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Larvicidal effects of methanol, acetone and hexane dried leaves extracts of *Azadirachta indica* against *Anopheles gambiae* sensu stricto (Diptera: Anophelinae) Giles, 1902

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Abstract
Insecticidal effects of methanol, acetone and hexane extracts from dried leaves of *Azadirachta indica* have been assessed on stage 4 larvae of *Anopheles gambiae s.s* Giles, 1902 at the laboratory of Zoology of the University of Ngaoundere. Biological tests carried out according to the WHO protocol revealed that the extracts obtained from the various organic solvents have larvicidal activities. The acetone extract was the most effective. The LC_{50} values were 51.34 ppm, 56.54 ppm and 65.37 ppm for acetone, methanol and hexane extract respectively. The LH_{50} values were 4 h 06 min 36 sec, 3h 34 min 59 sec and 4 h 29 min 34 sec respectively for the three extracts. Phytochemical screening revealed the presence of terpenoids, sterols, saponins, flavonoids, and tannins; secondary metabolites responsible for the insecticidal activity. Each of these extracts showed low toxicity compared to Temephos (positive control) at the WHO Recommended concentration of 0,012 mg/L.

Keywords: Larvicidal effects, extracts, *Azardirachta indica, Anopheles gambiae*, larvae, Ngaoundere, Cameroon

Introduction
In sub-Saharan Africa, malaria caused more damage than natural disasters and wars [2]. If it is true that since the years 2000 to 2016, there have been remarkable efforts or advances both in its prevention and its treatment; it remains also true that since 2017, we are witnessing the progression and the resurgence of the disease [3]. Today, Africa remains undeniably the continent most affected by this scourge, with 213 million victims, 93% of cases and 394,460 deaths, 94% of global mortality [2]. Children under five years and pregnant women are the most affected by malaria [2]. This heavy human toll is added the considerable socio-economic impact due to the expenses related to the treatment of the sick, the loss of income in productivity and the prevention of the disease in the most affected areas [3]. To reverse this trend, the World Health Organization recommends vector control through the use of mosquito nets impregnated with synthetic insecticides and indoor spraying. Unfortunately, this action is currently coming up against the phenomena of resistance and behavioral change in certain species of anopheles following the massive deployment of long-acting impregnated mosquito nets (MILDA) which are jeopardizing the overall strategy for controlling vectors of malaria. Faced with these obstacles, it is urgent to overlook vector control by redirecting not only the strategy towards the evolutionary stages of the vector, namely the larval stage, but also and above all by making use of available local plants in order to overcome the use of synthetic insecticides and significantly reduce mortality attributable to malaria [4]. Good management of breeding sites kills mosquito larvae before they reach the adult stage. This control technique offers a great advantage in that the larval stage is immobile and requires less effort to apply the control method. The techniques for managing breeding sites concern in particular the drainage or drying of sites, the application of larvicides in to the water or the introduction of animals that prey on mosquito larvae in aquatic environments. Several studies have been carried out on the insecticidal effects of local plants. However, the larvicidal activity of the extracts of these plants remains one of the important pillars of the plan adopted by the WHO to reduce the resurgence of malaria [5]. *Azadirachta indica* is a plant widely used in the northern regions of Cameroon for its repellent and insecticidal activity [6].
Economically, the leaves of *Azadirachta indica* are accessible to all for the prevention as well as the fight against malaria pathogen. The general objective of this work is to contribute to the fight against malaria by reducing the populations of vectors of *Plasmodium* spp. The specific objectives consisted in:

- Evaluate the mortality of the larvae in the different extract;
- Evaluate the effectiveness of extracts by determining CL_{50} and HL_{50};
- Determine the chemical composition of extracts.

Materials and Methods
Study site and plant collection
The leaves of *Azadirachta indica* used in this work were collected from Baladji I, (figure 1) a district of the town of Ngaoundere, Adamawa Region of Cameroon, in February 2021, a period during which the plant retains all its foliage in a Sahelian zone with a dry climate (MINEF, 1994). The leaves were harvested in the morning between 7 a.m. and 10 a.m. and dried in the laboratory for 7 days. Then leaves were crushed using a traditional mortar and the ground material was sieved using a sieve with tight mesh of 0.1mm caliber in order to obtain a very fine powder [4]. Powders were soaked in methanol, acetone and hexane during three days. Crude extracts were condensed in rotavapor apparentus.
The study area is presented in figure 1.

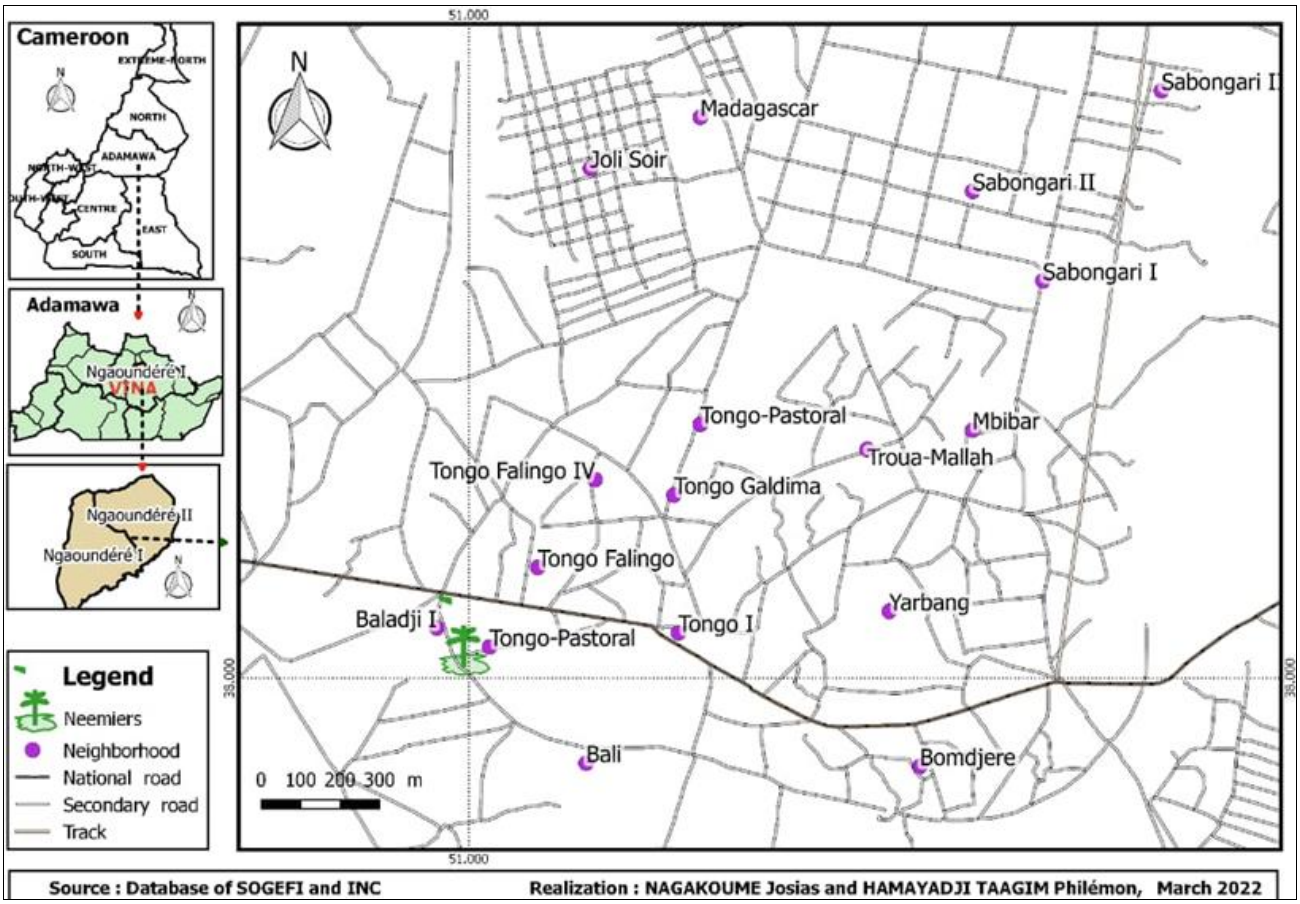

Fig 1: Position of *Azadirachta indica* plant in Baladji district in Ngaoundere

Eggs providing and breeding of larvae of *Anopheles gambiae*

To eliminate any slant that would have occurred with the wild strain and supplant the hypothesis of a possible resistance that would result from a permanent contact of the strains with *Azadirachta indica*, the strains domesticated at the malaria research laboratory for the Coordination and Organization of the Fight against Endemics in Central Africa (OCEAC) were used. The eggs of *Anopheles gambiae s.s* placed in petri dishes were soaked in trays 30 cm in diameter, containing spring water. As soon as they hatched, the larvae obtained were reared according to the WHO protocol [1] under laboratory conditions (27±2 °C; 76±4% RH).The stage 4 larvae were collected using a bulb pipette in batches of 25 and transferred to the 160 mL capacity bottles containing spring water for carrying out the biological tests. The biological tests are carried out on the larvae of stage 4 because of their maturity and their resistance compared to the other previous stages.

Preparation of the different extracts

The working method used was that proposed by the protocol of Gueye [8]. For each type of extract 500 g of *Azadirachta indica* powder were weighed using a GM-300P brand electronic scale and macerated in 2.5L of acetone for the acetone extract, in 2.5 L of methanol for the methanolic extract and in 2.5 L of hexane for the hexane extract for 72 hours. The mixtures were then filtered using Whatman N°1 paper placed in a funnel. The filtrates obtained were concentrated under a rotary evaporator set at temperatures of 65 °C, 68 °C and 69 °C respectively for the extract with acetone, methanol and hexane. The pasty-looking extracts thus obtained were used to carry out the larviciding tests. These different crude extracts were stored at -4°C in a refrigerator till they were used. After condensation, yield of extract was calculated using the following formula [7]:

$$\text{Yield (\%)} = \frac{\text{Weight of extracted metabolites}}{\text{Total leaf powder weight}} \times 100$$

Larviciding test

These tests consisted in assessing the mortality of young stage 4 larvae of *Anopheles gambiae* in the presence of dilute solutions of acetone, methanol and hexane extract of *Azadirachta indica* according to by the standard WHO protocol [1]. Thus, 25 young stage 4 larvae were collected using a Pasteur pipette and placed in 160 mL cups each containing 100mL of spring water. Preliminary experiments made it possible to select a range of concentrations for the actual tests. Concentrations of 50, 100, 150, 200 and 250 ppm were retained. Three repetitions were performed for each dilution. Two control cups were also made up under the same conditions as the test cups. The negative control contained only spring water while the positive control contained Temephos, a larvicide registered at the WHO [5] with a recommended concentration of 0.012 mg/L. The observations were made every two hours, for 12 hours of exposure with the different concentrations of the extracts and the device was maintained for up to 24 hours for the evaluation of the mortality of larvae. The larvae were declared dead if they do not make any movement even when touched or pinched with an entomological needle [4]. The determination of lethal concentration 50 (LC_{50}) and lethal hours 50 (HL_{50}) of the various extracts of *Azadirachta indica* on the larvae of *Anopheles gambiae* was carried out according to the method of Finney [9] from the regression lines obtained by transformation of the percentages of mortality into probit after 24 hours of exposure in relation to the decimal logarithm of concentrations and hours.

Photochemical screening

Phytochemical screening which is a qualitative analysis based on reactions of precipitation and/or staining made it possible to highlight the presence or absence of secondary metabolites such as polyphenols, flavonoids, saponins, tannins, alcaloids and terpenoids in the different organs of the plant according to Singleton's method [10].

Data analysis

Data analysis was done using descriptive statistics to calculate averages and percentages. Finney's formula (1971) was used for the determination of LC_{50} and LH_{50} values. Excel 2007 was used to create curves and regression lines. The Mann-Whitney U test was used for comparing independent variables.

Results

Yields

The extraction results showed that the yields of the extracts obtained (Table 1) vary from one organic solvent to another. The best yields were obtained with polar solvents, namely methanol (11.14%) and acetone (10%). The hexane extract showed the lowest yield that is 8.2%.

Table 1: Yields of different extracts of *Azadirachta indica*

Extracts	Total powder mass	Mass of the extracts obtained	Yields (%)
Methanol extract	500	55,7	11,14
Extract with acetone	500	50	10
Extract with hexane	500	41	8,2

Mortality rate of *Anopheles gambiae* larvae exposed to the different *Azadirachta indica* extracts

The data obtained made it possible to produce the curves of the mortality rates of the larvae of *Anopheles gambiae* exposed to the different concentrations of the different extracts of *Azadirachta indica* (Figures 3, 4 and 5). These three figures show that the mortality rate changes in function of the exposure time and the increase in extract concentration. For the acetone extract (Figure 3), the smallest concentration (50ppm) which became active after 2h where it induces 8% mortality, caused in 24h, 93.33% mortality. Concentrations 100, 150, 200 and 250 ppm caused 100% mortality in 24 hours of exposure. Figure 4 illustrates the mortality rate of *Anopheles gambiae* larvae to methanolic extracts of *Azadirachta indica*. The methanolic extract of *Azadirachta indica* also induced mortalities at all concentrations. From the second hour of exposure this mortality increases in 24 hours where it reaches 93.33%, 85.33%, 82.66%, 77.33% and 72% at respective concentrations of 250ppm, 200ppm, 150ppm, 100ppm and 50ppm. No concentration of the methanolic extract caused 100% mortality in 24 hours of exposure. For the hexane extract (Figure 5), the concentrations 150ppm, 200 ppm and 250ppm induced 100% mortality in 24 hours of exposure. The effectiveness of different extracts varies depending on the type of solvent used which reflects the polar character of methanol and acetone which have an affinity with certain polar compounds such as phenolic compounds. Hexane, which is a non-polar solvent, has an affinity with certain non-polar compounds such as the terpenoids responsible for the observed larvicidal effect. [Annick *et al*., 2011]. Very high mortality rates reaching 100% from the second hour of exposure were observed with Temephos, a positive control. Temephos is marketed under the name of Abate, a larvicidal organophosphate used in the treatment of water infested with the larvae of various insects.

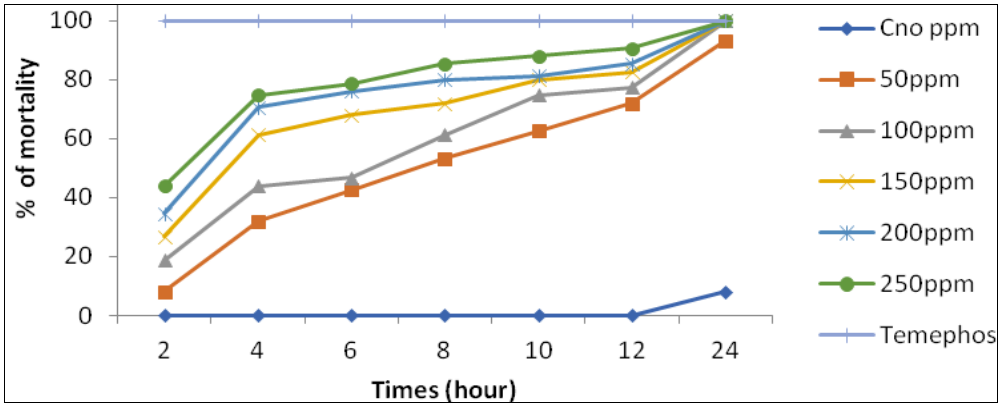

Fig 2: Mortality rate of *Anopheles gambiae* larvae exposed to the acetone extract of *Azadirachta indica*

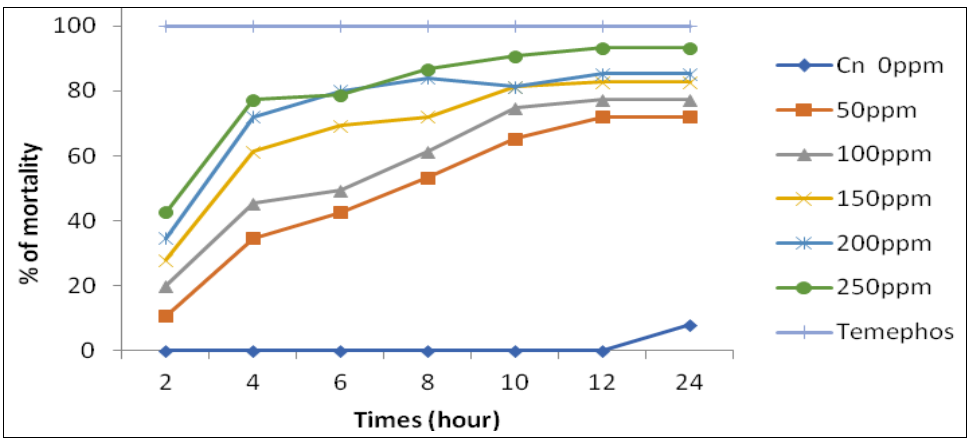

Fig 3: Mortality rate of *Anopheles gambiae* larvae exposed to the methanolic extract of *Azadirachta indica*

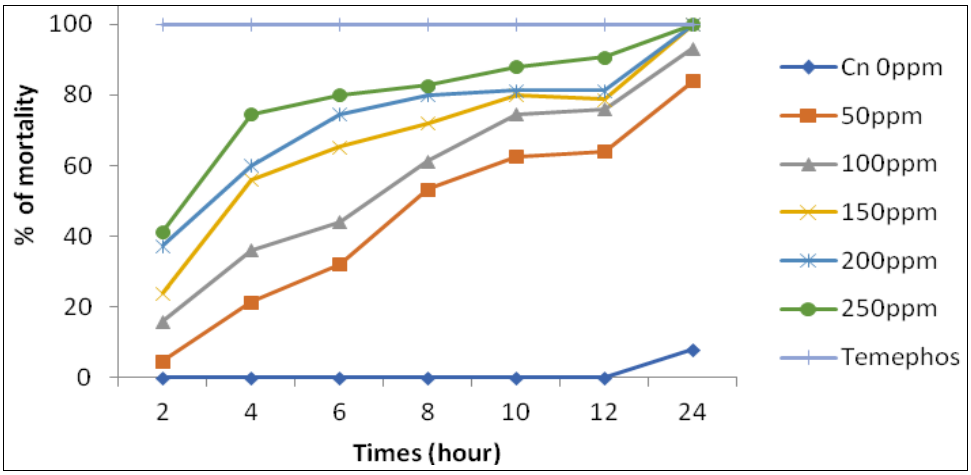

Fig 4: Mortality rate of *Anopheles gambiae* exposed larvae to the hexane extract of *Azadirachta indica*

Determination of lethal concentrations (LC_{50}) of the various extracts of *Azadirachta indica*

The LC_{50} were calculated and recorded in Table 2.

Table 2: Regression equations, CL_{50} of the various extracts of *Azadirachta indica*

Type of extracts	Regression equations	DF	R^2	LC_{50} (ppm)
Acetone extract	1,1412x + 3,048	4	0,98***	51,34[a]
Methanol extract	1,1973x + 2,9016	4	0,97***	56,54[a]
Hexane extract	1,3098x + 2,6222	4	0,99***	65,37[b]

R^2: coefficient of determination; Df: degree of freedom. The values followed by the same letters on the same column are not significantly different. ***=Very highly significant (p<0.001).

In the light of the data presented in Table 2, the acetone extract (LC_{50} = 51.34 ppm) and the methanol extract (LC_{50} = 56.54 ppm) have low LC_{50} compared to the hexane extract (LC_{50} = 65.37 ppm); this could explains its low biological activity on the larvae.

Determination of the lethal hours (HL_{50}) of the various extracts of *Azadirachta indica*

The LH_{50} were calculated and recorded in Table 3.

Table 3: Regression equations, LH_{50} of the various extracts of *Azadirachta indica*.

Type of extracts	Regression equation	DF	R^2	LH_{50} (ppm)
Acetone extract	y = 2,2868x + 3,5962	6	0,97***	4h 06min 36sec [b]
methanolic extract	y =1,4626x + 4,1893	6	0,93**	3h 34min 59sec [a]
Hexane extract	y = 2,1273x + 3,6119	6	0,99***	4h 29min 34sec [b]

R^2: coefficient of determination; Df: degree of freedom. The values followed by the same letters on the same column are not significantly different. ***=Very highly significant (p<0.001).
**=Highly significant (p<0.01).

The lethal hours 50 determined follow the same order of reactivity with 4h 06min 36sec for the acetone extract, 3h 34min 59sec for the methanolic extract and 4h 29min 34sec for the hexane extract. Although no concentration with methanolic extracts has reached 100% mortality, however, its extract has the lowest lethal hour 50, thus reflecting its effectiveness compared to other solvents. Also, this solvent has the greatest capacity to extract in sufficient quantity the various secondary metabolites [4].

Phytochemical screening

The results of phytochemical screening of the various extracts of *Azadirachta indica* are presented in Table 4.

Table 4: Results of phytochemical screening of extracts

Type of extract	Terpenoids	Sterols	Saponins	Tanins	Flavonoids
Acetone extract	+++	++	+++	+++	++
methanolic extract	+++	+++	+++	++	+++
Hexane extract	+++	++	++	++	+

+ in trace; ++: average presence; +++: strong presence

From Table 4, it appears that the different extracts of *Azadirachta indica* present secondary metabolites whose content differs from one extract to another. Terpenoids are abundant in all extracts.

Discussion

Extracts of the dried leaves of *Azadirachta indica* based on acetone, methanol and hexane showed larvicidal effects from the second hour of exposure. This mortality evolved over time and with the increase in extract concentrations. Moreover, the results of the work of Saotoing *et al.* [11] on the insecticidal effect of vegetable oils extracted from mature seeds of *Azadirachta indica* and *Khaya senegalensis* on *Anopheles gambiae* sl and Saotoing [12] on, *Calotropis procera* and *Boswellia dalzielii*, extracts with insecticidal effects revealed that the extraction yields vary from one plant to another and that the efficacy and/or the degree of toxicity of the extracts do not depend on the extraction yield. These variations can be linked to the genetic properties of the plants, their geographical origin, the conditions and duration of their storage, the period of harvest and the extraction methods applied. In Burkina Faso, Azevodo *et al.* [13] observed that extracts from different parts of the same plant or from different plants subjected to the same solvent give different yields. The insecticidal activity of *Azadirachta indica* extracts has been proved by several studies. Farou *et al.* (2006) [14] conducted a study on the effect of essential oils of *Azadirachta indica* in the pre-imaginal stages of *Culex quinquefasciatus* and confirmed that these oils have larvicidal and pupicidal effects. The present results corroborate those of Ndione [15] in Senegal who showed that with low doses

and for a short duration (2-3 g/L in 24 hours), extracts from the leaves of *Azadirachta indica* induced 100% mortality in *Culex quinquefasciatus* larvae. This same observation was made during the work of Wandscheer and Duquel [16] in Brazil which showed that *Aedes aegypti*, vector of the dengue virus, was sensitive to essential oils of *A. indica* of a dose of 0.05g/L. The mortality of the larvae could be explained by the presence of the azadirachtin contained in the neem extracts. Secondary metabolites such as terpenoids, sterols, saponins, tanins and flavonoids were found in the leaves of *Azadirachta indica* in varying amounts depending on the organic solvent used. Kalaivani [17] showed that the bioactivity of neem is due to azadirachtin (limenoid complex) which acts very effectively on mosquito larvae by targeting growth hormone. In addition, Kalaivani [17] revealed a strong larvicidal, pupicidal and adulticidal activity of azadirachtin on *Anopheles stephensi*, potential vector of malaria. Beyond the larvicidal effect, the metamorphosis and viability of adult mosquitoes were also affected. Phytochemical screening revealed that the three solvents used extracted equal amounts of the terpenoids contained in the leaves of *Azadirachta indica*. Furthermore, previous work has revealed that terpenoids are harmful to insects. For example, Park *et al.* [18] proved that hydrocarbon monoterpenes are very toxic molecules on mosquitoes in a closed environment in South Korea. These authors have also showed that terpenoids (bornyl acetate and terpenolene) are adulticidal for insects. Ngassoum *et al.* [19] showed that hydrocarbon monoterpenes and oxygenated sesquiterpenes have an insecticidal effect. Likewise, Seye *et al.* [20] in Senegal showed that neem leaf powder was more toxic at the larval stage (mortality between 86.1 and 100%) than at the pupal stage (between 14.5 and 95.9% of fledged adults). Azadirachtin was tested by Lundh [21] on a mite, the red poultry louse *Dermanyssus gallinae* and at different concentrations. On average, the treatment reduced the number of mites by 92%. Siriwattanarungsee *et al.* [22] also obtained with a concentration of 0.2% azadiractin, respective mortalities of 16.67% and 21.11% in larvae of *Chrysomya megacephala* F. and *Musca domestica* L. at stage 3. The same observation was made by Seljasen and Meadow [23] on the cabbage parasite *Mamestra brassicae* L where egg hatch is not affected, but the larvae do not survive and reach the second instar. Similar results were also obtained by Singh [24] in Indian on other insects such as Lepidoptera, Hemiptera, and ticks of the genus *Hyalomma Chougourou et al.* [25] also proved the larvicidal power of this plant on the house fly of *Musca domestica* L in Benin. The positive control (Temephos) induced a very high mortality rate reaching 100% mortality from the second hour of exposure. The work of Gusti *et al.* [26] showed that Temephos inhibits the growth of pre-imaginal stages, thus preventing them from becoming adults. Saotoing *et al.* [12] carried out a study in Cameroon with the vegetable oil of *Azadirachta indica* in the larvae of *Anopheles gambiae ss* and showed that this oil has a very significant biological activity on the larvae.

Conclusion

The present work has shown that the methanolic, acetonic and hexanoic extracts of *Azadirachta indica* have a larvicidal activity on *Anopheles gambiae ss*. The extracts from acetone and methanol were the most toxic and they contained terpenoids, sterols, saponins, flavonoids, and tannins in large quantities. *Azadirachta indica* contains azadirachtin and can therefore be highly recommended for its insecticidal effects in vector control. Methanolic extracts has the lowest lethal hour 50, thus reflecting its effectiveness compared to other solvents. The present study will be quite helpful in developing plant based anti- malarial vector and others borne diseases.

Conflicts of Interest

The authors declare that they have no conflict of interest concerning the publication of this article.

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