal carcinoma) on the one hand (Pelka et al., 2000), cells A-549 lung carcinoma cells and the HT-29 colon second (Carballo et al., 2002), we can say subject to further investigation, that the tested extracts are also exempt towards cytotoxic activity humans . This is also what justifies the high consumption of *L. taraxacifolia* by the populations of West African countries. The results recorded at the end of this study are encouraging in the sense of eradicating malaria through an anti-larval control using green chemistry. This control method can adjust the resistance of vectors of malaria issues on one hand and the protection of the environment on the other.

Conclusion

This work has shown that apart from its well known nutritional properties, *L. taraxacifolia* has a very interesting larvicidal power against *Anopheles gambiae*. This toxicity is almost negligible to the larvae of *Artemia salina*, aquatic species having physiological similarities with some human cells. The water-methanol extract which showed a marked larvicidal activity, is mainly composed of polyphenols, evidenced by the results from the phytochemical screening. In the integrated control framework against malaria vectors, *L. taraxacifolia* is a good candidate in the formulation of a bio-larvicidal. It is therefore necessary to continue this study to specifically identify the compounds responsible for the larvicidal activity at this legume edible.

Research Highlights

This study reveals that beyond its consumption as a legume rich in nutritional substances, *L. taraxacifolia* is also a good candidate in the bio-insecticide formulation against mosquito larvae.

The compounds responsible for this property are concentrated in the aqueous alcoholic extract which has no toxic effect on larvae of *Artemia salina* shrimp.

The offending compounds are polyphenols. The differential toxicity of these compounds on aquatic larvae tested is an encouraging result as to the preservation of animal species other than anopheles in treatment environment.

Limitations

The structure of the compounds responsible for the larvicidal activity of *L. taraxacifolia*, remains to be characterized.

The activity of the extracts studied could be compared to a bio-larvicide reference already used in the field.

Recommendations

It is necessary to purify, isolate and characterize bioactive polyphenols hydro-alcoholic extract of the plant. Perform routine tests in comparison with reference bio-larvicides and identify the biochemical mechanism of toxicity on Anopheles larvae to reach a better formulation.
This study deserves a monitoring and assistance by the authorities in charge of the management of the environment and the management of Benin's public health and related institutions.

Authors' Contribution and Competing Interests

The results published through this manuscript, from research to Ahouansou C.A., supervised by Fagbohoun L. and directed by Gbaguidi A. F. Tchètchè J. accompanied Ahouansou in operations linked to phytochemical screening. Kotchoni S. oversaw the work related to the bioassay conducted on the mosquitoes. The manuscript proposed by Ahouansou was corrected by Fagbohoun and Medegan Fagla S.R. and supervised by Gbaguidi. All authors read and approved the article.

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