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Lavidical activity of *Ziziphus lotus* hydro-ethanolic
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biochemical alteration

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ملخص

تهدف هذه الدراسة إلى تقييم النشاط الإبادي ليرقات البعوض للمستخلص الهيدروإيثانولي لنبات السدر (*Ziziphus lotus*) ضد يرقات *Culiseta longiareolata*، مع التركيز على التغيرات المورفومترية والكيميائية الحيوية. بلغ مردود استخلاص الأجزاء الهوائية لنبات السدر 24.17%.

تم تعريض يرقات الطور الرابع لتركيزات شبه مميتة (LC25) و (LC50) من هذا النبات لفترات زمنية مختلفة (24 ساعة، 48 ساعة، 72 ساعة). (شملت الدراسة تقييمات مورفومترية) قطر الرقبة والوزن (وتقييمات كيميائية حيوية) البروتين الكلي، الكربوهيدرات، والدهون. (أظهرت النتائج أن مستخلصات السدر تُحدث تغيرات ذات دلالة إحصائية في كل من شكل وكيمياء حياة اليرقات. بناءً على ذلك، تشير هذه البيانات إلى أن المستخلصات الهيدروإيثانولية لنبات السدر قد تمثل بديلاً آمناً بيئياً لمكافحة تجمعات البعوض.

الكلمات المفتاحية: *Ziziphus lotus*؛ نشاط إبادي لليرقات، *Culiseta longiareolata*، تغيرات مورفولوجية، تغيرات كيميائية حيوية

Abstract

This study aims to evaluate the larvicidal activity of the hydroethanolic extract of *Ziziphus lotus* against *Culiseta longiareolata* mosquito larvae, focusing on morphometric and biochemical changes. The extraction yield of the aerial part of *Ziziphus lotus* was 24.17%.

Fourth-instar larvae were exposed to sublethal concentrations (LC25 and LC50) of this plant over different time periods (24 hours, 48 hours, and 72 hours).

The study included morphometric assessments (neck diameter and weight) and biochemical assessments (total protein, carbohydrates, and lipids). The results showed that *Ziziphus lotus* extracts induce statistically significant changes in both the morphology and biochemistry of the larvae. Therefore, these data suggest that the hydroethanolic extracts of *Ziziphus lotus* may represent an environmentally safe alternative for controlling mosquito populations.

Keywords: *Ziziphus lotus*, larvicidal activity, *Culiseta longiareolata*, morphological changes, biochemical changes

Resumé

Cette étude vise à évaluer l'activité larvicide de l'extrait hydroéthanolique de *Ziziphus lotus* contre les larves du moustique *Culiseta longiareolata*, en se concentrant sur les changements morphométriques et biochimiques. Le rendement d'extraction de la partie aérienne de *Ziziphus lotus* était de 24,17 %.

Des larves du quatrième stade ont été exposées à des concentrations sublétales (LC25 et LC50) de cette plante pendant différentes périodes (24 heures, 48 heures et 72 heures). L'étude a inclus des évaluations morphométriques (diamètre du cou et poids) et des évaluations biochimiques (protéines totales, glucides et lipides)

Les résultats ont montré que les extraits de *Ziziphus lotus* induisent des changements statistiquement significatifs à la fois dans la morphologie et la biochimie des larves. Par conséquent, ces données suggèrent que les extraits hydroéthanoliques de *Ziziphus lotus* pourraient représenter une alternative écologiquement sûre pour contrôler les populations de moustiques.

Mots-clés : *Ziziphus lotus*, activité larvicide, *Culiseta longiareolata*, changements morphologiques, changements biochimiques

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Abbreviation	Meaning
<i>Z. lotus</i>	<i>Ziziphus lotus</i>
<i>C. longiareolata</i>	<i>Culiseta longiareolata</i>
LC	Lethal concentration
LC50	Lethal Concentration for 50% of larvae
LC25	Lethal Concentration for 25% of larvae
et al.	And others
R1,R2,R3	Replicates 1,2,3

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Introduction

1. Introduction

Culiseta longiareolata (Macquart,) is a common species of mosquito found throughout northern Africa, including the region of Tébessa (Algeria), and like many other mosquitoes, is capable of reproducing in a variety of different types of natural and artificial water. This species of mosquito has also been found to serve as a potential vector for disease agents such as the West Nile virus (Hazratian *et al.*, 2019). Several species of mosquitoes are among the most significant threats to public health globally, as over 500 million new infections of vector – borne pathogens are recorded each year. As a result, the World Health Organization has declared mosquitoes as « public enemy number one » for their role in transmitting various vector – borne diseases including malaria, dengue, chikungunya, yellow fever, filariasis, Japanese encephalitis, and West Nile virus to millions of people on a yearly basis in Tropic and Subtropics regions of the World (Ghosh *et al.*, 2012). The ongoing emergence of these disease – causing agents has resulted in ongoing and significant burdens to health care systems in many developing countries, including limitations on comprehensive vector control programs to limit the transmission of disease – causing agents (World Health Organization 2020).

Since the development of modern insecticides, the main approach to controlling mosquito populations has involved the use of synthetic chemical insecticides (e.g., organophosphates, pyrethroids, odorglorylenes, etc.) (Becker *et al.*, 2010). The continued and sometimes indiscriminate use of synthetic insecticides has produced many negative effects: the emergence of resistance to commonly used mosquito control products among vector species, biological magnification of pesticides through the food chain, environmental contamination from soil and water, and adverse effects on non-target organisms including humans (Ghosh *et al.*, 2012). As a result, scientist have begun to develop new insect control methodologies that are ecologically sound, biodegradable, and targeted against specific pests (Pavela, 2014). One possible solution to these problems is the use of plant derived insecticides (botanicals). Phytochemicals, or naturally occurring secondary metabolites, are produced by plants as a means to defend themselves from herbivory and environmental stresses. Phytochemicals have demonstrated a broad range of insecticidal activity against a variety of insect species (Ghosh *et al.*, 2012).

Unlike traditional synthetic insecticides which use a single active ingredient, botanical extracts are made up of complex mixtures of chemical compounds that work together to affect multiple physiological / bio – processes on a variety of pests, making them much less prone to

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developing resistance to the compound(s) found within the botanical extract, as well as being generally biodegradable, affordable and being non – toxic to non-target species (George *et al.*, 2014).

Insecticides derived from plants have been used for many years as part of agricultural traditions world-wide. Long before the invention of chemical insecticides, farmers used plant-based products to fight off insect pests attacking their crops (Isman, 2006). Today, there is renewed interest in using botanical insecticides as one option for being environmentally responsible regarding pest management and protecting humans and other species from potential harm. Using botanical insecticides has many unique benefits; such as being biodegradable in the environment, often having novel modes of action that are not subject to the resistance seen with synthetic chemicals, and can be produced in a local area at a relatively low cost compared to synthetic chemicals (Isman, 2020). There are many documented plant species with insecticidal properties including neem (*Azadirachta indica*), pyrethrum (*Chrysanthemum cinerariifolium*), and essential oil-producing plants (Pavela and Benelli, 2016). *Ziziphus lotus* (L.) Desf., also known as wild jujube or "Sedra", is a well-known member of the rich Mediterranean flora and has been studied due to its great adaptability to arid conditions (Bencheikh *et al.*, 2023) being found throughout North Africa, including Morocco, Algeria, Tunisia, and Libya, as well as parts of the Middle East and Southern Europe (Hafiz *et al.*, 2024). The phytochemistry of *Ziziphus lotus* has shown that it contains a number of phytochemicals that are considered to be bioactive. These include flavonoids, alkaloids, saponins and phenolics which provide a variety of biological activities (Bencheikh *et al.*, 2023). This evidence indicates that *Ziziphus lotus* has antioxidant, antimicrobial, anti-inflammatory and antidiabetic activities (Hafiz *et al.*, 2024). However, little is known about *Ziziphus lotus*'s ability to control mosquito vector populations as a larvicidal agent. Due to *Ziziphus lotus* phytochemicals, eco-friendly alternative insecticides are needed; therefore, the present study's goal was to investigate the larvicidal activity of *Ziziphus lotus* hydro-ethanolic extract against larvae of *Culiseta longiareolata* by observing the morphometric and biochemical changes. The determination of the lethal concentrations (LC₂₅ and LC₅₀) of the extract was the first step before studying the morphometric effects on larvae exposed to the sublethal concentrations (weight of larvae and their neck width as an indicator of body volume) after 24, 48, and 72 hours of exposure. After that, the biochemical properties (the quantity of total protein, carbs (glucosides) and lipids) of the treated larvae will be examined to confirm the mechanisms of the larvicidal effects exhibited by *Ziziphus lotus* and

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assist in the establishment of these plant-derived eco-friendly alternatives to using synthetic insecticides for controlling mosquito populations in Algeria.

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2. Material and mhetods

2.1. Ziziphus lotus Study

2.1.1. Geographical distrubtion of *Ziziphus lotus*

Ziziphus lotus is a drought-tolerant shrub that is native to North Africa, where it is broadly distributed across Morocco, Algeria, Tunisia, and Libya (Le Floc'h *et al.*, 2010). It is considered a typical Mediterranean species with limited extension into the northern Sahara (Ghedira, 2013). Beyond North Africa, its distribution extends to regions such as Yemen and the island of Socotra, as well as parts of the Middle East including Palestine, Syria, Turkey, and Cyprus. Additionally, it is present in southern Europe, particularly in Greece, Sicily, and southern Spain. In Algeria, *Ziziphus lotus* is widely spread in arid and semi-arid zones, especially in southern regions. It is commonly found in areas such as Ain Ouessara and Maessad (Djelfa province), characterized by arid climatic conditions, and in Taghit (Bechar province), which has a Saharan climate (Mounni, 2008 cited by Benammar, 2011).

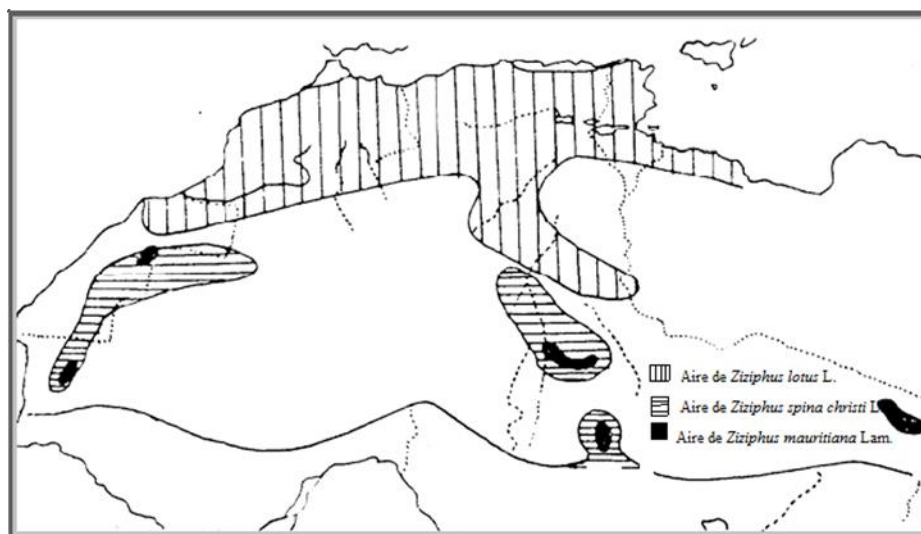


Figure 01.Distribution of *Ziziphus lotus* in Algeria (Quezel and Santa, 1962)

2.1.2. Botanical description of *Ziziphus lotus*

The *Ziziphus lotus* is a shrub that is slow-growing and has many thorns. This plant has a rounded shape, zigzagging branches, green spiny shoots, and bark that has fissures. The stems of the *Ziziphus lotus* have two different types of thorns that occur in pairs: there is a long

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straight thorn that can be 1.8-2.5 cm in length and an additional short thorn , which may be curved as well . (Messaad and Belhadj, 2025 ; Torres-García *et al.*, 2021)

The leaves are small, measuring approximately 1–2 cm in length and about 7 mm in width, with an oval to oblong or elliptical shape (Bayer and Butter, 2000). They are smooth and glossy on both surfaces and exhibit three prominent longitudinal veins originating from the petiole (Baba Aissa, 1999). The stipular thorns are unequal, with one being long, straight, and upward-oriented, and the other shorter and curved downward like a hook.

The flowers of *Ziziphus lotus* are small but conspicuous, yellow in color, and composed of star-shaped sepals and small petals surrounding a superior bisexual ovary. Flowering typically occurs in June (Baba Aissa, 1999 ; Nour and Dilmi, 2017). The fruit is an oval to elongated drupe, similar in size and shape to an olive. It is initially green, turning yellow and then dark red or orange-brown upon ripening in October (Bayer and Butter, 2000). The fruit has a slightly sweet taste, and its seeds, which are often separated from the pulp, may reach up to 1.05 cm in length (El Aloui *et al.*, 2014). Compared to fruit trees such as apple or peach, *Ziziphus lotus* possesses a relatively less developed root system (Mengjun, 2006). As a result, it exhibits slow growth and generally begins fruit production around the age of four years. Its productive lifespan can extend from 20 to 25 years (Maraghni *et al.*, 2010) (Figure 1).

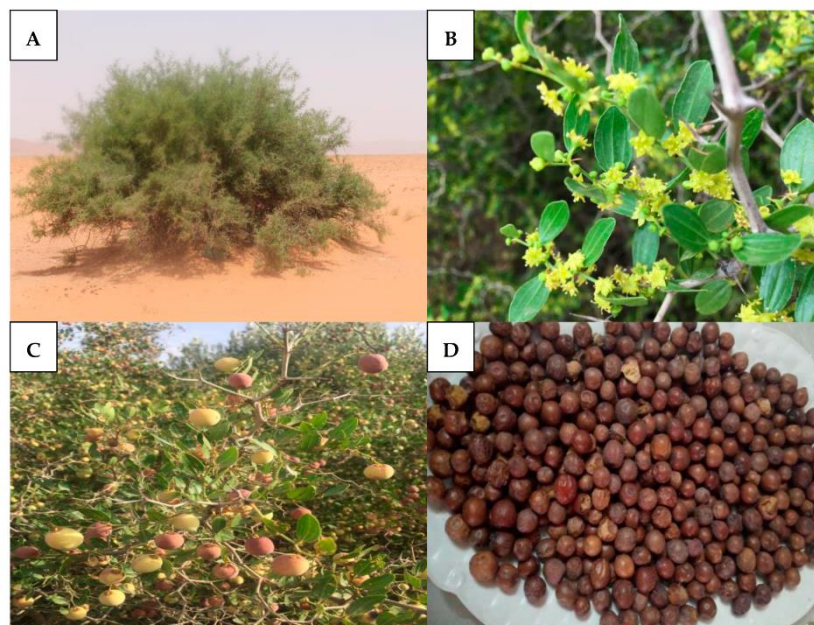


Figure 02. *Ziziphus lotus* (A) ,Fowers (B) ,Fruits(C D) (Alla *et al.*, 2025)

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2.1.3. Classification

The taxonomic classification of *Ziziphus lotus* reflects its position within the group of flowering plants. It belongs to the family Rhamnaceae, which comprises numerous species well adapted to arid and semi-arid environments. As a member of the order Rosales, *Ziziphus lotus* shares several morphological and genetic characteristics with other dicotyledonous plants. Its classification is established based on key morphological features, including leaf arrangement, floral structure, and fruit type, in addition to molecular evidence provided by recent phylogenetic studies. The complete taxonomic hierarchy of *Ziziphus lotus* is presented in the table below (Mabberley, 2017).

Table 01 . Classification of *Ziziphus lotus* (Gheedira, 2013)

Kingdom	Plantae (Plant Kingdom)
Phylum	Magnoliophyta (Angiosperms)
Subphylum	Magnoliophytina (Angiosperms)
Class	Magnoliopsida (Dicotyledons)
Subclass	Rosidae
Order	Rhamnales
Family	Rhamnaceae
Genus	<i>Zizyphus</i>
Species	<i>Zizyphus lotus</i> L

2.1.4. Chemical Composition of *Ziziphus lotus*

2.1.4.1 Terpenes

Terpenes are a class of hydrocarbons formed through the assembly of two or more isoprene units (Belhachat, 2019). They represent a large and diverse group of natural metabolites commonly found in plants. Due to their structural diversity and biological activities, plant-derived terpenes are widely utilized in various fields, including the food industry, cosmetics, pharmaceuticals, and biotechnology (Jiang *et al.*, 2016).

2.1.4.2 Alkaloids

Alkaloids are naturally occurring nitrogen-containing compounds, typically characterized by the presence of one or more nitrogen atoms within a heterocyclic ring structure. These compounds exhibit significant pharmacological properties. Moreover,

alkaloids have the ability to react with heavy metal salts, which facilitates their detection and identification using specific reagents such as Mayer's and Dragendorff's reagents (Krief, 2003).

2.1.4.3. Flavonoids

Flavonoids are natural polyphenolic compounds that serve as important plant pigments and are considered nearly ubiquitous in the plant kingdom. They occur either in free form or as glycosides. Flavonoids are distributed in various plant parts, including roots, stems, wood, leaves, flowers, &

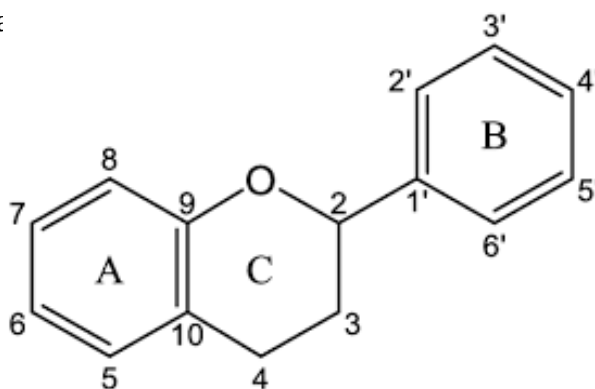


Figure 03 . Basic structure of flavonoides (Harbone, 2000)

2.1.4.4. Tannins

Tannins are highly hydroxylated phenolic compounds characterized by a wide range of chemical structures. These secondary metabolites, found in higher plants, may have molecular weights of up to 20,000 Da. A key property of tannins is their ability to form complexes with proteins and carbohydrates. They are commonly located in the vacuoles of parenchymal cells or in specialized cells such as idioblasts. Tannins are widely present in plant-derived foods, including legumes, cereals, fruits, nuts, and vegetables, as well as in beverages such as tea, wine, cocoa, and cider (Montes-Ávila *et al.*, 2018 ; Hassanpour *et al.*, 2011) Figure 3 .

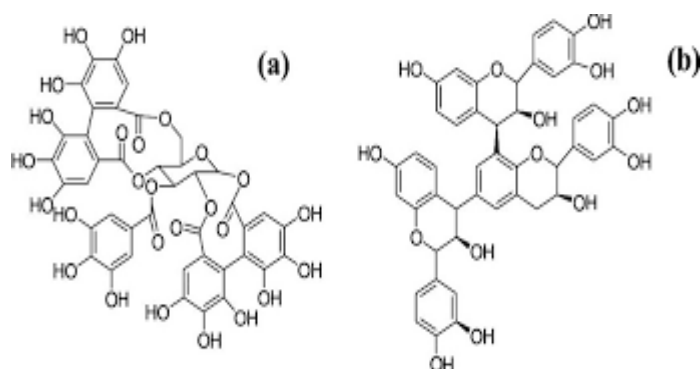


Figure 04 . Structure of (a) hydrolysable (b) condensed tannins (Naczka, 2006)

2.1.5. Biological properties of *Ziziphus lotus*

Studies have demonstrated various biological activities for *Ziziphus lotus* (L.) Desf., and many of its biological properties have been examined ; the majority of these activities can be ascribed to the number of secondary metabolites (specifically phenolics, flavonoids, tannins, and alkaloids) contained in the plant (Bencheikh *et al.*, 2023).

2.1.5.1. Antioxidant Activity

The antioxidant activity of *Ziziphus lotus* can be attributed to free radical scavenging ability ; *Ziziphus lotus* also has strong free radical scavenging ability. Rais *et al.* (2021) reported that *Ziziphus lotus* fruit's ethyl acetate extract had significant DPPH free radical scavenging ability, with an IC₅₀ of 79.61 µg/mL. Hamdaoui *et al.* (2025) demonstrated that *Ziziphus lotus* extracts also exhibited successful ABTS free radical neutralisation with an IC₅₀ of 18.49 ± 1.47 µg/mL. Prominent types of phenolic compounds (e.g. rutin, hyperin, isoquercitrin, and resveratrol) were responsible for this antioxidant capability (Alla *et al.*, 2025) ; one potential explanation for how larvicides work is by inducing oxidative stress, as large quantities of reactive oxygen species can perturb cellular homeostasis within mosquito larvae.

2.1.5.2. Enzyme Inhibition Activity

The hydro-ethanolic extract derived from the *Ziziphus lotus* plant has been shown to inhibit enzymes. One important enzyme that has been identified and inhibited by this extract is acetylcholinesterase (AChE). This enzyme is naturally targeted by neurotoxic agents (Bencheikh *et al.*, 2023), and when inhibited, acetylcholine accumulates at the synaptic

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junctions, resulting in prolonged nerve impulses, paralysis, and the eventual death of insect larvae, as well as other types of invertebrate. Furthermore, this extract is also believed to modify the levels of various detoxifying enzymes, which include glutathione S-transferase (GST) and esterases (Alla *et al.*, 2025), and this change in the activity of these enzymes will assist us with the development of our understanding of how the extract acts as a larvicidal agent.

2.1.5.3. Antimicrobial Activity

Research has shown that *Ziziphus lotus* extract exhibits inhibition toward a variety of pathogenic bacterial cultures. Rais *et al.* (2023) demonstrated how oil extracted from *Z. lotus* fruit exhibited an inhibition zone of approximately 15 ± 0.1 mm against staphylococcus aureus and 14 ± 0.2 mm against Escherichia coli. Furthermore, Bencheikh *et al.* (2023) noted the extract demonstrated active against methicillin-resistant staphylococcus aureus (MRSA). The minimilal inhibitory concentration (MIC) was approximately 5 mg/ml. An example of biologically active compound is one that inhibits bacterial growth but does not directly correlate with larvicidal action but rather establishes the existence of such biologically active agents present that exhibit effectiveness of disrupting biological systems.

2.1.5.4. Other Biological Properties

There are several biological activities exhibited by *Ziziphus lotus* that have no mention above, including aspiratory (anti-inflammatory, analgesic, anti-ulcer, and hepatoprotective) effects (see Messaad and Belhadj, 2025).

2.1.6. Ethnobotanical uses of *Ziziphus lotus*

Ziziphus lotus L., commonly referred to as “ Sedra ” or “ Nbgh ” in various areas of North Africa, is considered a part of the traditional medicine in dry and semi- dry climates (such as Algeria, Tunisia ; Morocco ; and United Arab Emirates) and has been utilized in this manner for centuries (Bencheikh *et al.*, 2023). Many therapeutic applications exist for many parts of the *Ziziphus lotus* plant , including the fruit , leaves , roots, and seeds , depending on the method of extraction by which they were prepared through decoction, infusion, or maceration (Bencheikh *et al.*, 2023). The Nbeg (fruit) of *Z. lotus* has long been consumed both fresh and dry by humans and animals alike as food and as herbal medicine due to its culinary applications ; this fruit has also had therapeutic uses in the treatment of various gastrointestinal

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conditions (such as diarrhea and ulceration in the stomach) due to its antispasmodic and antiulcer effects and for helping to relieve various respiratory problems (such as bronchitis and tuberculosis) (Bencheikh *et al.*, 2024) through older and more traditional means utilizing *Z. lotus* .

Traditionally , *Z. lotus* leaves were made into infusion or decoctions to treat fever, insomnia and various liver disease disorders (eg . hepatic colic , liver congestion) . In addition to these uses , the leaves of the plant have historically been used as a topical agent to enhance wound healing (Elachouri *et al.*, 2024). Many of these traditional uses have stimulated recent pharmacological studies , which have confirmed the therapeutic capabilities of *Z. lotus* .

2.2. Preparation of hydro-ethanolic extract from Sidr (*Ziziphus lotus*)

To prepare the extract, ethanol and distilled water were first mixed in a 1000 mL beaker with thorough stirring to ensure homogeneous blending. Dried Sidr (*Ziziphus lotus*) leaf powder was then added, and the mixture was stirred using a mechanical agitator to facilitate the extraction of bioactive compounds from the plant material .

After stirring, the mixture was filtered using Whatman No. 1 filter paper to separate the liquid extract from solid residues. The filtered extract was then transferred to a rotary evaporator set at 55°C to remove the solvents and concentrate the extract

Finally, the concentrated extract was placed in a drying oven to ensure complete drying and preservation before further testing and analysis (Figure 4).

The yield of the plant extract is the ratio between the weight of the extract and the weight of the dry plant material. It was evaluated from 4 extractions and expressed as a percentage using the following formula :

$$Y = (W_e / W_p) \times 100$$

Where :

Y = Yield of the extract (%)

W_e = Weight of the extract (g)

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Wp = Weight of the dry plant material (g)



Figure 05 . Steps of extraction

2.3. *Culiseta longiareolata* study

2.3.1. Presentation of the target species (*Culiseta longiareolata*)

Culiseta longiareolata is a species of the family Culicidae within the subfamily Culicinae (Khaligh *et al.*, 2020). This insect thrives in warmer climates and undergoes complete metamorphosis. As a member of the order Diptera and the family Culicidae, it ranges from 3 to 5 mm in length and is characterized by a small body, long slender legs, and narrow membranous wings (Ferede *et al.*, 2022). The species is readily identifiable by key morphological features: non-plumose antennae, a short nearly conical siphon, and an anal saddle with an interrupted segment (Chorfi and Sedira, 2016).

2.3.2. Taxonomic position

Table 02 . The systematic position of *Cs longiareolata* (Dris, 2019).

Kingdom	Animalia
Sub-Kingdom	Metazoa
Phylum	Arthropoda
Sub-Phylum	Hexapoda
Super-class	Protostomia
Class	Insecta
Sub-class	Pterygota
Infra-class	Neoptera
Super-order	Endopterygota
Ordre	Diptera
Sub-order	Nematocera
Infra-family	Culicomorpha
Family	Culicidae
Genus	Culiseta
Species	<i>Culiseta longiareola</i> (Aiten 1954)

2.3.3. Life cycle of *Culiseta longiareolata*

The complete metamorphosis of *Culiseta* mosquitoes includes four distinct and successive stages (figure 05) :

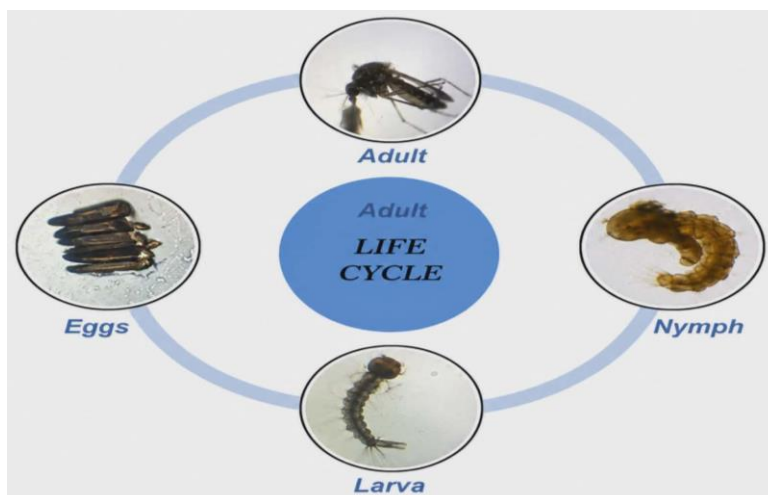


Figure 06 . life cycle of *Culisita langiareolata*

2.3.3.1. Egg stage

Female *Culiseta* mosquitoes deposit their eggs in rafts on calm or static water bodies. As a general rule, the eggs tend to hatch within 1 to 3 days when temperature and moisture conditions are adequate (Becker *et al.*, 2010)



Figure 07 . Mosquito eggs

2.3.3.2. Larval stage

Commonly referred to as « wrigglers, » mosquito larvae are primarily aquatic and feed on filamentous algae and microorganisms. This developmental phase typically lasts between 5 and 14 days, depending on environmental conditions (Harbach, 2023).

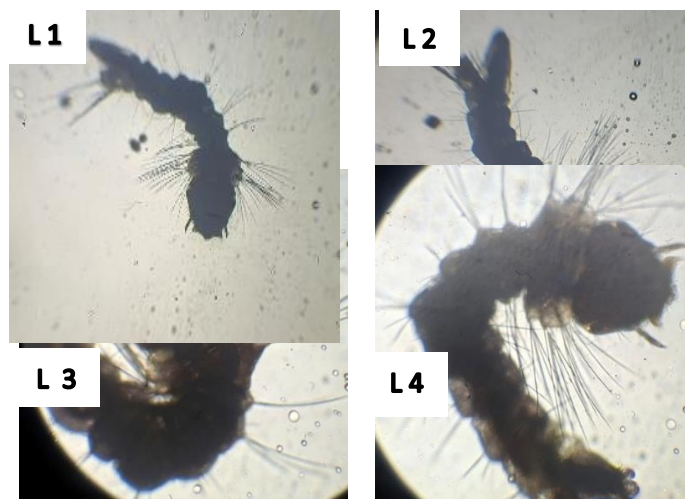


Figure 08 . Larval stage of *Culiseta longiareolata*

2.3.3.3. Pupal stage

The pupal stage is a non-feeding but mobile phase. The pupae, also known as « tumblers, » have a transitional period of approximately 1 to 4 days before emerging as adult mosquitoes (Becker *et al.*, 2010).

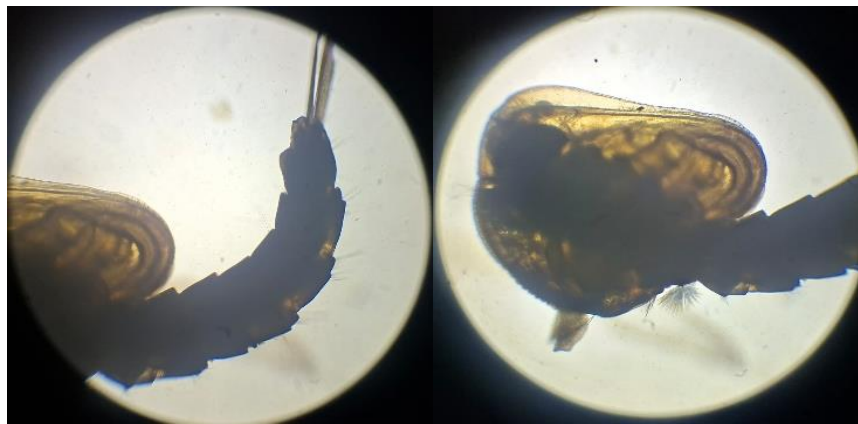


Figure 09 . Nymhh *Culiseta langiareolata*

2.3.3.4. Adult stage

After the pupal stage, the adult *Culiseta* mosquitoes rest on the water surface. Male *Culiseta* sustain themselves on plant juices, whereas females require a blood meal before laying subsequent eggs. Adult mosquitoes may live anywhere from a few days to a couple of weeks (Darsie and Ward, 2005).

2.3.4. Collecting, Rearing, and Breeding conditions

Specimens of larva were collected around Tebessa city, specifically from the Hammamet districts. (Figure 09).



Figure 10. The collect locations

After they were collected, the larvae were placed into the laboratory where they were raised in plastic containers with 150 mL of dechlorinated tap water. They had a diet which consisted of 75% biscuit powder and 25% baking powder available at all times. Water was replaced every 48 hours to help eliminate microbial growth and to maintain an ideal rearing environment (Dris 2019). Wigglesworth (1972) has shown that in order for females to produce optimal numbers of eggs after developing as larvae, they must receive a high amount of protein in their larval food source compared to carbohydrate-only (sugar) food sources . Figure 10 summarizes the rearing conditions .

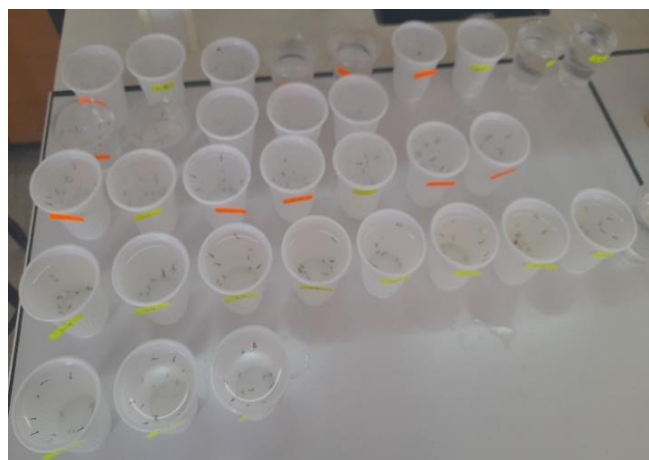


Figure 11 . Breeding of mosquitoes in the laboratory

2.4. Morphometric Study

Morphometric analysis was performed by measuring biomass (wet weight) and estimated body volume of individual larvae from two parameters. The thoracic diameter of mosquitoes increased continuously throughout the intermolt period and had significant correlations to protein, lipid, and carbohydrate synthesis (Timmermann and Briegel, 1998). Measurements of the thoracic width of the larva were taken using a calibrated binocular stereomicroscope (Becker *et al.*, 2010) and the weight of the larvae was taken using a precision balance (Zuharah *et al.*, 2016). The linear relationship between the linear morphological traits of mosquito larvae (i.e. thoracic width) and body mass has been further validated through additional studies confirming the thoracic width as a reliable predictor of body mass for different species (Mocq *et al.*, 2024).

2.5. Determination of Biochemical Constituents

The Controls and treated larvae exposed to *Ziziphus lotus* (LC₂₅ or LC₅₀) hydro-ethanolic extraction in the L₄ larval stage, were collected and subjected immediately after collection to 1 ml of 20% Trichloroacetic Acid (TCA) to stop enzymatic degradation and denaturation of the proteins (Shibko *et al.*, 1967). Proteins (total), total carbohydrates and total lipids were extracted using a sequential extraction procedure developed by Shibko *et al.*, (1967).

Both control (LC₂₅ = 1.13 mg/ml) and treated (LC₅₀ = 1.48 mg/ml) (Nebba, 2025) larval (L₄) stages of the larvae were placed into 1 mL of 20% trichloroacetic acid (TCA) and subsequently subjected to ultrasonic homogenization and centrifugation (3,000 rpm; 4°C; 10 min) resulting in supernatant I and pellet I. The carbohydrates present in supernatant I were assayed according to Duchateau and Florkin (1959). To extract lipids, 1 mL of ether/chloroform (1:1; v/v) was added to pellet I and was centrifuged (5,000 rpm; 10 min); supernatant II was collected for quantification of lipids using Goldsworthy *et al.* (1972). Protein in pellet II (dissolved in 0.1 N sodium hydroxide) was determined by Bradford (1976) according to the extraction sequence described by Shibko *et al.* (1967).

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2.6.Statistical analysis

The number of individuals tested in each series is given with the results. Data are presented as the mean $\pm$ standard deviation (SD). The significance between different series was tested using one-way analysis of variance (ANOVA) at 5% level followed by Dunnett's multiple comparaisn test. All statistical analyses were performed using Prism 8.0 for Windows (GraphPad Software Inc., www.graphpad.com with a significant level $p < 0.05$).

Results and discussion

3. Results and discussion

3.1 Results

3.1.1. Yield of *Ziziphus lotus* hydro-ethanolic extract

the extraction of the plant was prepared following the protocol described by Merghem *et al* (1995). The *Ziziphus lotus* hydroethanolic extract has a green color and yielded 24.17% .

3.1.2. Impact of *Ziziphus lotus* hydro-ethanolic extract on mosquito growth

3.1.2.1. Effect of *Ziziphus lotus* hydro-ethanolic extract on the weight growth of *Culiseta longiareolata*

The results of changes in the body weight of *Culiseta longiareolata* fourth instar larvae are shown in Figure 12 . These results show a significant difference between the control and treated series ($p=0.01$).

Comparison of the means between the control and treated series using the Dunnett test revealed a significant decrease in the body weight of the 4 larvae at 72 h for the series treated with LC25 and LC50.

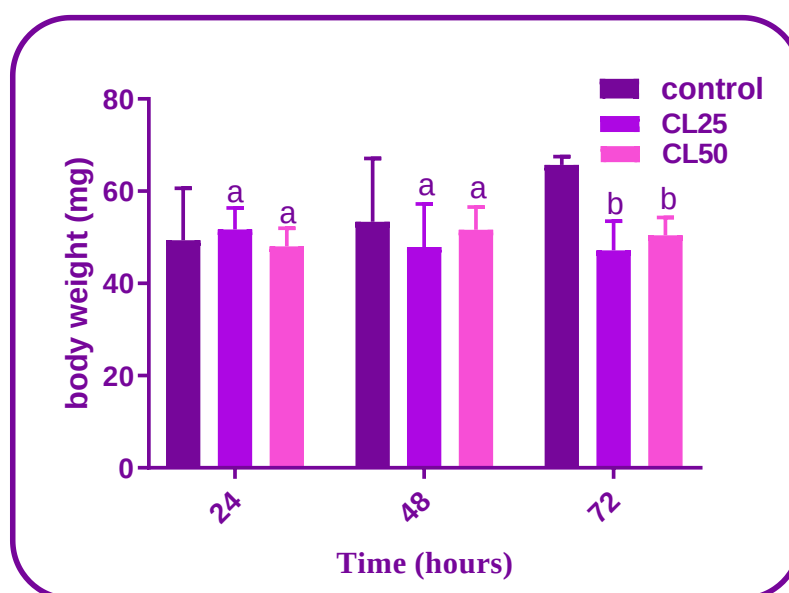


Figure 12: Effects of *Ziziphus lotus* hydro-ethanolic extract on weight (mg) against fourth instar larvae *Cs. longiareolata*: ($m \pm SD$, $n = 5$ pools each containing 10 individuals).

3.1.2.2. Effect of *Ziziphus lotus* hydro-ethanolic extract on the linear growth of *Culiseta longiareolata*

For the stage (L4), the results obtained show that the body volume decreases in a very highly significant manner ($p < 0.0001$) when comparing the three series during 24, 48 and 72 hours after treatment (Fig. 17)

Comparison of the means by the Dunnett test shows that the treatment with the two concentrations causes a significant reduction during the three times compared to the control series.

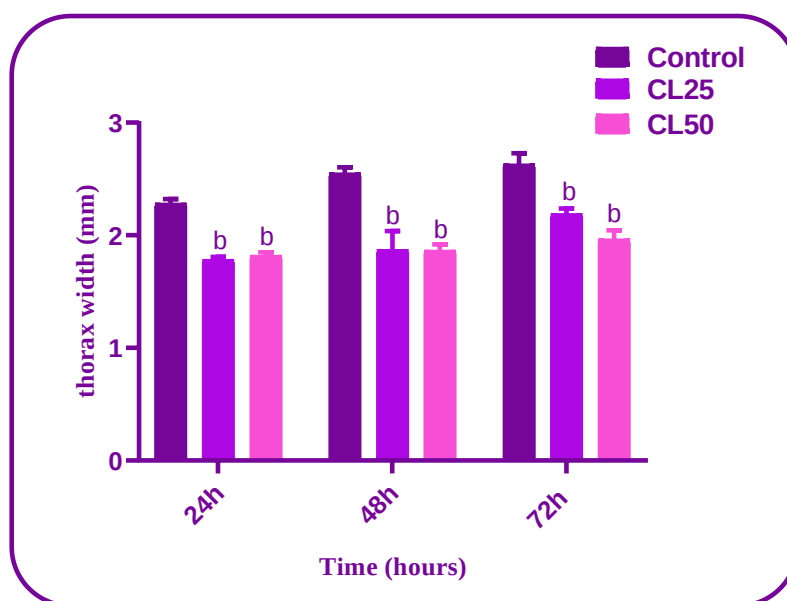


Figure 13 : Effects of *Ziziphus lotus* hydro-ethanolic extract on thorax width (mm) against fourth instar larvae *Cs. longiareolata*: ($m \pm SD$, $n = 5$ pools each containing 10 individuals).

3.1.3. Biochemical changes (Impact on Protein levels and Enzyme activities).

3.1.3.1. Impact of *Ziziphus lotus* hydro-ethanolic extract on the biochemical composition of *Culiseta longiareolata*

The LC_{25} and LC_{50} concentrations of the hydroethanolic extract of *Ziziphus lotus* were administered to newly molted fourth-instar larvae of *Culiseta longiareolata*.

The impact of the treatment on the biochemical composition of the larvae, including carbohydrate, lipid, and protein contents, was assessed 24, 48, and 72 h after exposure.

3.1.3.2. Effect on total protein content

The assay results are presented in Figure 14. Statistical analysis revealed a highly significant difference in protein content between the control and treated groups ($P = 0.0004$), whereas no significant variation was observed among the three exposure periods (24 h, 48 h, and 72 h) ($P = 0.4557$).

Multiple comparison analysis showed that treatment induced a highly significant reduction in protein content at 48 h and 72 h compared with the control group.

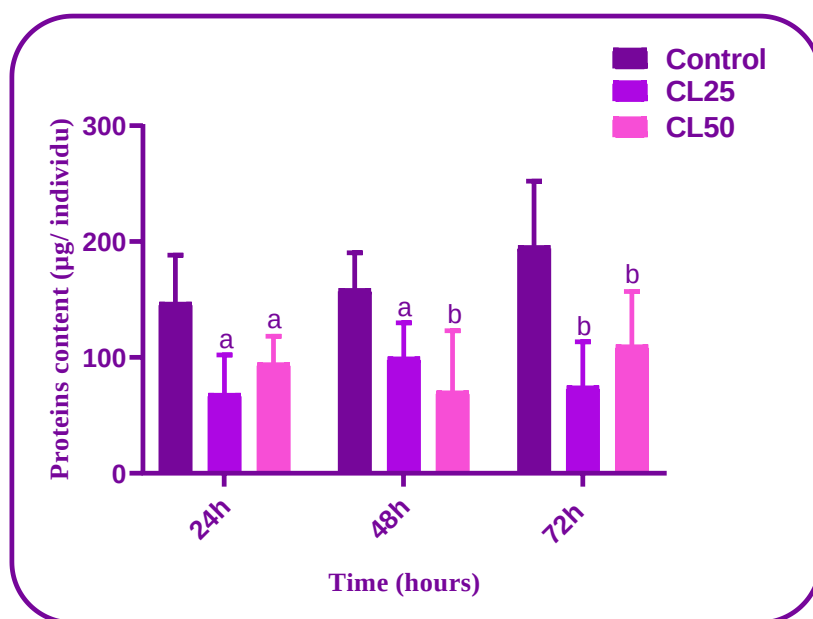


Figure 14: Effects of *Ziziphus lotus* hydro-ethanolic extract on the proteins content (µg/individu) against fourth instar larvae *Cs. longiareolata*: ($m \pm SD$, $n = 5$ pools each containing 10 individuals).

3.1.3.3. Effect on total carbohydrate content

The hydroethanolic extract of *Ziziphus lotus* caused a highly significant difference between the control and treated groups ($P = 0.0012$), while exposure time also had a significant effect ($P = 0.0154$).

Post hoc multiple comparisons revealed a highly significant reduction in the treated groups (LC₂₅ and LC₅₀) at 48 h and 72 h relative to the control.

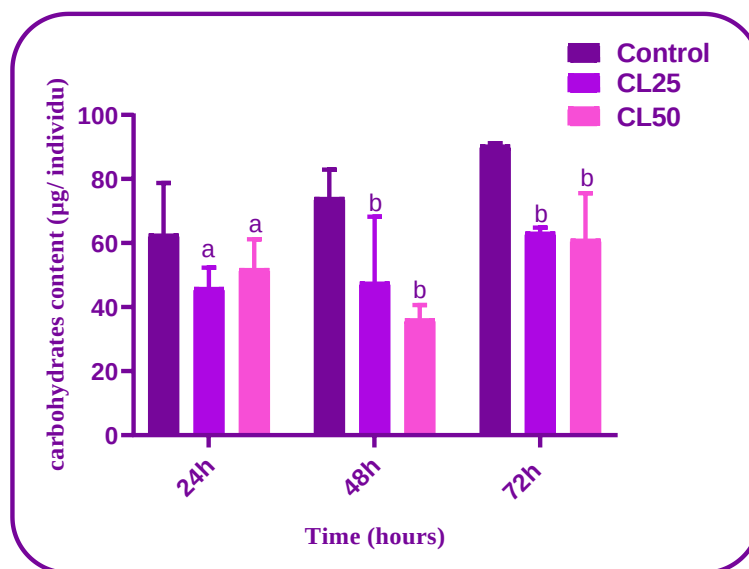


Figure 15: Effects of *Ziziphus lotus* hydro-ethanolic extract on the carbohydrates content (µg/individu) against fourth instar larvae *Cs. longiareolata*: ($m \pm SD$, $n = 5$ pools each containing 10 individuals).

3.1.3.4. Effect on total lipid content

In the treated groups, lipid content decreased significantly ($P = 0.0355$), while a highly significant variation was observed among the three exposure periods (24 h, 48 h, and 72 h) ($P = 0.0006$).

Multiple comparison analysis revealed a significant reduction in lipid content at 24 h, 48 h, and 72 h following treatment compared with the control group.

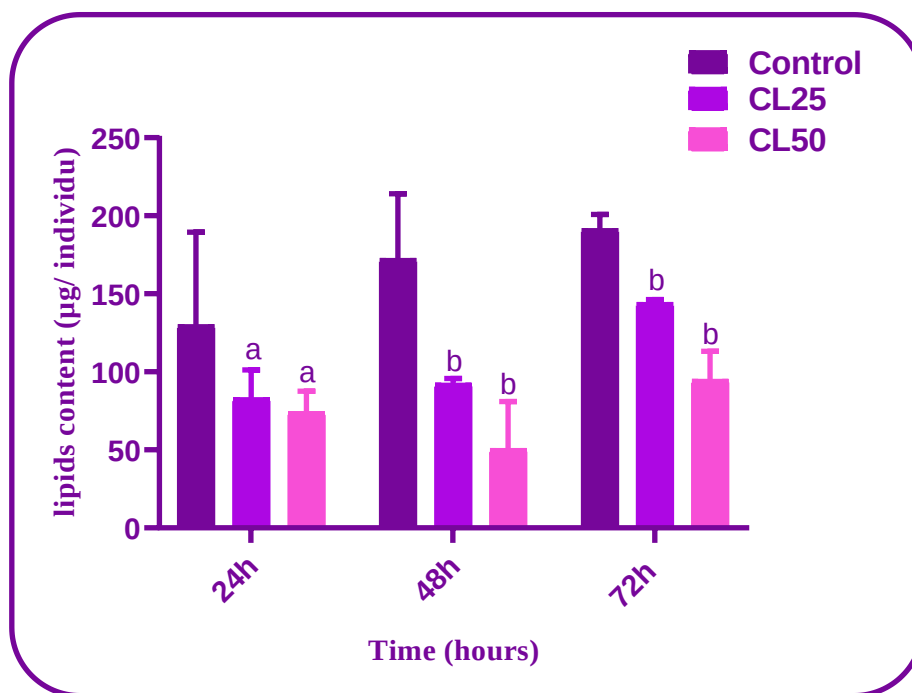


Figure 16: Effects of *Ziziphus lotus* hydro-ethanolic extract on the lipids content (µg/individu) against fourth instar larvae *Cs. longiareolata*: ($m \pm SD$, $n = 5$ pools each containing 10 individuals).

3.2 Discussion

3.2.1. Extract yield

The hydro - ethanolic extract of *Ziziphus lotus* leaves provided 24.17% , which indicates an efficient extraction process - this is consistent with recent findings carried out on the same plant parts from a variety of locations in Algeria . The yield obtained in the present study is close to the value of 21.8% reported by Boulekbache - Makhlouf *et al.* , (2020) for leaves obtained from Tissemsilt (located in north - western Algeria) via an ethanol extraction method . On the other hand , leaves obtained from the Sahara region of Béchar have produced a much higher yield of 35.6% (Gherib *et al.*, 2025), likely due to increased climatic stress stimulating the accumulation of extractable metabolites (Bencheikh *et al.*, 2023). An intermediate yield of 17.5% has been reported for the Djelfa region (Bouزيد *et al.*, 2018). Results from this study provide a yield value (24.17%) within ranges between (10 - 36%) recorded for *Z. lotus* leaves within Algeria ; therefore , geographical origins and extraction conditions have been assumed as contributing to the observed differences in yield .

3.2.3. Morphometric alterations

Results of the present study support the conclusion that the hydro - ethanolic extract of *Ziziphus lotus* has a marked inhibition of growth in *Culiseta longiareolata* fourth instar (L4) as indicated by the several metrics measured (i.e. body weight, thorax width, and body volume) . Body weight and thorax width values decreased significantly across three treatment periods (24, 48, and 72 hours) , while all measures exhibited statistically significant differences ($p < 0.0001$ for body volume ; $p = 0.01$ for body weight) between treated and control groups (Dunnett 's post - hoc comparisons) . All three metric values exhibited a pronounced increase in suppression over time , providing evidence of a cumulative physiological disruption as opposed to a temporary stress response. Such delayed but progressive effects are found in many instances when herbal - derived bioactive materials exert an influence on the disruption of endocrine regulation, nutrient uptake and / or cuticular growth / development in immature insect stages (Rattan 2010 ; George *et al.* 2014).

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The decrease in thoracic width and weight indicates that there has been some sort of failure in normal somatic development (most likely caused by disrupted molting and / or tissue differentiation) . Larval development and metamorphosis in mosquitoes are under strict hormonal control by the endocrine system , particularly by ecdysteroids and juvenile hormone. Plants produce a variety of secondary growth inhibitors , including saponins, flavonoids, tannins, and alkaloids ; many of these compounds have been described in *Ziziphus* species . These plant - derived compounds inhibit or interfere with the hormonal pathways that control the growth of mosquitoes and can cause stunted development and incomplete larval growth (Bencheikh *et al.*, 2023; Rattan, 2010).

Additionally , the continued reduction in body size and linear measurements suggests a systemic reduction in metabolism (i.e. , reduced metabolic efficiency) for this population cohort . Decreased feeding, poor digestion, and / or altered midgut enzyme activity are possible explanations for the observed phenotypic effects on this cohort . Several plant extracts have demonstrated antifeeding action or disrupt the integrity of the gut and therefore reduce the efficiency with which nutrients are absorbed and utilize those nutrients for growth (Elhag, 2009). The same mechanisms have been found in mosquitoes that were exposed to plant - derived extracts and were primarily affected by the reduction in biomass accumulation due to toxicity (George *et al.*, 2014).

Biochemical analyzes of the treatment revealed significant reductions in total proteins , glucosides (carbohydrates) and lipids of treated larvae (Shibko *et al.*, 1967; Bradford, 1976; Duchateau & Florkin, 1959). The reduction in protein levels suggests that larvae are either inhibiting protein synthesis or activating proteolytic pathways as a result of toxic stress, both of which lead to decreased body - weight and slow growth (Bencheikh *et al.*, 2023). The decrease in glucosides (carbohydrates) indicates that if larvae are depleting their energy stores to detoxify the xenobiotic compounds, fewer resources are available for growth. Similarly, the decrease in lipid provides evidence of either (1) using stored lipids to aid in membrane repair and detoxifying , or (2) the presence of direct damage to lipid-rich tissues (Elhag,2009; Dris and Bouabida, 2023). These biochemical modifications provide a metabolic rationale for the reductions seen in morphometric parameters .

Our findings closely match those of Dris and Bouabida (2023), who reported that *Mentha piperita* essential oil caused a significant decrease in both body length and head capsule width of *Culiseta longiareolata* . Other studies have also shown similar impacts of *Azadirachta indica* extracts on several mosquito species where there were reductions in weight and retardation of

Results and discussion

growth (George *et al.*, 2014). The strength of the statistical relationship between body volume ($p < 0.0001$) and weight ($p = 0.01$) may indicate that the extracts have a greater effect on the structural growth of the mosquitoes through cuticle deposition and sclerotization compared to the accumulation of biomass, or may simply reflect lower variability in the mean size of thoracic width. An important consideration from an applied perspective, these strong inhibitory effects of larval development will play a significant role in controlling mosquito populations because any disruption to larval development will decrease the probability that larvae will enter the pupal stage and emerge as adults; leading to a reduction in mosquito populations. Several botanicals, such as *Z. lotus* extracts, may provide an environmentally friendly alternative to synthetic insecticides, especially in areas where there is widespread resistance to conventional insecticides (Becker *et al.*, 2010; George *et al.*, 2014). The conclusion can be drawn from the evidence that *Ziziphus lotus* hydro-ethanolic extract contains important larvicidal and growth-regulatory activity that has a considerable effect on a number of different morphological (body weight, thorax width, body volume) and biochemical (protein, glucoside, lipid) characteristics for larvae of *Culiseta longiareolata*. Furthermore, these effects are dependent on the amount of time that has elapsed since the larvae were treated and also vary according to how much of the extract is used. Finally, the findings are supported by the literature on the use of botanical insecticides. Additional studies should focus on separating the active phytochemicals that account for these effects, and further investigating their exact method of action at both the biochemical and molecular levels.

3.2.4. Biochemical alterations

The current research investigated how sublethal concentrations (LC_{25} and LC_{50}) of *Ziziphus lotus* hydro-ethanolic extracts would affect the biochemical make-up of *Culiseta longiareolata* fourth instar larvae. The findings indicated significant declines in the total protein, carbohydrate and lipid levels in the treated larvae as compared to the control group, suggesting a major disturbance of their energy metabolism and the biosynthesis of these nutrients. In particular, the total protein level was compared to the initial average of both groups and showed a highly significant reduction of p -value = 0.0004 over the treated groups, particularly at 48 and 72 hours post-exposure. This could be the result of various mechanisms. For one, the bioactive compounds from the extract, such as flavonoids and polyphenolic compounds, may produce oxidative stress, resulting in a denaturation of proteins and

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subsequent degradation (Bencheikh *et al.*, 2023). The increased activity of proteolytic enzymes (e.g. caspases and proteasomes) , which occur as part of the stress response pathway of a cell , will also result in higher rates of protein " catabolism " (Cacciola *et al.* , 2022). The extract also interferes with the process of protein synthesis by decreasing availability of free amino acids or interfering with ribosome function (Rattan, 2010).

A lack of significant difference between exposure durations ($p = 0.4557$) indicates that protein depletion happens quickly after exposure and remains constant over time, therefore indicating an early and long term toxic effect on the animal . The carbohydrate levels showed a significant decrease in the treated groups ($p = 0.0012$) with a significant effect of exposure time ($p = 0.0154$) and the greatest decrease occurred at 48 and 72 hours . Carbohydrates as primarily glycogen and trehalose are primarily the major stored energy source of insect larvae and their depletion indicates that larvae exposed to the extract are utilizing their energy reserves to detoxify xenobiotic compounds (Dris and Bouabida, 2023). The depletion of these energy reserves will affect growth, development and metamorphosis. The time - dependent nature of the decrease in carbohydrate indicates that the demand for energy will continue to increase over time , as the larvae continues to metabolize and / or eliminate the toxic substances contained in the extract (George *et al.*, 2014).

There was a considerable decrease in the lipid levels in the treated groups ($p=0.0355$), while a very significant difference in duration ($p=0.0006$) was detected through 24 h , 48 h and 72 h . Lipids are the long - term energy reserve and the structural component of cell membranes. Lipid depletion indicates that stored lipids were being used as energy sources when carbohydrates were depleted , or that the fat body or other lipid - laden tissue had been directly damaged (Elhag, 2009). The saponin component of *Z. lotus* extracts may contribute to this damage by interacting with lipid membranes to increase permeability and induce cell lysis (Bencheikh *et al.*, 2023). Additionally , fatty acid loss is enhanced due to the possibility of unsaturated fatty acids oxidizing internally as a result of oxidative stress (Rattan, 2010). The simultaneous loss of all three classes of primary metabolites suggests a general disruption of metabolism rather than a targeted disruption to one pathway , as is the case with the mechanism of action of botanical insecticides that generally contain complex mixtures of active compounds that act on multiple physiological pathways simultaneously (George *et al.*, 2014). The biochemical changes observed offer a metabolic explanation for the changes in morphology seen previously including significant decreases in body weight ($p = 0.01$) and thoracic width ($p < 0.0001$). Larvae that use energy for detoxification instead of growth reduce protein synthesis

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, result in poor cuticle deposition, and deplete lipid reserves which leads to decreased growth rate of the larVa (Dris and Bouabida, 2023). These results support previous reports of the effects of botanical larvicides on mosquito larvae . Dris and Bouabida (2023) reported that larvae of *Culiseta longiareolata* when treated with essential oil of *Mentha piperita* had significantly lower reserves of protein , carbohydrate , and lipid combined with morphometric changes similar to those reported in the current study. In addition , other studies have reported that multiple plant extracts resulted in depletion of energy reserves in mosquito larvae resulting in inhibited growth and increased mortality rates (Elhag, 2009; George *et al.*, 2014). The consistent results across various plant species support that the disruption of primary metabolism is likely a common mechanism by which botanical larvicides function .

There are many limitations to this research . For example , only total protein carbohydrates and lipids were measured ; exact molecular species were not identified , the studies took place under controlled laboratory conditions which does not represent the large number of different types of sites where mosquitoes breed in nature , and finally there was no investigation into the long - term effects of sublethal exposures on adult emergence of mosquitoes , fecundity, or vector competence . Therefore it can be concluded that the extract from *Ziziphus lotus* has a considerable effect on the larvae of *Culiseta longiareolata* due to alterations in the biochemical composition (i.e. decrease in protein, sugar and fat stores) therefore providing proof of disruption in their systemic energy metabolism which will affect the morphometric arguments seen , thus supporting the larvicidal properties of *Z. lotus*. These data indicate that *Z. lotus* has potential for being an environmentally friendly method of controlling mosquitoes in Algeria (Bencheikh *et al.*, 2023).

Conclusion

4 . Conclusion

In this report , we present results from an investigation of the larvicidal properties of a hydro - ethanolic extract of *Ziziphus lotus* leaves against fourth - instar larvae from *Culiseta longiareolata*, based upon morphometric and biochemical parameters . The hydro - ethanolic extract yielded a total of 24 . 17 % . Larvae exposed to all sub - lethal concentrations of hydro - ethanolic extract significantly reduced ($P = 0.01$) larval weight and proportionately more thorax width ($P < 0.0001$) after 72 hours, showing that both the growth inhibitory effects were time - and dose - dependent . The differences in the total protein ($P = 0.0004$), carbohydrate ($P = 0.0012$) and lipid P ($P = 0.0355$) contents of treated larvae vs . non - treated larvae were also significant , demonstrating that there is a disruption in energy metabolism and biosynthetic pathways in the larvae as a result of treatment with the *Ziziphus lotus* hydro-ethanolic extract .

The findings demonstrate that the hydro - ethanolic extract from *Z. lotus* has multiple mechanisms for acting on mosquito larvae : inhibition of larval growth through reduced weight and thorax width ; depletion of protein , carbohydrate and lipid energy reserves . The bioactive secondary metabolites known to exist in *Z. lotus* (alkaloids, saponins, flavonoids and tannins) which have been identified previously , are likely responsible for this activity by causing neurotoxicity (through inhibition of acetylcholinesterase as a neurotransmitter) and metabolic stress (by inducing detoxification enzymes). There are limitations to the current study . Only fourth-instar larvae from one mosquito species (*Culiseta longiareolata*) were examined in a laboratory setting . The active compounds that led to the observed effects were not isolated or identified . The long-term consequences of the effects on emergence from pupae , fecundity and insect vector capacity remained unmeasured , but nevertheless , the results indicate that *Z. lotus* extract is larvicidal for mosquitoes .

Future studies should seek to isolate and identify active compounds responsible for the larvicidal effect (chromatographic techniques such as HPLC, GC - MS) and test the extract against multiple mosquito species and various larval stages while also evaluating the toxicity of the extract on non-target organisms (beneficial insects, aquatic animals). The creation of a stable formulation of the extract (emulsion or powder) for use in the field would allow for *Z. lotus* - based larvicides to be integrated into sustainable mosquito control programs. In conclusion, the hydro - ethanolic extract of *Ziziphus lotus* roots has demonstrated significant larvicidal activity against *Culiseta longiareolata* larvae through both morphologically and

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biochemically altering them . This plant can be used as a valuable, environmentally friendly alternative to synthetic insecticides for integrated vector management schemes within Algeria.

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