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Evaluation of Lousicidal Activity of *Ziziphus mauritiana* Alkaloids and *Eucaylptus camaldulensis* Terpenoids Leaves Extracts in Chickens: An *In Vitro* Study

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Abstract

The present study aimed to highlight and assess the *in vitro* poultry anti-lice properties of *Ziziphus mauritiana* alkaloids and *Eucalyptus camaldulensis* terpenoids leaves extracts. *In vitro* poultry antilouse effects of the extracts of various concentrations (1 mg/mL, 5 mg/mL, and 10 mg/mL) using filter paper contact bioassay was applied. Results revealed that lice mortality was concentration-dependent for both studied extracts. The median lethal concentration (LC50) values were 3.687 and 1.045 for alkaloids and terpenoids extracts respectively. The results indicate that terpenoids extract has strong lousicidal activity. Further steps should be pursued to make these extracts to be tested *in vivo* then may be used as a novel treatment for poultry lice in future, allowing lice control in a less aggressive way to the environment.

Keyword: *Ziziphus mauritiana, Eucalyptus camaldulensis*, anti-lice extract, poultry lice, alkaloids, terpenoids.

Introduction

Poultry lice (order: Phthiraptera) are one of the important poultry ectoparasites. They are small, mostly 3 mm long, wingless straw-coloured insects (Figure 1.A), having chewing mouthparts and feed on dry skin scales, feather parts and blood. Poultry lice generally parasitize on a single bird and complete their life cycle there, and die within a few days to a week if out of a host [1,2]. Poultry lice can't survive on humans or on non-avian domestic pets. The poultry lice infestation tends to be greatest during autumn and winter. Lice eggs bundle up to the size of a marble and attach to feather base [1].

Phthiraptera includes over 5000 species globally, chewing lice (Bird lice) represents 88% of them [3]. Poultry can be infested by more than 40 species of chewing lice [4], causing skin irritation, weight loss, restlessness, general weakness, and probability of secondary infections [5,6] or serve as reservoir of some pathogens just like rickettsia [7]. There are six species of lice infested the chickens in north of Iraq: body lice (*Menacanthus stramineus*), feather shaft lice (*Menopon gallinae*), chicken head lice (*Cuclotogaster heterographus*), fluff lice (*Goniocotes galline*), large chicken lice (*Goniodes gigas*) and wing lice (*Lipeurus caponis*) [8]. The most pathogenic are hematophagous species *Menacanthus stramineus* and *Menacanthus cornutus*; which cause anemia, heavy skin lesions or even death [9] leading to an economic loss.

Now-a-days resistance to available synthetic drugs is a major problem. Therefore, phytotherapy becomes excessively used as insecticides and pesticides due to they are easily extractable, ecofriendly, biodegradable, and having low or no toxicity against mammals and birds as the same time they are effective against broad spectrum of insects and pests [10,11]. There are several studies showed the insecticidal activity of *Eucalyptus* essentials oils [12,13,14]. The pesticide properties of these plants are due to presence of various complex chemical substances of different composition which occur as secondary metabolites [15]. Secondary metabolites and essential oils have various degree of lipophilicity, relative hydrophilicity, and volatility at room temperature [16]. In the current study *Ziziphus mauritiana* and *Eucalyptus camaldulensis* were selected because they are commonly available in Iraq.

Ziziphus mauritiana belongs to the family Rhamnaceae [17]. The name *Ziziphus* is related to an Arabic word and ancient Greeks used the word ziziphon for the jujube [18]. About 40 species of *Ziziphus* are available, *Ziziphus mauritiana* is very common [19].

Also *Eucalyptus* (family Myrtaceae), represented by over 700 species distributed throughout the world. In fact, *Eucalyptus* oil has been known for hundreds of years as antibacterial, antifungal and antiseptic in nature and it ranks top in quality and has advantages over essential oil from other tree crops [20]. The composition of *Eucalyptus* essential oils is very dissimilar, so their properties are extremely diverse even conflicting [21].

As known, synthesis of secondary metabolites in plants is determined by certain genetic characteristics (genotype) of the plant, physiological conditions, as well as edaphoclimatic factors. Thus, the chemical composition of plant extracts varies according to the plant origin [22,23], as well as cultivation of plant materials for extraction may imply fluctuation of the results [24].

Relatively, few studies were done on poultry lice available in Middle East [25]. To the best of our knowledge the poultry anti-lice activity of *Ziziphus mauritiana* alkaloids and *Eucalyptus* terpenoids of leaves extracts has not been investigated yet. Therefore, this study aimed to evaluate the possible *in vitro* poultry anti-lice properties of these secondary metabolites.

Materials & Methods

Collection of lice. Poultry lice were collected from naturally infested chickens taken from Najaf bird market. Identification of the lice was done by using insect identification keys [26,27,28]. Lice were collected from the predilection sites of the animals by blunt pointed forceps to avoid any harm to lice and host. Adult lice were examined for activity and morphological integrity under the dissecting microscope, the damaged ones were discarded. Institutional ethical and animal care guidelines were applied during sampling.

Collection of plant samples. *Ziziphus mauritiana* (Rhamnaceae) and *Eucalyptus camaldulensis* (Myrtaceae) fresh leaves were collected from Najaf governorate/ Iraq in October 2016. The samples were positively identified in the herbarium of college of Education for Girls. In the lab, leaves samples were properly washed with distilled water, then were shed dried at room temperature for several days. The plant samples were ground using blender to obtain leaves powder then stored in refrigerator at 4° C till used.

Phytochemical Screening

Alkaloids test. Alkaloids were identified by using Dragendroff's reagent. (0.5) mL of *Ziziphus* aqueous extract in a test tube, equal volume of the reagent were added; appearance of orange precipitate was taken as positive result for presence of alkaloids [29].

Terpenoids test. Two mL of the extract of *Eucalyptus* were mixed with 2 mL of chloroform and 3 mL of concentrated sulfuric acid then heated for about 2 minutes. Grayish colour indicates to the presence of terpenoids [29].

Extraction

Extraction of crude alkaloids. Ten grams of *Ziziphus* powder were extracted in soxhlet extractor with 200 mL of 95% ethanol for 24 hours then extract was dried by rotary evaporator. Dried material then was dissolved in 5 mL of ethanol, then 30 mL of 20% sulfuric acid were added to the alcoholic extract, rotary evaporator was used again, then little amount of 10% ammonium hydroxide were added to the residual acid solution till pH was equal to 9. Then this solution was extracted by separating funnel further four times with 10 mL of chloroform. The yield of chloroformic extract (inferior layer) was added to 10 grams of anhydrous sodium sulfate for demoisturizing. Finally, extract was filtered then re-evaporated using rotary evaporator again. Alkaloids were collected and kept at -20° C till used [30]. The extraction was repeated to obtain enough alkaloids.

Extraction of crude terpenoids. Twenty grams of dry weight of *Eucalyptus* were added to soxhlet extractor with 200 mL of chloroform as a solvent, for 24 hours at 40° C. Chloroform was evaporated by rotary evaporator, the extract was collected and kept at -20° C till used [31]. The extraction was repeated to obtain enough terpenoids.

Preparation of test concentrations. Three concentrations for alkaloids and terpenoids were prepared separately. One gram of each extract was dissolved in 10 mL of dimethyl sulfoxide

(DMSO), then the volume was completed to 100 mL by adding distilled water and 0.2 mL of tween-20 to prepare 10mg/mL stock solution. Two diluent concentrations (5, 1) mg/mL were prepared according to the equation (N1V1=N2V2). Control treatment was prepared from 10 mL of DMSO and 90 mL of distilled water and 0.2 mL of tween-20.

***In vitro* filter paper bioassay of lousicidal activity**. Lice were divided into groups and were distributed in Petri dishes (6 cm in diameter) lined with Whatman no. 1 filter paper. Three mL of each concentration of extracts were pipetted onto individual filter papers. Filter paper contact bioassay method was used to evaluate the toxicity of different concentrations of present extracts [32]. The experiment was carried out in triplicate, six lice/ replicate (total n=18 for each concentration of the extract). Lice were incubated at 25° C in darkness. Adult mortality were noticed every 5 minutes for 120 minutes by dissecting microscope. The lice were regularly stimulated with a needle to determine their viability, lice showing no movement and complete absence of any vital signs were considered dead [33].

Statistical analysis. All experiments were performed in triplicate. Significant differences among the concentrations and the lethal time for each concentration were analyzed by least significance difference (LSD). Significant differences between the extracts were analyzed by T-test. Median lethal concentration (LC50) were calculated by Probit regression analysis. The results were represented as mean± standard deviation (SD). *p*-value < 0.05 was considered significant.

Results & Discussion

Biological control means destruction or elimination of pest or unwanted populations by natural means; phytotherapy represents one of them. The present study focuses on lousicidal effect of alkaloids of *Ziziphus*, and terpenoids of *Eucalyptus* extracts. To our knowledge the lousicidal effects of these extracts has not been previously reported on poultry.

The chicken louse *Menacanthus stramineus* under the dissecting microscope (40x) shown in Figure (1.A). Large amount of lice on the feathers of infested chicken during sampling (Fig.1.B). Filter paper contact bioassay to assess the toxicity of extracts (Fig.1.C). Finally, the two dried extracts, alkaloids of *Ziziphus*, and terpenoids of *Eucalyptus* (Fig.1.D).

The current study appeared that *Eucalyptus* terpenoids extracts caused higher lice mortality than *Ziziphus* alkaloids extracts for all concentrations as shown in Table (1). The statistical analysis observed that the concentration 10 mg/mL was significantly different for both extracts ($p< 0.05$). The higher extract concentration, the better mean mortality of lice. Also, terpenoids revealed significant differences at the concentrations (1 and 5) mg/mL as shown in Table (1).

Terpenoids extract of *Eucalyptus* revealed higher mortality rates in all concentrations. This result was in agreement with the data noticed by [34] that proved pediculocidal activity of *Eucalyptus* essential oils. Terpenes of *Eucalyptus* showed to be effective against head lice [35]. Monoterpenes (1,8-Cineole and α-Pinene) were the most effective essential oils of *Eucalyptus globulus* against head lice [36]. *Eucalyptus spp* have been shown to release large amounts of terpenes into the atmosphere [37], some of these terpenes are insects repelling, for example, 1,8-cineole, α-terpineol and bicyclogermacrene [38]. Limonene demonstrated insecticidal activity in a mechanical way by penetrating the cuticle of the insect, by respiration, and through the digestive system [39]. Their low molecular weight can pass through the cuticle of louse up to the trachea causing the death by suffocation [16].

Other *Eucalyptus* terpenoids were Vinidiflorol and α-Penine, which have certain characteristics to be used as insecticides [40], these compounds prevent transmitting of nervous impulses in adults beetles *Callosobruchus maculatus* by inhibition of acetyl choline hydrolysis [41]. As suggested by [42], the mode of delivery of the essential oils and compounds was likely by vapor mode via respiratory system or spiracles blocking. Other study showed that fumigation tests of some monoterpenoids against female lice were more effective in closed cups than in open ones, indicating to the vapor phase action [36] .The

cytotoxic activity of terpenoids is due to the containment of phenols, aldehydes and alcohols in their structure [43]. In many cases, terpenoids have been shown to act synergistically to get the overall pesticidal effect [44].

The lipophilicity of the products and the rate of diffusion through the cuticle are important factors which could affect the contact toxicity results [45,46]. The density and the molecular structure also affect the penetration rate. Essential oils with higher densities are generally more toxic than those with low densities [47]. Also, degradation of the essential oils component, movement of the compound to target site, and the ability of the insect to excrete the compound [48]; all these factors may cause the insecticidal effect.

Alkaloids display antiparasitic and antimicrobial properties. Recent research has proven that they are not toxic to the organisms that produce them, but toxic toward foreign organisms or cells and it is selective. Alkaloids can alter DNA, selectively deform cells [49]. More than 100 cyclopeptide alkaloids have been isolated from various plants of the genus *ziziphus* [50]. The cyclopeptide alkaloids of *Ziziphus* showed antibacterial and antifungal activity [51]. Phyto-chemically, six alkaloid compounds were demonstrated in *Ziziphus mauritiana*. The highest concentration was Iusiphine followed by Coclaurine [52]. These two compounds may be mainly responsible for the lice mortality in the present study.

Lethal time for the each concentration of the applied extracts significantly differed. In control group, the lice still alive even after the 120 min treatment period. The highest concentration of both extracts (10 mg/mL) demonstrated strong lice toxicity, and its activity tended to be time-dependent (Table 2). With regard to the concentration-response data, the median lethal concentration values (LC50) were 3.687 and 1.045 of alkaloid and terpenoid extracts respectively (Table 3). This observation indicates that terpenoid extract is more effective than alkaloid extract against poultry lice.

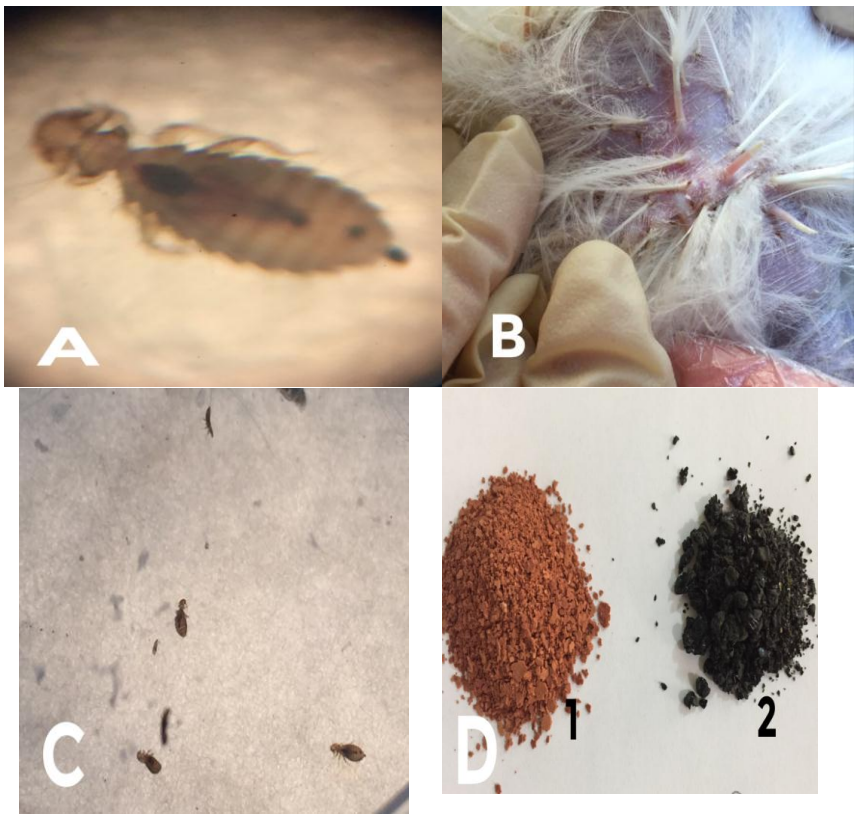

Figure 1. *Menacanthus stramineus* 40x (A). Lice infestation in a chicken (B). Filter paper contact bioassay(C). Dried crude extracts:1-alkaloids of *Ziziphus* 2-terpenoids of *Eucalyptus*.

Table 1. Poultry lousicidal activity of *Ziziphus* alkaloids and *Eucalyptus* terpenoids extracts *in vitro*

Concentration mg/mL	Alkaloids	Terpenoids	T-test
	Mortality, mean± SD		
Control	0.0±0.0	0.0±0.0	
1	1.67±1.25	3.0±0.82	1.98*
5	3.0±0.0	4.67±0.94	2.2*
10	4.33±0.47	5.33±1.89	1.1
LSD	1.6*	2.8*	

*Indicates a significant difference

Table 2. Lethal time of poultry lice treated with *Ziziphus* alkaloids and *Eucalyptus* terpenoids extracts *in vitro*

Concentration mg/mL	Alkaloids				Terpenoids			
	Lethal time, min							
	15	30	60	120	15	30	60	120
Control	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0	0±0.0
1	0±0.0	0.67±0.47	0.67±0.94	0.33±0.47	0.67±0.47	1.33±0.94	0.67±0.47	0.33±0.47
5	0.33 0.47 ±	1.0±0.0	0.67 ±0.47	1.0 ±0.0	0.67 ± 0.94	1.33 ± 1.25	1.67± 1.25	1.0± 0.82
10	0.67±0.47	1.0±0.82	1.33±0.94	1.33±0.47	0.67 ± 0.47	1.0± 0.0	1.67± 1.70	2.0± 0.0
LSD	0.29*	0.33*	0.46*	0.7*	0.25*	0.33*	0.8*	1.33*

Numbers represent mean± SD. * Indicates a significant difference.

Table 3. Probit analysis of the toxicity (LC50) of the *Ziziphus* alkaloids and *Eucalyptus* terpenoids extracts *in vitro*.

Extract	LC50 [a]	Regression equation	Slope †	Pearson-χ^2
Alkaloids	3.687	y=0.8482x- 3.6745	1.119	1.6*
Terpenoids	1.045	y=0.8292x- 4.128	1.198	1.9*

[a] Median lethal concentration. † Slope of the regression lines. * Indicates a significant difference.

Conclusions and Recommendations

Eucalyptus camaldulensis terpenoids and *Ziziphus mauritiana* alkaloids showed potential lousicidal activity. *Eucalyptus camaldulensis* terpenoids was more effective

lousicide, since its LC50 was less. The mortality of lice was observed within relatively short period of time; these data suggested them to be promising bio-control candidates against poultry lice. Further *in vivo* studies are needed to confirm these findings.

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