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Research Article

Changes in Physicochemical and Biological Properties at the Developmental Stages (egg, larvae and pupa) of Lucilia sericata (Meigen, 1826)

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ABSTRACT

Qualitative dynamics and compositional changes during developmental stages in different insect species form the basis of techniques used in forensic and medical studies. Their discovery has a great importance both in terms of elucidating the developmental processes of Lucilia sericata and revealing its different potentials. In this study, the chemical composition of extracts obtained from Lucilia sericata eggs, larvae and pupae were analysed by Fourier Transform Infrared Spectroscopy (FTIR) and DPPH (2,2-diphenyl-1-picryl hydrazyl) antioxidant analysis and protein denaturation tests were performed to determine their biotherapeutic properties. Structural morphology was examined by Scanning Electron Microscopy (SEM) and as a result of all analyses, the potential of the extracts obtained from pupae and eggs in different medical and cosmetic fields other than larval use was revealed.

Keywords: *Lucilia sericata*, larva, FTIR, antioxidant, antiinflammatory.

1. INTRODUCTION

Lucilia sericata larvae, also called 'WuGuChong' in traditional Chinese medicine[1], are currently used as an alternative approach when surgery is not beneficial in the treatment of necrotic wounds, including diabetic ulcers, postoperative wounds and burns, and in the treatment of many chronic diseases.[2] Anthropological evidence suggests that widely dispersed cultures such as the Mayans of Central America, Australian aborigines and the hill people of Myanmar (formerly Northern Burma) all used maggots in such wound treatments.[3] Over time, the emergence of explosive devices in warfare significantly complicated the management of battlefield injuries using traditional surgical techniques. During the Napoleonic Wars, the American Civil War and the First World War, military doctors observed that wounds accidentally infested with maggots healed rapidly with minimal scarring.[3,4] This led to the use of larvae hatched from sterilised eggs to debride the wound in order to remove necrotic tissue in the treatment of chronic wounds, bedsores, severe burns and bone infections, a procedure referred to as larval therapy. Larval therapy was used as a common procedure in medicine until the general use of antibiotics began in the 1940s.[5] After a hiatus of about 50 years, the clinical use of surgical maggots has been reintroduced in the treatment of such diseases when antibiotics are ineffective and surgery is inadequate or refused by the patient. It is believed to aid wound debridement and healing through multiple mechanisms, including the liquefaction of necrotic tissues by proteases, the consumption of necrotic tissue by larvae as a food source, the alteration of wound pH from acidic to beneficial alkaline levels via ammonia secretion, and the removal of bacteria from the wound[6] through serous exudate induced by the larvae's irritating effect. Research to fully understand these processes is still ongoing. Understanding how the in vitro biological activities of *L. sericata* change depending on developmental stages serves as a preliminary step in understanding how enzyme activities and antimicrobial

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properties change, which is very important and necessary to optimise treatment processes.[7] Not only in terms of therapeutic activity, but also physicochemical analyses of the developmental stages are of great importance in forensic entomology. The examination of physicochemical changes during the transition stages from egg to larva and pupa contributes to the processes of understanding which stage the fly population on the corpse is in and optimising them.[8] The larvae of *L. sericata* at different stages, which are mostly used in research studies, overshadow the research potential of products that can be obtained from other developmental stages of the species. The needs and biological functions of the fly are different at each developmental stage. Therefore, the chemical compounds produced or secreted in this process also vary, which directly affects the magnitude of different biological effects such as antioxidant, anti-inflammatory and antibacterial.[9] For example, enzymes and proteins necessary for embryonic development are secreted at the egg stage. Extraction at the egg stage may contain promising bioactive components, especially for the prevention of protein denaturations. Since the larval stage is the most active feeding process, the bioactive components to be obtained here will have high proteolytic content and antioxidant activity is likely to be high.[10] On the other hand, the content and possible biological functions of the extract to be obtained from the pupal stage are a mystery for the literature. The most important reason for preferring the extracts rather than using the organism itself for different purposes is the aim to benefit from high bioresistance and maximum effect.[11] This study aimed to carry out physicochemical and biological characterisations of the extracts obtained from different stages of *L. sericata* species in order to contribute to the discovery of therapeutic potentials.

2. EXPERIMENTAL

2.1. Materials

Phosphate buffered saline (PBS) (pH 7.4) were purchased from Sigma Aldrich (St. Louis, MO, USA). Ethanol (CAS no. 920.026.2500) was purchased from ISOLAB. All the chemicals and solvents used in the study were of analytical grade.

Lucilia sericata Rearing and Sample Collection

In AYBÜ GETAT insectarium, which has a temperature of 24-27°C and 40-60% humidity and a photoperiod cycle of 12 hours day and 12 hours night with a timer system, adult flies are housed in 40x40x40 cm wire cages surrounded by tulle.

Lucilia sericata specimens (egg, larvae and pupa) were collected randomly and right after, the samples were taken to the research laboratory in 70% ethanol and distilled water (1:1, v: v) solution for sterilization.[12] The pictures of egg, larvae, pupa and fly specimens were taken by a stereomicroscope (Figure 1).

Figure 1. Stereomicroscope images of the developmental stages of *L. sericata* (Left to right: Adult, pupa, larva, egg).

2.2. Methods

2.2.1. Extraction of the Samples

The samples were extracted using the same method to minimise experimental error and to provide a holistic approach.[13] Eggs, larvae and pupae were crushed with a mortar and pestle, weighed 1 g, added 100 ml PBS (pH 7.4) and left in a 37°C incubator for 1 hour. Afterwards, the extract samples were allowed to cool down then filtered with filter paper and stored at +4°C.

2.2.2. DPPH (2,2-diphenyl-1-picryl hydrazyl) Antioxidant Activity

The antioxidant capacities of the samples were determined according to the El-Garawani et al. Method.[14] This method is based on measuring the scavenging effects of antioxidants on stable and synthetic DPPH (2,2-diphenyl-1-picryl hydrazyl) radical. Approximately 1 mL of samples of varying concentrations were mixed with 1 mL (0.2 mM) of methanolic DPPH solution and incubated in the dark for 30 min at room temperature. The absorbance of the samples was measured at 517 nm with a spectrophotometer (Shimadzu UV-1800 spectrophotometer, Japan) at the end of the incubation period. Ascorbic acid was used as standard and methanol was taken as blank. Values were calculated as % inhibition and analyses were performed in 3 replicates.

2.2.3. Anti-inflammatory Activity – Protein Denaturation Assay

Temperature-induced protein denaturation was performed according to a modified method by Zhang et al. [15] In summary, 5 µL aliquots of the samples at various concentrations were added to test tubes containing 500 µL of a 0.4% (w/v) collagen buffer solution (pH 6.4, prepared with acetic acid). Ibuprofen

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was used as a positive control. The reaction mixtures were heated up to 70°C for 30 min and then cooled at room temperature for 20 min. The turbidity of the solutions (protein denaturation level) was measured at 275 nm using a spectrophotometer. The percentage of precipitation inhibition (protein denaturation) was calculated relative to the negative control using the following formula,

$$\textbf{\% Protein Denaturation Inhibition} = \left[\frac{Ac - As}{Ac}\right] \times 100 \quad (1)$$

where Ac and As are the absorbance of the control and the sample, respectively.

2.2.4. Fourier Transform Infrared Spectroscopy (FTIR) Analysis

FTIR analysis was conducted to determine the chemical functional groups of the bioactive compounds present in the extract samples. The extracts were placed on a plate in the FTIR spectroscope and analyzed across a wavenumber range of 550 to 4,000 cm^{-1} with a resolution of 4 cm^{-1}. The FTIR spectra were recorded in triplicate, and the functional groups were identified based on data from the literature.

2.2.5. Scanning Electron Microscopy (SEM) Analysis

Images for SEM analysis of larvae, pupae, and eggs that underwent heat treatment were collected. Initially, the samples were secured onto SEM stubs using double-sided tape and then coated with gold for 2 minutes using a LEICA ACE-200 sputter coater. Subsequently, the samples were analyzed using a HITACHI SU5000 FE-SEM, and digital images were recorded in the electron microscopic imaging laboratory of MERLAB at Ankara Yildirim Beyazit University.

2.2.6. Statistical Analysis

The results are presented as mean ± SD of replicates. Data were analyzed using one-way ANOVA, and differences between means were evaluated with the Tukey–Kramer test ($p \leq 0.05$) utilizing the GraphPad Prism 10.0 software for Windows.

3. RESULTS and DISCUSSION

3 .1. Antioxidant Activity Results

DPPH radical inhibition test is a widely used method to evaluate antioxidant activity and provides easy comparability. The scavenging effects of extracts obtained from different developmental stages of *L. sericata* larvae were measured and the results are presented in Figure 2. The scavenging activity of all the extracts showed a linear increase with the concentration of the added extracts. The scavenging effect of the extracts on DPPH radical was as follows, larva>pupa>egg and EC_{50} values were found as follows, 0.473± 3.07, 1.017± 2.5 and 1.732± 5.307 µg/ml, respectively. On the other hand, the EC_{50} value for ascorbic acid as a positive control was 0.9 µg/ml. Larval extracts showed a remarkable effect compared to ascorbic acid at the same concentration.

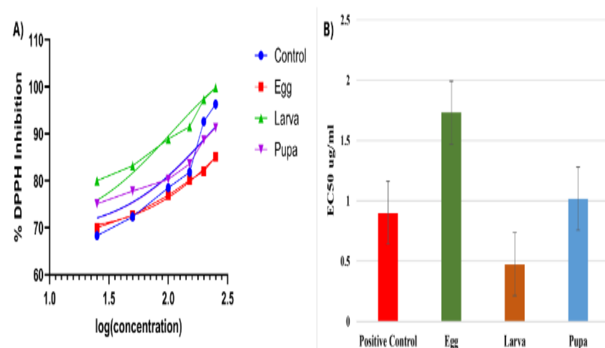

Figure 2. Antioxidant activity of various extracts from *L. sericata*. Ascorbic acid was used as positive control. Values are mean ± standard deviation (n = 3) and $p \leq 0.05$.

3.2. Anti-inflammatory Activity Results

The results obtained after heat-induced collagen denaturation assay presented in Figure 3. The highest activity was 70.2% at the highest concentration in the larval extract compared to the control group. The ranking of protein denaturation inhibitory activity was determined as larva>pupa>egg and the results were in parallel with high antioxidant activity. According to EC_{50} data, the value found as 1.805± 3,57 µg/ml for positive control was determined as 2.302± 0,8, 4.462± 1,7 and 5.926± 3,06 µg/ml for larva, pupa and egg, respectively.

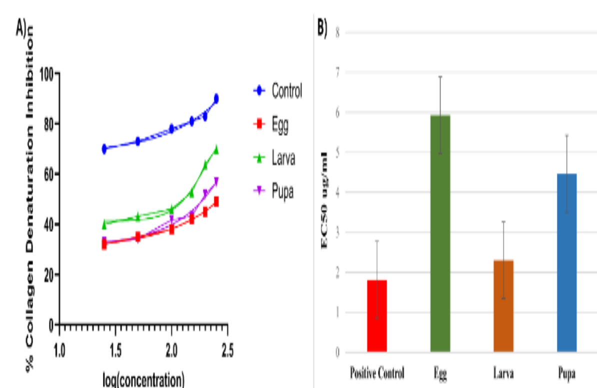

Figure 3. Protein denaturation inhibition activity of various extracts from *L. sericata*. Ibuprofen was used as positive control. Values are mean ± standard deviation (n = 3) and $p \leq 0.05$.

Research studies on *L. sericata* have mostly focused on the larvae of the species, which are also used in LDT, and include analyses of secrations. In their study, Borkataki et al. determined that oxidative stress in the

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wound area of *L. sericata* larvae decreased in direct proportion to the decrease in lipid peroxidation.[16] In another study, the IC_{50} value was determined as 75.28 mg/ml as a result of DPPH antioxidant activity analysis performed on extracts of *L. sericata* larvae obtained by a similar method to our study.[17] This numerical difference is thought to be caused by the usage of methanol in the preparation of the extracts. On the other hand, there is no research study in which species-specific extracts of eggs and pupae were obtained and their antioxidant activities were evaluated. However, there are entomological research studies in which different biological activities of eggs and pupae of different insects other than *Lucilia* species were examined.[18] Anti-inflammatory studies on the larvae of *L. sericata* species are not based on its secretion, but mostly on in vivo wound analyses. In a study about transcriptome analysis of wound-associated keratinocytes, endothelial cells, fibroblasts and monocytes, it was determined that larval secretions had a negative effect on proinflammatory cytokines such as TNF-α and IL-12 production, while increasing the expression levels of the anti-inflammatory cytokine IL-10, further supporting immunomodulatory activity.[19] A comparable anti-inflammatory effect was noted in a study where larval secretion products successfully decreased lipopolysaccharide-induced IL-8 release by fibroblasts.[20] The reduction of pro-inflammatory cytokine levels by larval extract was also determined by in vivo analyses performed on acutely traumatised rats.[21]

3.3. FTIR Results

FTIR analyses were studied to identify the chemical composition of the extracts and changes according to different stages of the *L. sericata*. FTIR spectra of different extracts are shown in Figure 4.

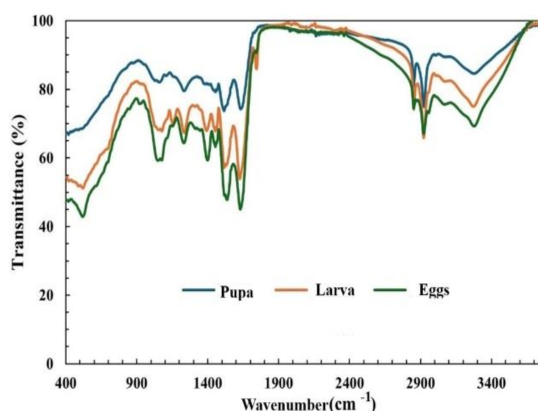

Figure 4. FTIR spectrums of egg, larva and pupa extracts.

The spectra obtained show that peaks were obtained at similar points for all 3 samples, but their intensities varied in different extracts. Peaks obtained between 500-1000 cm^{-1} are generally fingerprint peaks and are not used to obtain specific information about the chemical structure. In the 1400-1450 cm^{-1} range, deformation (bending) vibrations of methyl (CH_3) and methylene (CH_2) groups were observed. In the 1200-1300 cm^{-1} range, intense peaks due to stretching vibrations of the C-O bond in esters, alcohols, ethers and carboxylic acid derivatives were determined. In the 1500-1550 cm^{-1} range, amide II vibrations in proteins are generally observed. These peaks represent a combination of N-H bending vibrations and C-N stretching vibrations and point to structural proteins. Between 1800-2500 cm^{-1} the peaks of larvae and eggs peaked, giving peaks that were slightly more intense than those of the pupal extract. Peaks in this range usually indicate vibrations of triple bonds, especially C≡C (alkynes) and C≡N (nitriles). Asymmetric stretching vibrations of carbonyl groups (C=O) also produce peaks close to 1800 cm^{-1}. This region usually contains characteristic vibrations of triple bonds and some special functional groups, which explains the increased intensity of the peaks in the larva as the most developed organism of *L. sericata* compared to the pupa and egg forms. The most important factor that causes all these antioxidant and anti-inflammatory effects is undoubtedly the chemical profile rich in bioactive components, and according to the FTIR analysis results, it is understood that the amount and type of chemical fingerprint components it contains vary depending on the developmental stage of *L. sericata*. The high pupal extract composition obtained from the results can be attributed to the fact that the organism completes its entire development in the pupal stage and now transitions to the adult fly stage.[22] Significant structural differences were observed between egg, larva and pupa samples in the results obtained from FTIR spectra. A broad peak in the 3300-3400 cm^{-1} range is evident in all three samples, and this region is associated with O-H or N-H stretching vibrations, indicating the presence of proteins or water contents.[23] Peaks in the 2800-3000 cm^{-1} region are C-H stretching vibrations, mainly due to fatty acids, and in this region the spectra of eggs, larvae and pupae were observed close to each other.[24] Peaks in the 1500-1700 cm^{-1} range are associated with Amide I and Amide II bands in protein structures, while the 1000-1300 cm^{-1} region can be associated with carbohydrates and lipids.[25] These FTIR spectra confirm that there are changes in the amount of protein, lipid and carbohydrate components during the developmental stages.

3.4. SEM Results

The surface morphology of the samples obtained directly from *L. sericata* eggs, larvae and pupae were examined by SEM (Figure 5). The morphological structures of the organism in developmental stages are different from each other and there is a morphological adaptation according to the life cycle. As seen in Figure 5 A and B, there are mouth hooks and gas exchange cavities in the anterior and posterior parts of the larva, while in the egg structure of Figure 5C, there are

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stingray-like structures between the outer membrane and the developing embryo. Finally, in Figure 5D, the pointed parts and arrow on the outer part of the pupa indicate a chitin-chitosan structure and a hardened structure on the outer part.

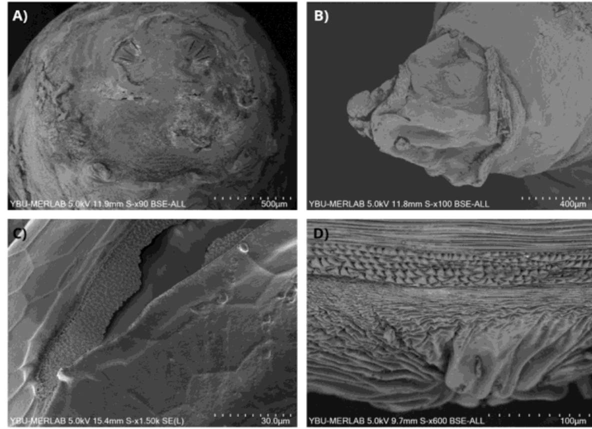

Figure 5. A) Posterior of larvae, B) Anterior of larvae, C) Egg structure and D) Outer morphology of the pupa sample.

4.CONCLUSION

There is growing interest in exploring the roles of different types of bioactive components of insects, especially those with antioxidant, antiinflammatory and antimicrobial properties. In recent years, organic extracts from various sources have been widely used in traditional medicine and pharmaceutical industry and are generally considered safe. Among natural sources, insects are popular research subjects of recent years due to their bioactive products effective as a new class of antioxidants with potential clinical value for humans. Among them, especially the larvae of different species of flies belonging to the Calliphoridae (including *Lucilia* species) have been used throughout human history for medical, food, cosmetic and various other purposes. It is of great importance to reveal the biological and physicochemical properties of the organic components of the traditionally used *L. sericata* species at different developmental stages in order to develop their potential for use and to optimise the processes. The results highlight that extracts from *L. sericata* larvea, egg and pupae exhibit anti-inflammatory properties, aiding in wound healing. It is of great importance to emphasise the potential of each developmental stage of *L. sericata* species and to investigate it as a biotherapeutic raw material in cosmetic and pharmaceutical industry studies. More in vitro and in vivo studies are needed to investigate the different extracts of the species in a wider range.

Conflict of Interest

There is no conflict of interest with any person, institution, company, etc.

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