EUROPEAN JOURNAL OF BIOLOGY

Owner on behalf of the Istanbul University Faculty of Science: Yesim Oktem – Publication Type: Biannually – Printed at: İlbey Matbaa Kağıt Reklam Org. Müc. San. Tic. Ltd. Şti. 2.Matbaacılar Sitesi 3NB 3 Topkapı/Zeytinburnu, İstanbul, Turkey www.ilbeymatbaa.com.tr, Sertifika No: 17845 – Printing Date: June 2019

Publishing Company
Istanbul University Press
İstanbul Üniversitesi Merkez Kampüsü,
34452 Beyazıt, Fatih / Istanbul - Turkey
Phone: +90 (212) 440 00 00

İSTANBUL UNIVERSITY PRESS

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The scope of the journal includes but not limited to; botany, zoology, hydrobiology, animal and plant systematics, ecology, environmental biology, microbiology, radiobiology, molecular biology, biochemistry, genetics, biotechnology, physiology, toxicology, cell biology, cancer biology, neurobiology, developmental biology, stem cell biology, regenerative and reparative biology, nanobiotechnology, system biology, tissue engineering, biomaterials, and omic sciences.

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Instructions to Authors

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- Grant information and detailed information on the other sources of support,
- Name, address, telephone (including the mobile phone number) and fax numbers, and email address of the corresponding author,
- Acknowledgment of the individuals who contributed to the preparation of the manuscript but who do not fulfil the authorship criteria.

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Statistical analysis to support conclusions is usually necessary. Statistical analyses must be conducted in accordance with international statistical reporting standards. Information on statistical analyses should be provided with a separate subheading under the Materials and Methods section and the statistical software that was used during the process must be specified.

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Tables should be included in the main document, presented after the reference list, and they should be numbered consecutively in the order they are referred to within the main text. A descriptive title must be placed above the tables. Abbreviations used in the tables should be defined below the tables by footnotes (even if they are defined within the main text). Tables should be created using the "insert table" command of the word processing software and they should be arranged clearly to provide easy reading. Data presented in the tables should not be a repetition of the data presented within the main text but should be supporting the main text.

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Review Article	5500	250	No limit	5	6
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Images should not be labeled (a, b, c, etc.) to indicate figure subunits. Thick and thin arrows, arrowheads, stars, asterisks, and similar marks can be used on the images to support figure legends. Like the rest of the submission, the figures too should be blind. Any information within the images that may indicate an individual or institution should be blinded. The minimum resolution of each submitted figure should be 300 DPI. To prevent delays in the evaluation process, all submitted figures should be clear in resolution and large in size (minimum dimensions: 100 × 100 mm). Figure legends should be listed at the end of the main document.

All acronyms and abbreviations used in the manuscript should be defined at first use, both in the abstract and in the main text. The abbreviation should be provided in parentheses following the definition.

When a drug, chemical, product, hardware, or software program is mentioned within the main text, product information, including the name of the product, the producer of the product, and city and the country of the company (including the state if in USA), should be provided in parentheses in the following format: "Discovery St PET/CT scanner (General Electric, Milwaukee, WI, USA)"

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Limitations, drawbacks, and the shortcomings of original articles should be mentioned in the Discussion section before the conclusion paragraph.

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Book Section: Suh KN, Keystone JS. Malaria and babesiosis. Gorbach SL, Barlett JG, Blacklow NR, editors. Infectious Diseases. Philadelphia: Lippincott Williams; 2004.p.2290-308.

Books with a Single Author: Sweetman SC. Martindale the Complete Drug Reference. 34th ed. London: Pharmaceutical Press; 2005.

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Conference Proceedings: Bengisson S. Sothemin BG. Enforcement of data protection, privacy and security in medical informatics. In: Lun KC, Degoulet P, Piemme TE, Rienhoff O, editors. MEDINFO 92. Proceedings of the 7th World Congress on Medical Informatics; 1992 Sept 6-10; Geneva, Switzerland. Amsterdam: North-Holland; 1992. pp.1561-5.

Scientific or Technical Report: Cusick M, Chew EY, Hoogwerf B, Agrón E, Wu L, Lindley A, et al. Early Treatment Diabetic Retinopathy Study Research Group. Risk factors for renal replacement therapy in the Early Treatment Diabetic Retinopathy Study (ETDRS), Kidney Int: 2004. Report No: 26.

Thesis: Yılmaz B. Ankara Üniversitesindeki Öğrencilerin Beslenme Durumları, Fiziksel Aktiviteleri ve Beden Kitle İndeksleri Kan Lipidleri Arasındaki Ilişkiler. H.Ü. Sağlık Bilimleri Enstitüsü, Doktora Tezi. 2007.

Epub Ahead of Print Articles: Cai L, Yeh BM, Westphalen AC, Roberts JP, Wang ZJ. Adult living donor liver imaging. Diagn Interv Radiol. 2016 Feb 24. doi: 10.5152/dir.2016.15323. [Epub ahead of print].

Manuscripts Published in Electronic Format: Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis (serial online) 1995 Jan-Mar (cited 1996 June 5): 1(1): (24 screens). Available from: URL: http:/ www.cdc.gov/ncidodlElD/cid.htm.

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Contents

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RESEARCH ARTICLE

Eur J Biol 2019; 78(1): 1-4

Benedenia sp. Infestation Along with Motile *Aeromonas* Septicemia in Common Stingray (*Dasyatis pastinaca* Linnaeus, 1758)

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Please cite this article as: Turgay E, Yardimci RE, Karatas S. *Benedenia* sp. Infestation Along with Motile Aeromonas Septicemia in Common Stingray (*Dasyatis pastinaca* Linnaeus, 1758). Eur J Biol 2019; 78(1): 1-4.

ABSTRACT

Objective: The aim of this study was to determine the cause of mortalities occurring in common stingray (*Dasyatis pastinaca*) individuals held in a public aquarium.

Materials and Methods: Moribund common stingrays (approx. 1 m in length and 2 kg in weight) that had previously been caught by trawling and placed in the quarantine tanks in a public aquarium were examined to determine the cause of mortalities.

Results: The affected common stingrays had haemorrhages on the ventral side of the body. Internally, the liver was pale and pseudotubercules were observed in the spleen. In the examined specimens, monogenean parasites were observed in blisters on the skin of the dorsal side of the body. According to their morphological, physiological and biochemical characteristics as well as gene sequence analysis, the parasites were identified as *Benedenia* sp., and the bacterial isolates were identified as *Aeromonas hydrophila*.

Conclusion: In this study, *Benedenia* sp. is reported initially, along with motile *Aeromonas septicemia* in common stingray.

Keywords: Common stingray, *Dasyatis pastinaca*, benedenia, *Aeromonas hydrophila*

INTRODUCTION

The common stingray (*Dasyatis pastinaca*), which is a member of the family Dasyatidae, is distributed throughout the North-eastern Atlantic Ocean, the Mediterranean Sea and along the African coast southwards to Senegal. It is also a coastal species, which enters coastal lagoons, shallow bays and estuaries. In the North Aegean Sea, the common stingray is caught by trawl fisheries (1) and occasionally (as part of small-scale commercial concerns), by bottom trawl, gillnet, beach seine, bottom longline and trammel net fisheries. While so many studies on the biology and ecology of the common stingray exist in other countries, this kind of information is limited in Turkey. In one such study, the diet of common stingray was studied by Saglam et al. (2). In that study, the specimens were collected by bottom trawling at depths of 20-40 m in the southeastern Black Sea between June 2007 and May 2008. In the results of the study, no difference was detected between sexes and diet of small and large individuals, but significant differences were found between seasons. Yigin and Ismen (3) studied age, growth and reproduction of the common stingray in the North Aegean Sea. Only one case report of parasite infestation in Turkey exists in Turkey, by Diamant et al. (4). They reported subcutaneous spindle-shaped inclusions on the stingray's dorsal surface as the earliest clinical signs of a microsporidian infection in the common stingray in Iskenderun Bay. The purpose of this study was to determine the cause of mortalities occurring in common stingray held in quarantine under stressful conditions.

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Submitted: 30.11.2018 • **Revision Requested:** 17.12.2018 • **Last Revision Received:** 18.12.2018 • **Accepted:** 19.12.2018

MATERIALS AND METHODS

The common stingrays (*Dasyatis pastinaca*) (20 specimens, approx. 1 m in length and 1-2 kg in weight) were caught by trawling in Edremit Bay in the Aegean Sea, and then transported to Istanbul and placed into the quarantine tanks of a public aquarium. A few days later, mortalities were observed in the stingray population as well as severe feeding difficulty. The water parameters of the aquarium were as follows: temperature was 24°C, salinity was 28, pH was 8, dissolved oxygen concentration was 7,6 mg/L and oxidation reduction potential was 175 mV. Two moribund common stingrays were sampled in line with standard methods (5). During dissection, all internal organs and the skin, as well as the body cavity and the fins, were examined for parasites. The obtained parasites were first examined under a dissection microscope and the isolated parasites were fixed and preserved according to standard protocols. The bacteriological samples from the liver, spleen and skin lesions were streaked onto Marine Agar (MA) (Difco, Detroit, MI, USA) plates and the plates were incubated at 22°C for 72 hours. The morphological and physiological characteristics of the bacterial colonies from each plate were determined using conventional biochemical and physiological tests as well as rapid identification kits (API 20E) (BioMérieux, Marcy-l'Etoile, France). Molecular diagnostic methods were also used for the identification of both parasite samples and bacterial strains. The isolates on the MA plates were inoculated into Marine Broth (MB) (Difco, Detroit, MI, USA) and incubated overnight at 22°C, then total DNA was extracted from liquid cultures or directly from ethanol fixed parasite samples using the Pure Link™ Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For bacterial identification, a 540-bp-long fragment of the 16S rRNA gene was amplified and sequenced using the universal bacteria primer set: primer S-D-Bact-0008-a-S-20 and primer S-*-Univ-0536-a-A-18 (6). To identify the parasites, the universal eukaryotic primer set F-566 and R-1200 was used to amplify and sequence a part of the 18S rRNA gene (7).

RESULTS

During the parasitological examination, monogenean parasites were observed in blisters on the skin of the dorsal side of the body (Figure 1). Affected common stingrays had haemorrhages on the ventral side of the body and on the cloaca (Figure 1). Internally, a pale liver or haemorrhages in the liver and pseudotubercules in the examined fish spleen were observed (Figure 1). The parasites were identified as *Benedenia* sp. following the diagnostic keys outlined by Yamaguti (8). The anterior part of the parasite had a pair of attachment organs and the opisthaptor had a marginal extension surrounding the haptor as a skirt (Figure 2). On the bacteriological examination side; creamy, round, raised, entire colonies of 2–3 mm diameter were formed on the MA. These bacterial isolates appeared as Gram-negative, motile chemo-organotrophic (fermentative) rods and were oxidase and catalase positive. According to their morphological and biochemical characteristics (Table 1) and 16S rRNA and 18S rRNA gene sequencing results, the bacterial isolates that obtained from

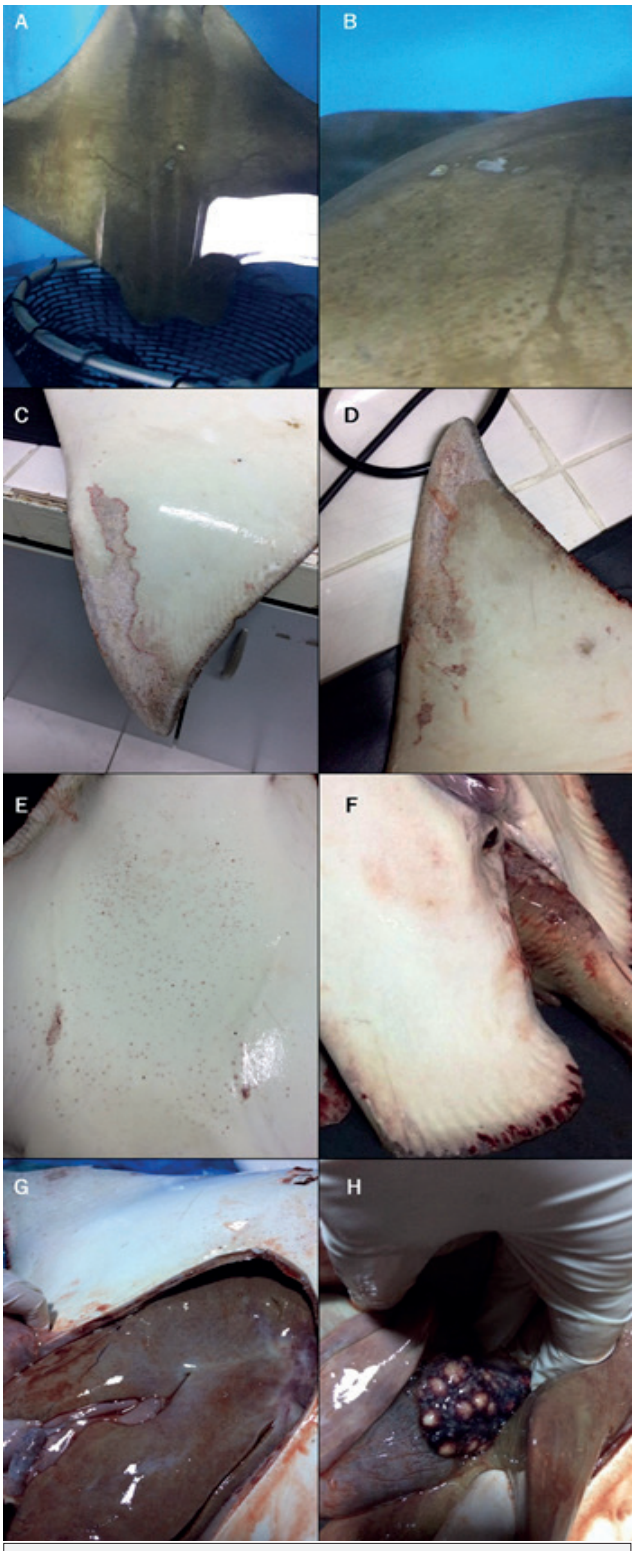

Figure 1. Affected common stingray, (A) *Benedenia* sp. on the dorsal side of the body, (B) blister on the skin, (C, D) haemorrhages on the ventral side of pectoral fins and skin lesions, (E) petechial haemorrhages on the ventral side of body, (F) haemorrhages on the cloaca, tail and pelvic fins, (G) haemorrhages in the pale liver, (H) pseudotubercules in the spleen.

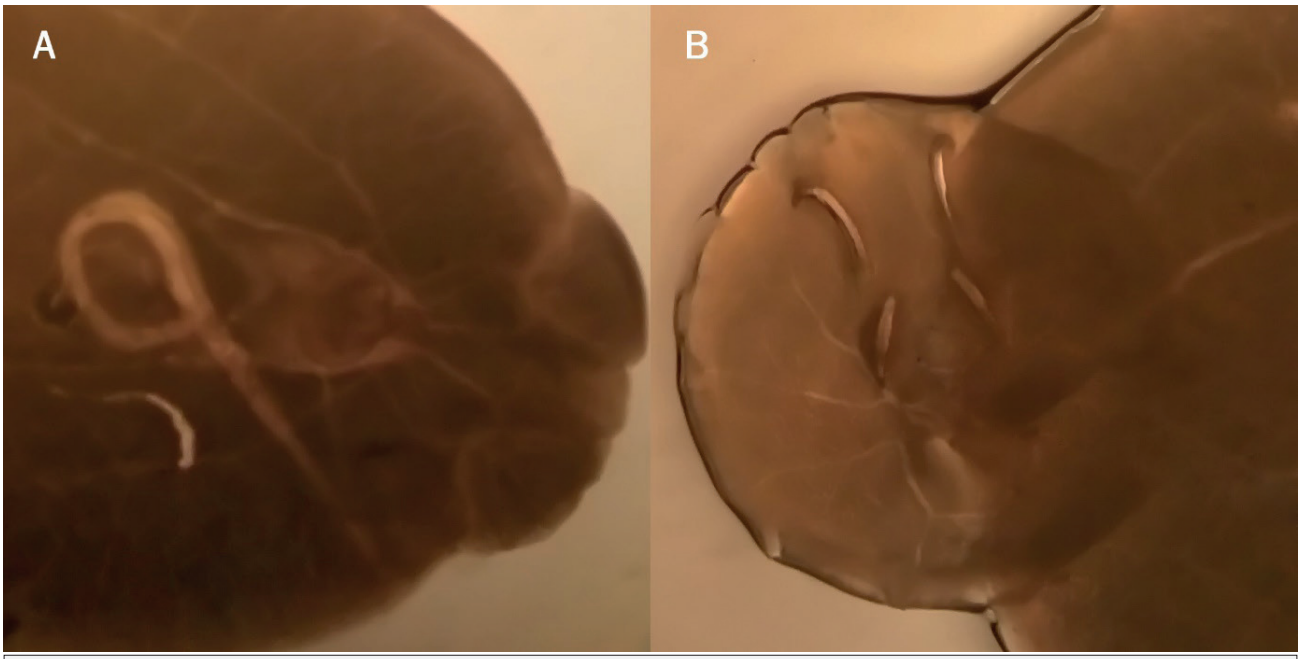

Figure 2. *Benedenia* sp. (A) attachment and copilatory organs at the anterior part (x25), (B) marginal hooks on the opisthaptor.

Table 1. Morphological and phenotypical characteristics of the isolated strain

Morphology	**rods**	**Citrate**	**+**
Motility	+	Degradation of urea	-
Gram staining	-	H_2S production	-
Catalase	+	ONPG	+
Cytochrome oxidase	+	MacConkey	+
O/129 resistance (150µg)	R	Glucose	+
O/F	F	Sucrose	+
Indole	+	Inositol	-
Voges Proskauer reaction	-	Maltose	+
Methyl red	+	Esculine	+
Arginine dihydrolase	+	Nitrate reduction	+
Lysine decarboxylase	-	Gelatin hydrolase	+
Ornithine decarboxylase	-	Growth at 37°C	+

Key: -: Negative, +: Positive, F: Fermentative, R: Resistant

the liver, spleen and skin lesions, were identified as *Aeromonas hydrophila*, whereas monogeneans were identified as *Benedenia sp.* (acc. no. MK106094).

DISCUSSION

Monogenean fish parasites are commonly found in both marine and freshwater environments and live on a wide range of aquatic animals including fishes, however, most of them are ectoparasites with relatively low fecundity and direct life cycles and they usually infect a single host species (9). Although monogenean parasites are usually not present in high numbers on individual fishes in the wild, they may cause more destructive effects in animals that are held in captivity like those in public aquaria as a consequence of increased stressors-mostly due to spatial restriction and overcrowding (10, 11).

The monogenean *Benedenia epinepheli* was first discovered by Yamaguti (12) and then this parasite was re-described and reported by Ogawa et al. (13). *Benedenia* species previously found in fish were as follows (with fish host and isolated tissue/organ information): *B. epinepheli* from gills, fins, eyes and body surface from *Epinephelus akaara*, *Conger myriaster* and *Paralichthys olivaceus* (12); *B. sekii* from body surface of *Pagrus major* (12); *B. seriolae* from *Seriola lalandi* (12) and from skin in *Seriola quinqueradiata* (14); *B. rohdei* from gills and *B. lutjani* from skin of *Lutjanus carponotatus* (15); *B. sciaenae* from *Sciaena umbra* and *Umbrina cirrosa*, *B. monticellii* from gill filaments of *Liza aurata* (16, 17). Despite the large number of the parasite isolation reports in teleost fish, almost none of them were isolated from diseased hosts. Additionally, reports in elasmobranch species like stingrays are very limited.

The only *Benedenia sciaenae* infestation in Turkey, has been reported in cultured meagre (*Argyrosomus regius*) (18). In that study, they reported haemorrhagic lesions and scale losses on the skin as clinical findings. Similarly, our examined stingrays

only showed haemorrhagic lesions on the skin externally, but additionally we observed the parasites in blisters on the skin.

Aeromonas hydrophila is one of the ubiquitous and well known fish pathogens of both marine and freshwater fish species and is mostly considered an opportunistic pathogen (19). *A. hydrophila* involving infections in fish may cause several distinct pathological conditions but has mostly been associated with causing haemorrhagic septicaemia (20). In accordance with this, moribund stingrays had haemorrhages on their body and also internal haemorrhages were observed in the liver.

CONCLUSION

Low host specificity, ubiquitous distribution and the high susceptibility of marine aquarium fish are factors which are indicative of the parasite's great potential to become a disease problem among cultured fish. In this study, *Benedenia* sp. is reported initially along with motile *Aeromonas* septicemia in the common stingray.

Peer-review: Externally peer-reviewed.

Acknowledgements: This study was approved by the Istanbul University Local Committee on Animal Research Ethics (decision year 2016).

Conflict of Interest: The authors have no conflict of interest to declare.

Funding Information: There are no funders to report for this submission

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ISTANBUL UNIVERSITY PRESS

RESEARCH ARTICLE

Eur J Biol 2019; 78(1): 5-10

Cyanide Removal in Electroplating, Metal Plating and Gold Mining Industries' Wastewaters by Using *Klebsiella pneumoniae* and *Klebsiella oxytoca* Species

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Please cite this article as: Avcioglu NH, Seyis Bilkay I. Cyanide Removal in Electroplating, Metal Plating and Gold Mining Industries' Wastewaters by Using *Klebsiella pneumoniae* and *Klebsiella oxytoca* Species. Eur J Biol 2019; 78(1): 5-10.

ABSTRACT

Objective: In this study, cyanide containing wastewaters of electroplating, metal plating and gold mining industries were selected in order to investigate the usability of *Klebsiella pneumoniae* and *Klebsiella oxytoca*.

Materials and Methods: In order to investigate their cyanide removal capability *K. pneumoniae* and *K. oxytoca* cultures both, separately and with sterile crude extracts were used.

Results: It is observed that cyanide in electroplating wastewater was effectively removed by the cultures and sterile crude extracts of *K. pneumoniae* (both 100%) and *K. oxytoca* (100% and 70%, respectively). Additionally, culture of *K. pneumoniae*, mixture of *K. pneumoniae* and *K. oxytoca* cultures, sterile crude extract of *K. oxytoca* and mixture of *K. pneumoniae* and *K. oxytoca* sterile crude extracts degraded cyanide efficiently (94%, 96%, 90% and 93%, respectively) in metal plating wastewater.

Conclusion: It is determined that *K. pneumoniae* and *K. oxytoca* cultures and crude extracts can be a promising alternative for cyanide removal in wastewaters of different industries.

Keywords: *Klebsiella pneumoniae, Klebsiella oxytoca*, cyanide, wastewater, biodegradation

INTRODUCTION

Cyanide consumption and cyanogen waste production are approximately 1.5 million tones and 14 million kg per year respectively in worldwide (1). Furthermore, cyanide is an important inhibitor of cytochrome oxidase c and some other metalloproteins and it binds with methemoglobin irreversibly in blood. Therefore, removal of cyanide especially in industrial wastewaters by using effective methods is an important issue in order to protect the health of living organisms (2-5). In this respect, researchers focused on the treatment of this toxic compound by biological treatment methods. Accordingly, biological systems are being used in the removal of cyanogen compounds. Based on the investigations obtained from the literature, various plants such as *Sorghum bicolor* and *Linum usitassimum var. omega-gold* (6), *Zea mays* (7), various fungi such as *Aspergillus niger* (8), *Cryptococcus humicolus* (9), *Fusarium lateritium* (10, 11), *Fusarium oxysporum* (12, 13), *Fusarium solani* (14, 15), *Rhizopus arrhizus* (16), *Scenedesmus obliquus* (17), *Trichoderma sp.* (18) and *Trichoderma harzianum* (19) were found as cyanide degrading species. Additionally, *Agrobacterium tumefaciens* (20), *Azotobacter vinelandii* (21), *Bacillus nealsonii* (22), *Klebsiella oxytoca* (2, 4, 5), *Paracoccus sp.* (23), *Pseudomonas fluorescens* (24-26), *Rhodococcus sp.* (27, 28), *Serratia marcescens* (29, 30), *Serratia odorifera* (31), *Halomonas sp.* (32) are some of the cyanide degrading bacteria. Accordingly, cyanide removal is directly carried out with some metabolic pathways (hydrolytic, oxidative, reductive and substitutional/transfer reactions) which include cyanide degrading enzymes. By means of these enzymatic pathways, bacteria/fungi detoxify cyanide to nontoxic chemicals as ammonia (33). In this respect, in this study cyanogen waste forming industrial processes' wastewaters

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Submitted: 24.07.2018 • **Revision Requested:** 19.10.2018 • **Last Revision Received:** 17.01.2019 • **Accepted:** 06.02.2019

(electroplating, metal plating and gold mining) were prepared and cyanide removal efficiencies of *Klebsiella pneumoniae* and *Klebsiella oxytoca* cultures, sterile crude extracts and mix of the cultures and sterile crude extracts of them were investigated.

MATERIALS AND METHODS

Bacterial Strains

In this study, *K. oxytoca* and *K. pneumoniae*, which were determined as similar to *K. oxytoca* ATCC 13182 and *K. pneumoniae* ATCC 700721 by 16S rRNA analysis in our previous study (34), were used. *Klebsiella* strains were inoculated in to the Brain Heart Infusion Broth media containing 10% glycerol and stored at -20°C for later analysis.

Synthetic Wastewater

Electroplating, metal-plating and gold mining industries' wastewaters were selected in order to investigate the cyanide biodegradation capability of cultures, sterile crude extracts, mix cultures and mix sterile crude extracts of *K. pneumoniae* and *K. oxytoca* species. Accordingly, synthetic wastewaters were prepared as follows; (as mgL^{-1}) 1.2 Ag, 1.2 Cd, 7.0 Cr, 4.5 Cu, 4.1 Ni, 0.6 Pb, 4.2 Zn and 5.0 CN for electroplating wastewater, (as mgL^{-1}) 0.43 Ag, 0.69 Cd, 2.77 Cr, 3.38 Cu, 3.98 Ni, 0.69 Pb, 2.61 Zn and 1.2 CN for metal-plating wastewater and (as mgL^{-1}) 10.0 As, 0.02 Cd, 0.1 Cr, 400.0 Cu, 40.0 Fe, 0.05 Hg, 20.0 Mn, 50.0 NH_4, 10.0 Ni, 0.1 Pb, 6.0 Sel, 2.0 Ag, 100.0 Zn, 1000.0 CN and 2000.0 SCN- (thiocyanate) for gold mining industry wastewater (35).

Biodegradation Studies

Firstly, *K. pneumoniae and K. oxytoca* were incubated into the enrichment media (Luria Bertani Broth) at 37°C, 150 rpm for log phase and after the incubation period cultures of *Klebsiella* species were adjusted to OD_{600}= 1.0 and mix cultures of *K. pneumoniae and K. oxytoca* were formed by combining these species' cultures with the proportion of 1:1 (v/v) in order to use in the biodegradation studies of cyanide containing wastewaters.

Sterile crude extracts of *K. oxytoca* and *K. pneumoniae* species were prepared as follows; enriched *Klebsiella* cultures were inoculated into cyanide biodegradation media containing g/L: 1 glucose, 0.5 K_2HPO_4, 0.5 K_2HPO_4 and 0.05 $MgSO_4$ (24) and at the end of the incubation period, cultures were centrifuged at 4000 rpm for 5 minutes and were sterilized by 0.45 µM cellulose acetate filter (Millipore) to form sterile crude extracts of *Klebsiella* species. Mix of the sterile crude extracts of *K. pneumoniae* and *K. oxytoca* species were formed by combining these species' sterile crude extracts with the proportion of 1:1 (v/v).

Cultures, sterile crude extracts and mixtures of them were inoculated into the wastewaters with the concentration of 1:10 (v/v) and incubation was performed in optimal conditions at 25°C, 150 rpm for 3 days for *K. pneumoniae* and at 30°C, 100 rpm for 5 days for *K. oxytoca*. The experiment was performed in triplicate. Additionally, cyanide containing wastewater which doesn't contain bacteria was used as control in all experiments.

Analysis of Biodegradation Products

Residual cyanide concentration and ammonia formation in synthetic wastewaters were assessed by using picric acid method (36) and Nesslerization method (37) respectively. Additionally, growth of *K. pneumoniae*, *K. oxytoca* cultures separately and mix cultures of *K. pneumoniae* - *K. oxytoca* species in these wastewaters were measured by using spectrophotometer (Shimatzu-UV 1700) at 600 nm.

RESULTS

Cyanide Removal by Using Bacterial Cultures and Crude Extracts

Cyanide removal capabilities of cultures and sterile crude extracts of *K. pneumoniae* and *K. oxytoca* species in electroplating, metal plating and gold mining wastewasters were investigated in this study. As a result, it is found that *K. pneumoniae* culture degraded the cyanide content in electroplating and metal plating industries' wastewaters with efficiencies of 100% and 94%, respectively (Figures 1a and 1b). However, this strain was not effective in the biodegradation process of cyanide in gold mining industry wastewater (Figure 1c). Additionally, when biodegradation results are evaluated for *K. oxytoca*, it is found that results are in parallel with the biodegradation ability of *K. pneumoniae* culture and accordingly this strain also fully degraded the cyanide content in electroplating wastewater,

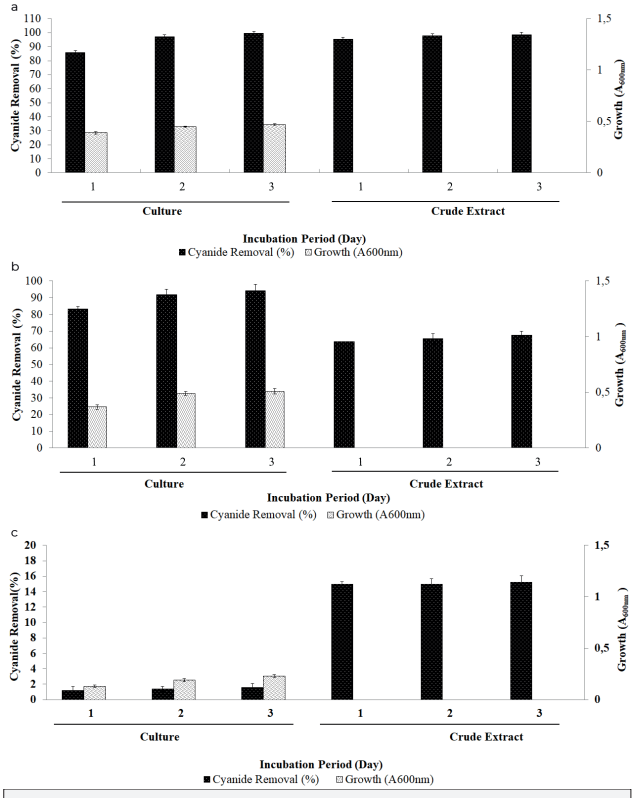

Figure 1. Cyanide removal in electroplating (a), metal plating (b) and gold mining (c) wastewaters with culture and crude extract of *K. pneumoniae*.

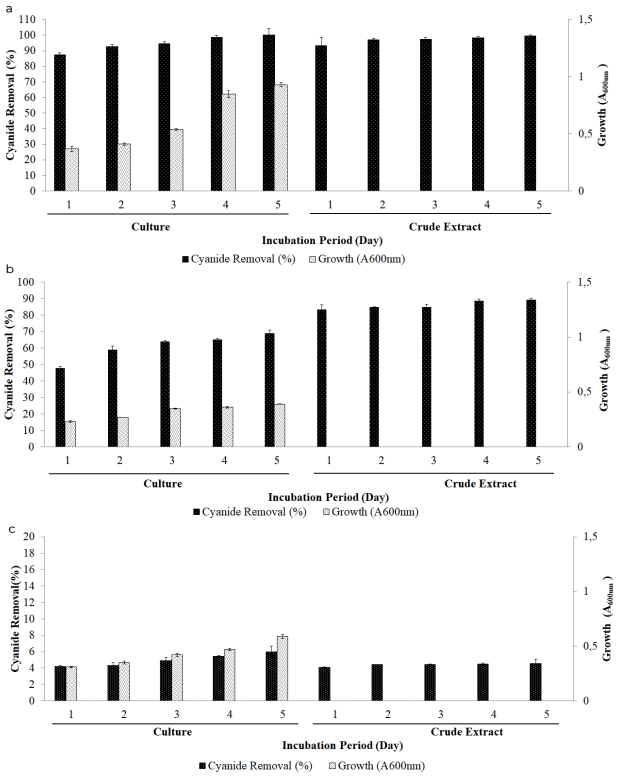

Figure 2. Cyanide removal in electroplating (a), metal plating (b) and gold mining (c) wastewaters with culture and crude extract of *K. oxytoca*.

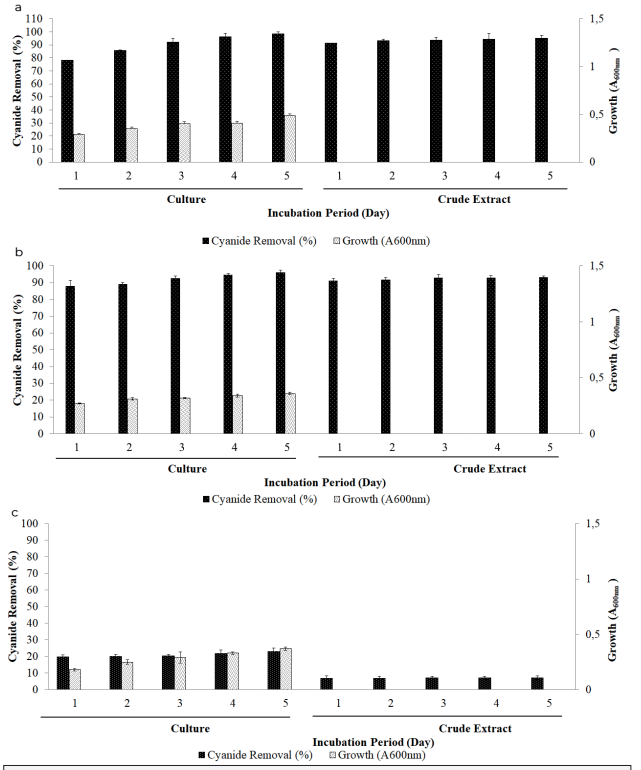

Figure 3. Cyanide removal in with culture and crude extract of *K. pneumoniae* and *K. oxytoca* in electroplating (a), metal plating (b) and gold mining (c) wastewaters.

70% of the cyanide content in metal plating wastewater and it was not effective in the biodegradation of cyanide content in the gold mining wastewater (Figure 2). Furthermore, when crude extracts are used for the removal of cyanide, it is found that sterile crude extract of *K. pneumoniae* fully degraded cyanide in electroplating wastewater and degraded 65% of the cyanide content in metal plating industry wastewater (Figures 1a and 1b). Besides, sterile crude extract of *K. oxytoca* degraded both of these wastewaters with the efficiencies of 100% and 90%, respectively (Figures 2a and 2b).

Cyanide Removal by Using Mix Cultures and Mix Crude Extracts

Mix cultures and mix crude extracts of *Klebsiella* species were formed in order to evaluate the interactions between these *Klebsiella* species in different cyanide removal processes. Accordingly, it is observed that mix of cultures and mix of crude extracts of *K. pneumoniae* and *K. oxytoca* species are also effective in the biodegradation of cyanide in electroplating and metal plating wastewaters (Figures 3a and 3b). Besides, when these species are combined with each other, synergetic interaction between these *Klebsiella* species was observed and biodegradation ability increased to 23% in gold mining wastewater (Figure 3c).

Ammonia Formation

In this study, ammonia was observed as a final product of cyanide biodegradation process. Accordingly, it is examined that ammonia was formed by both of *Klebsiella* species' cultures in electroplating wastewater and were formed by *K. pneumoniae* and *K. oxytoca* cultures in metal plating wastewater during cyanide biodegradation processes. Although the least cyanide biodegradation capability of all *Klebsiella* species was observed in gold mining wastewater, by means of including 1000 mg/l CN and 2000 mg/L SCN- in its content, the most amount of ammonia (6.9 mg/L and 4.68 mg/L) were formed by *K. pneumoniae* and *K. oxytoca* cultures, respectively.

Ammonia formation in wastewaters during cyanide biodegradation processes by using mix cultures and mix crude extracts of *Klebsiella* species were also investigated. As a result, it is observed that, 0.51 mg/L, 0.12 mg/L and 7.9 mg/L ammonia were formed in cyanide removal processes of electroplating, metal plating and gold mining industries respectively by using mix cultures of *Klebsiella* species and 0.49 mg/L, 0.12 mg/L and 7.9 mg/L ammonia were formed in cyanide removal processes by using their mix of crude extracts.

DISCUSSION

Cyanide removal by using biological methods seems more advantageous and environmentally friendly than chemical ones as they form nontoxic end products (2, 5, 38). Accordingly, microorganisms, which are being investigated for cyanide removal efficiencies, must be able to live in wastewaters

containing heavy metals and some other toxic chemicals and also synthesize enzymes for cyanide removal processes.

In all over the world, up to 20% of cyanogen wastes originate from electroplating industries (39). Additionally, electroplating wastewater is an important cyanogen waste former by means of using and forming sodium and potassium salts of cyanide in its production processes (40). In this study, *K. pneumoniae* and *K. oxytoca* species' cultures fully degraded the cyanide content in electroplating wastewater and 94% and 70% of the cyanide content in metal plating wastewater respectively. Some of other researches, *B. safensis, B. licheniformis* and *B. tequilensis* strains which were isolated from electroplating wastewater, degraded 200 and 400 CN^-/L cyanide with the efficiencies of 65.5% and 44.3% respectively (41) and *P. montelii* which was also isolated from wastewater, degraded 93% of the cyanide content in wastewater (40). Therefore, in the present study, *Klebsiella* species are found to be effective agents for the removal of cyanogen wastes for further treatment processes.

In this study, as presented above, biodegradation of cyanide by using *Klebsiella* species in gold mining wastewater was also investigated and it is observed that both of the *Klebsiella* species were not effective in this wastewater (Figures 1c and 2c). This is probably due to the fact that, the content of this wastewater contains not only heavy toxic ions (As, Cd, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Sel, Mg and Zn) and high concentrations of cyanide compounds (SCN-, CN^-), and it also contains NH_4^+ which is known as a competitive agent in cyanide biodegradation. Accordingly, cyanide removal by *K. pneumoniae and K. oxytoca species* was not effective in the removal of cyanide in this wastewater because of the presence of these chemicals. In parallel with our research, it is found that the presence of ammonia in the biodegradation media directly affects the biodegradation ability of microorganism as it utilizes ammonia instead of cyanide inside the medium (41).

Additionally, ion effect on cyanide biodegradation capability of microorganisms was also investigated in some other researches and it is observed that growth amount and cyanide biodegradation ability of *Burkholderia cepacia* inhibited in the presence of 1mM Cu and Fe ions and biodegradation is also directly related with the presence of Ni, Co, Mn and Mo ions (42) and it is determined that Pb and Cd and Fe ions inhibited this process approximately 20% and 30-35% respectively (43). In a similar study concentrated on ion effect on cyanide removal, it was determined that although Cu and Ca ions directly affected the bacterial growth and cyanide biodegradation, Mg and Mn ions affected only growth (44). Additionally, Ibrahim *et al.* (30) found that Hg ion shows toxic effect on the growth of *Serratia marcescens* and cyanide biodegradation with this bacterial strain dropped sharply from 92% to 24.7% in the presence of this ion. As a result, all wastewaters investigated in this study contain different concentrations of various ions and *K. pneumoniae* and *K. oxytoca* species achieved cyanide removal. Therefore, these results directly demonstrate the usability of these *Klebsiella* species in the treatment processes of ion containing cyanogen wastes.

Alternatively, purified enzymes from bacterial crude extracts can be used in biodegradation studies instead of their cultures. When purified enzymes are used, crude extracts directly degraded toxic compounds effectively in a short time. In this respect, sterile crude extracts of *Klebsiella* species were used in order to investigate the ability of these unpurified extracellular substances in biodegradation processes of different wastewaters. No purification method was used for purification of these sterile crude extracts to obtain cyanide degrading enzymes. In this respect, these extracellular substances may contain not only enzymes but also biodegradation products and some other unusable chemicals. Therefore, this study pointed out the usability of unpurified extracellular products of *Klebsiella* species in cyanide biodegradation and it is obvious that if it is purified, the efficiency of biodegradation will be much higher. Additionally, it can be noted that, *Klebsiella* enzymes can be used solely for cyanide biodegradation and alternative immobilized systems can be developed in future researches.

Antagonistic and synergetic interactions between bacteria are being used in environmental studies in order to treat various toxic chemical compounds in literature. Therefore, mix cultures of *Klebsiella* species' and mix of their crude extracts were formed and biodegradation abilities of these mixes were also investigated in this study. As a result, it is observed that mix cultures and mix crude extracts of *Klebsiella* species are also effective in cyanide biodegradation processes in electroplating and metal plating industries' wastewaters. Additionally, synergetic interaction between these species was also examined in the cyanide biodegradation of gold mining industry' wastewater. In the literature, it is observed that when *Citrobacter sp.* MCM B-181, *Pseudomonas sp.* MCM B-182, *Pseudomonas sp.* MCM B-183 and *Pseudomonas sp*. MCM B-184 strains are used solely, cyanide removal efficiencies of these strains are found as 92.5%, 88.4%, 73.2% and 68.8%, respectively but with a consortium and by using a reactor, cyanide removal increased to 99% , and when *Bacillus sp*., *Klebsiella sp*., *Pseudomonas sp*. and another *Pseudomonas sp*. are used, cyanide biodegradation amount increased to approximately 98% by means of synergetic interactions between these strains (44). In another study, it is observed that when a consortium included *B. subtilis* and *P. stutzeri* strains are used cyanide biodegradation increased from 66.9 % to 88.5 % (45). According to Razanamahandry et al. (46) enzymes which are synthesized by consortia are durable for a long time and the usage of these consortia in degradation studies can increase biodegradation efficiencies. Therefore, this research demonstrated the importance of synergetic interactions between microorganisms in environmental researches.

CONCLUSION

To sum up, the results of this research demonstrated the usability of cultures, unpurified sterile crude extracts and mixes of cultures and crude extracts of *K. oxytoca* and *K. pneumoniae* species in cyanide removal processes of electroplating and metal plating industries' wastewaters efficiently. Additionally,

different than other researches, this study also determined the importance of sterile crude extracts of *K. oxytoca* and *K. pneumoniae* species in the biodegradation of cyanide. The results are promising for the future researches on cyanide removal by using bacterial strains and their crude extracts.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of study: N.H.A, I.S.B.; Data Acquisition: N.H.A.; Data Analysis/Interpretation: N.H.A.; Final Approval and Accountability: N.H.A, I.S.B.; Drafting Manuscript: N.H.A.; Critical Revision of Manuscript: N.H.A.; Technical or Material Support: N.H.A., I.S.B.; Supervision: N.H.A., I.S.B.

Conflict of Interest: The author has no conflict of interest to declare.

Financial Disclosure: Hacettepe University Scientific Research Projects Coordination Unit 014 D03 601 004.

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RESEARCH ARTICLE

Eur J Biol 2019; 78(1): 11-22

The Effects of Atorvastatin on the Migraine Pathophysiology in Nitroglycerin Induced Migraine in Ovariectomized Rats

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Please cite this article as: Hicyilmaz AC, Akgun-Dar K, The Effects of Atorvastatin on the Migraine Pathophysiology in Nitroglycerin Induced Migraine in Ovariectomized Rats. Eur J Biol 2019;78(1): 11-22.

ABSTRACT

Objective: Migraine is one of the common primary headaches which affects the majority of the population. However, in spite of increasing information on migraine pathophysiology and various researches, the underlying mechanisms of migraine attacks still not completely understood. Statins have antioxidant, anti-inflammatory neuroprotective effects and they might be a useful drug for curing neurodegenerative disorders. There are several studies suggesting nitric oxide (NO) causes migraine attacks in migraine pathogenesis. We aimed to investigate the effects of atorvastatin (AT) which is a member of the statin family, on migraine pathophysiology in ovariectomized female rats in which migraine was stimulated using nitroglycerine (NTG).

Materials and Methods: In the study, ovariectomized adult Sprague Dawley female rats were divided into 4 groups; control, NTG, AT, NTG+AT. We examined immunohistochemically inducible nitric oxide (iNOS), endothelial nitric oxide (eNOS), neuronal nitric oxide (nNOS), matrix metalloproteinase-2 (MMP-2), β-catenin expression on brain sections and biochemically c-fos, calcitonin gene-related peptide (CGRP), β-catenin, calcium-binding protein B (S100B), NO, total antioxidant capacity (TAS) and total oxidant capacity (TOS) levels.

Results: Our results showed that AT was immunohistochemically increasing eNOS and nNOS levels, and reducing iNOS. In brain homogenates AT was reducing S100B, CGRP, c-fos, total Nitrite-Nitrate, β-catenin but increasing TAS and TOS. There was no change in MMP-2. Also, our findings showed that AT could inhibit astroglial activity with its anti-inflammatory effect and showed a protective effect towards blood-brain barrier and reduced the risk of possible neurodegenerative diseases in rats with migraine.

Conclusion: Our findings showed that AT could inhibit astroglial activity with its anti-inflammatory effect and showed a protective effect towards blood-brain barrier and reduced the risk of possible neurodegenerative diseases in rats with migraine. Our results will provide a significant contribution to studies in this field.

Keywords: Migraine, nitroglycerin, atorvastatin, ovariectomized, rat

INTRODUCTION

Migraine, one of the common primary headaches which affect the majority of the population, is generally characterized with nausea, vomiting, sensitivity to sound and light, along with a unilateral and severe headache (1). Migraine is a brain disorder that affects 11% of the population and is common in women (2). Generally, the activation and sensitization of trigeminovascular pain path (which is believed to be associated with migraine headaches), and cortically spreading depression are seen neurophysiologically together in migraines. However, in spite of increasing information on migraine pathophysiology and various pieces of research, the underlying mechanisms of migraine attacks are still not completely understood.

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Submitted: 08.11.2018 • **Revision Requested:** 30.11.2018 • **Last Revision Received:** 30.01.2019 • **Accepted:** 06.02.2019

Gonadal hormones, especially estradiol, play a role in migraine formation. In women, where headaches are three or four times more common, migraine may be triggered by a sudden decrease in the plasma levels of estrogen in the premenstrual period (3). Neurobiological mechanisms related to the effects of estrogen on migraine are controversial. It has been shown to decrease especially in the trigeminal system nociception with estrogen in mice (4). It is known that migraine is two to three times more common in women than men. In women migraine is higher in the reproductive age where the serum estrogen level is highest, whereas it is lower before puberty and after the menopause (5). The results of studies in women indicate that migraine attacks are positively associated with the menstrual cycle (6). The results obtained from the studies show a positive relationship between migraine attacks and different serum estrogen levels. Estrogen plays an important role in the pathogenesis of migraine but its molecular basis is not well understood.

Additionally, it is thought that they have neuroprotective effects, although statins have a few side effects they might be a useful drug for curing neurodegenerative disorders. In addition to the cholesterol-lowering properties of statins, their antioxidant, anti-inflammatory effects and also their effects on NO have been shown (7-11). Atorvastatin (AT), which is a member of the statin family, has been used because of its neuroprotective and anti-inflammatory effects in a variety of nervous system diseases (9, 12, 13).

Systemic administration of nitroglycerin (NTG) to migraine patients typically causes aura-free attacks after a few hours but not in healthy volunteers (14, 15). NTG also increases the expression of neuronal nitric oxide (nNOS) in spinal trigeminal neurons (16). The mechanisms that explain how NTG and NO affect migraine are not known for certain. Headaches are affected by changes in estrogen levels during pregnancy and menopause (17, 18). The regulatory effects of estrogen on migraine are controversial. Systemic NTG administration activates nociceptors in the caudal spinal trigeminal nucleus in rats (19). NO stimulates the trigeminal C and Aδ afferents, and the second type of nociceptors is responsible for this stimulation. Calcitonin gene-related peptide (CGRP) is a key molecule in primary nociceptive afferents, released by NO mediated mechanisms in animals (20). CGRP increases in jugular blood during migraine attacks (21). Several studies are suggesting NO causes migraine attacks in migraine pathogenesis (12, 22, 23).

It is known that the Wnt/β-catenin signaling pathway is involved in brain development and neurodegenerative diseases. In a study it was shown that CGRP is able to regulate β-catenin signaling by induction of RNA synthesis of β-catenin (24). NO was shown to attenuate the β-catenin/TCF signal in colon cancer cells and β-catenin degradation mediated this event (25). This study is consistent with the possibility that upregulation of Wnt/β-catenin may contribute to the macrocephaly associated with autism and some aspects of the behavioral phenotype (26). It was observed that NTG (10 mg/kg, s.c.) significantly increased plasma NO and CGRP concentrations and c-fos expression in the trigeminal nucleus caudalis (TNC) (27). NTG increases the expression of some key molecules such as c-fos (28-30) CGRP release increases c-fos expression (31). Increased serum calcium-binding protein B (S100B) concentration in patients with migraine has been reported (32,33). Brain specific proteins are involved significantly in neuropathology due to intracellular and extracellular functions. Measurement of the astroglial protein S100B, which is one of these proteins, especially in the serum, is used to show the blood brain barrier (BBB) damage index (34). It is reported that in a study of 151 patients with migraine that there was no difference between total antioxidant capacity (TAS) and total oxidant capacity (TOS) between patients and controls (35). Yilmaz et al. (36), reported that TOS levels increased in migraineurs compared to control, TAS levels did not change. Alp et al. (37) suggest that TOS is increased in migraine without aura, TAS is decreased. The results of the same study show that the vasoactive regulator is a new mechanism and the neurohormonal effect of CGRP is regulated by matrix metalloproteinase-2 (MMP-2) (38). In one review, statins are reported to lower cholesterol, increase endothelial nitric oxide (eNOS), disrupt β-amyloid production and serum apolipoprotein E levels, modulate learning associated receptors and MMPs, reduce reactive oxygen species, and increase cerebral blood flow (39). In this study, we aimed to investigate the effects of AT on migraine pathophysiology induced by NTG in ovariectomized female rats.

MATERIALS AND METHODS

Animals

Animal experiments Bezmialem University Foundation was approved by the Animal Care and Use Committee (Date: 29.10.2015, No: 2015/32). In the study, 42 3-month old adult Sprague Dawley female rats were placed in cages in groups of two or four in a temperature-controlled room (22 ± 3 ° C). Standard pellet and tap water were fed ad libitum.

Experimental Procedure

In order to minimize estrogen activity, ovaries of three-month-old female rats were removed under ketamine (35 mg / kg i.p) and xylazine (5 mg / kg i.p) anesthesia. A month later, 4 experimental groups were created. The 1st Group was the Control (n=10): Received a single dose saline 0,5 ml/kg intraperitoneally; The 2nd Group AT (n=10): Received 10 mg/kg AT intraperitoneally (Lipitor, Pfizer); The 3rd Group NTG (n=11): Received 10 mg/kg a single dose NTG (Glyceryl Trinitrate DBL 50 mg/10 ml Ampul, Orna) intraperitoneally; The 4th Group NTG and AT (n=11) Intraperitoneally received single dose 10 mg/kg NTG followed by a single dose 10 mg/kg AT administration. Four hours after NTG or vehicle injections the brains of 3 rats in each group were removed and postfixed for structural histological and immunohistochemistry examinations. The brains of 7 rats in each group, were dissected for biochemical analysis. We observed pain symptoms immediately after the injection (1,40).

Light Microscopy

Following the experimental study, blood samples from the heart were taken under anesthesia, the animals were decapitated, and

the brains were quickly removed. The brains were fixed in 10% neutral formalin (pH = 7.2) for 24 hours and embedded in paraffin using the routine paraffin method (41). 4 µm thickness sections were stained with hematoxylin-eosin (HE) and examined for general histological appearance under a light microscope.

Immunohistochemical Staining Procedure

The tissue slices that were mounted on poly-L-lysine slides were air-dried and deparaffinized. The sections were immunohistochemically stained with iNOS (rabbit Pub Neomarker, RB-1605-P dilution 1: 100), eNOS(rabbit Pab Neomarker, RB-1711-P dilution 1: 100), nNOS (Santa Cruz, rabbit polyclonal, SC-648 dilution 1: 100) MMP-2 (Santa Cruz, mouse monoclonal, SC-7963 dilution 1: 100), β-catenin (Santa Cruz, mouse monoclonal, SC-7963 dilution 1: 200) as previously indicated (10). eNOS and MMP-2 +4 °C 16 hours and iNOS, nNOS and β-catenin reacted with tissue samples for one hour at room temperature. The peroxidase activity was demonstrated using a 3-amino-9-ethylcarbazole substrate kit (Lab Vision, TA-004-HAC) and the sections were counterstained with Mayer's haematoxylin. The control tissue sections were used as positive controls. Slides were photographed and examined using the Kameram 390CU Imaging system (Mikro System Ltd. Turkey).

Biochemical Analysis

Total brain samples were separated and homogenized in PBS. Homogenized samples and blood were centrifuged. c-fos, CGRP, β-catenin, S100B levels, total nitrite/nitrate levels, TAS and TOS in plasma and tissue homogenates were measured by using ELISA kits [Hangzhou Eastbiopharm-CK-E30032 for c-fos, E30375, CGRP., E91556, β-catenin., E91555, S100B., E10868, total nitrite/nitrate., Siemens ADVIA 1200, TAS (42) and Siemens ADVIA1200, TOS (43) as previously described.

Statistical Analysis

Values are reported as mean ± SEM. Statistical analysis was performed using GraphPad Prism version 6,0 for Windows (GraphPad Software, San Diego, CA, USA). One-way ANOVA for repeated measurements with a Bonferroni post-hoc test was used for comparisons; p-value of <0.05 was considered statistically significant.

RESULTS

We weighed the animals at the beginning of experiment. The animals gained weight after one month compared to the time before ovariectomization. During the experimental time, pain-related symptoms such as decreased water and food intake, erection in the hairs, mastication movement, hump position, increase in susceptibility to pain were observed in previously determined pain criteria in NTG injected rats (44, 40).

Histological Findings

Histologically, all the experiment groups had expansions of the blood vessels in the brain, but it was more apparent in nitroglycerin-treated rats.

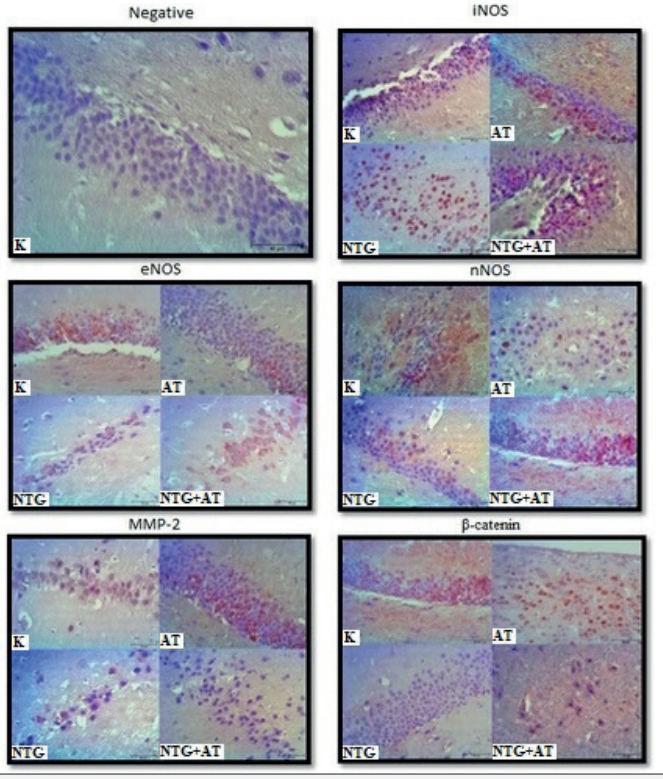

Figure 1. Immunohistochemical detection of iNOS, eNOS, nNOS, MMP-2 and β-catenin staining in total brain slices of rats in control (K) and experimental groups (NTG: Nitroglycerine, AT: Atorvastatin and NTG+AT: Nitroglycerine+Atorvastatin) (Bar:40 µm).

Table 1. Immunostaining intensity in total brain slices of rats in control and experimental groups were assessed by semiquantation of iNOS, eNOS, nNOS, MMP-2 and β-catenin on arbitrary four-point scale (NTG: Nitroglycerine, AT: Atorvastatin and NTG+AT: Nitroglycerine+Atorvastatin) (0= not detectable, += weak, ++= mild and +++= intense,++++=high intense , +++++= very high intense).

Groups	iNOS	eNOS	nNOS	MMP-2	ß-catenin
Control	+	+++++	+++++	+	+++
AT	++	+++	++	+++	+++
NTG	+++++	+	+	+	+
NTG+AT	+++	+++	++++	+	++

Immunohistochemical Findings

The results of MMP-2, iNOS, eNOS, nNOS and β-catenin immunohistochemical staining in the control and experimental groups rats are given in Figure 1. Reaction intensities of iNOS, eNOS, nNOS, MMP-2 and β-catenin positive cells in the total brain sections of rats in all groups are given in Table 1. According to our data, the reaction intensity in iNOS positive cells increased respectively in Control, AT, NTG+AT, NTG; the reaction in eNOS positive cells increased respectively in NTG, AT, NTG + AT, Control; the reaction in nNOS positive cells increased respectively in NTG, AT, NTG+AT, Control; the reaction in MMP-2 positive cells increased respectively in NTG, NTG+AT, Control, AT; the reaction in β-catenin positive cells increased respectively in NTG, NTG+AT, AT, Control.

Biochemical Findings

c-fos Levels in Plasma Samples

Plasma c-fos levels were detected in control group (4.068±0.911), AT group (4.111±0.675), NTG group (4.343±0.582) and NTG+AT group (4.544±0.994). NTG and NTG+AT groups had higher plasma c-fos levels than the control group, but it was not statistically significant (Figure 2).

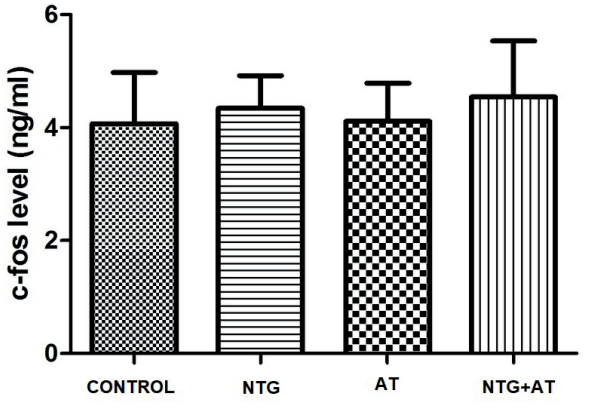

Figure 2. c-fos levels in plasma samples (NTG: Nitroglycerine, AT: Atorvastatin and NTG+AT: Nitroglycerine+Atorvastatin).

c-fos Levels in Brain Homogenate Samples

In the control group brain homogenate c-fos level was (57.596±17.571), in the AT group (48.887±18.252), in the NTG group (66.730±8.859) and in the NTG+AT group (56.209±17.979).

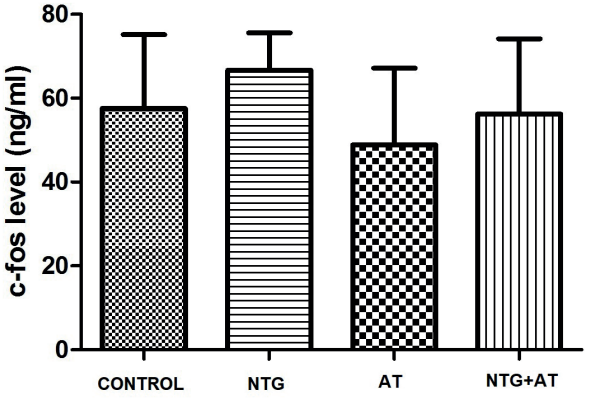

Figure 3. c-fos levels in brain homogenate samples (NTG: Nitroglycerine, AT: Atorvastatin and NTG+AT: Nitroglycerine+Atorvastatin).

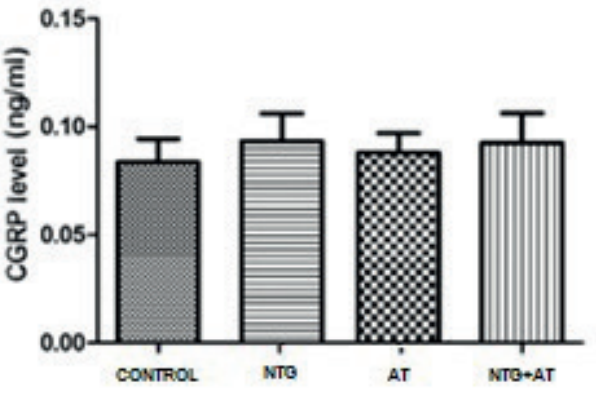

Figure 4. CGRP levels in plasma samples (NTG: Nitroglycerine, AT: Atorvastatin and NTG+AT: Nitroglycerine+Atorvastatin).

Brain homogenate c-fos level was less in the AT group than the control group. c-fos level in brain homogenate was higher in the NTG group than in the control and was less NTG+AT group than in the NTG group, but it was not statistically significant (Figure 3).

CGRP Levels in Plasma Samples

Plasma CGRP level was in the control group (0.084±0.011), AT group (0.088±0.009), NTG group (0.093±0.013) and NTG+AT group (0.011±0.014). Plasma CGRP levels were higher in the AT and NTG groups than the control group, whereas it was low in the NTG+AT group but not statistically significant (Figure 4).

CGRP Levels in Brain Homogenate Samples

In the control group brain homogenate CGRP level was (1.267±0.294), in the AT group (1.176±0.397), in the NTG group (1.394±0.285) and in the NTG+AT group (1.114±0.276). Brain homogenate CGRP levels were found lower in AT and NTG+AT groups than control group whereas in NTG group was found to be higher than the control group but it was not statistically significant (Figure 5).

β-catenin Levels in Plasma Samples

In the control group plasma β-catenin level was (7.806±0.874), in the AT group (8.089±0.689), in the NTG group (8.677±1.138) and in the NTG+AT group (9.125±1.130). Plasma β-catenin levels in other experimental groups were found higher than the control group. In the plasma, NTG+AT administration increased significantly β-catenin levels ($p<0.05$) (Figure 6).

β-catenin Levels in Brain Homogenate Samples

In the control group brain homogenate β-catenin levels was (96.776±28.600), in the AT group (93.569±28.549), in the NTG group (105.874±25.722) and in the NTG+AT group (87.018±22.421). Brain homogenate β-catenin levels were found lower in AT and NTG+AT groups than in the control

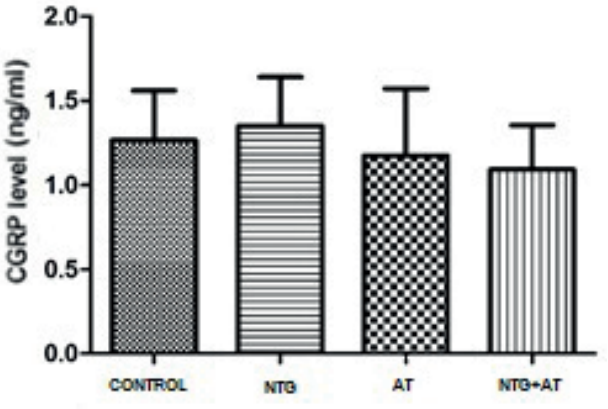

Figure 5. CGRP levels in brain homogenate samples (NTG: Nitroglycerine, AT: Atorvastatin and NTG+AT: Nitroglycerine+Atorvastatin).

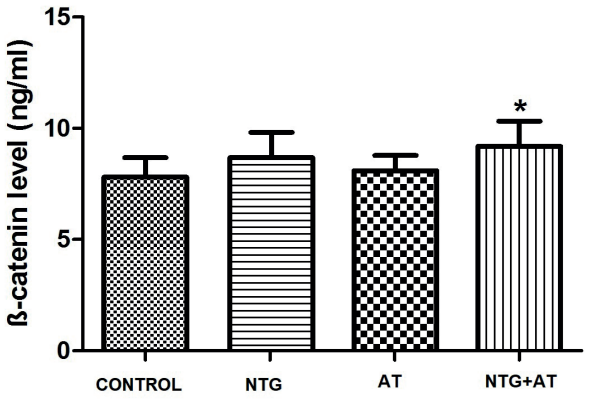

Figure 6. β-catenin levels in plasma samples. * Statistical significance compared to control group (*$p<0.05$) (NTG: Nitroglycerine, AT: Atorvastatin and NTG+AT: Nitroglycerine+Atorvastatin).

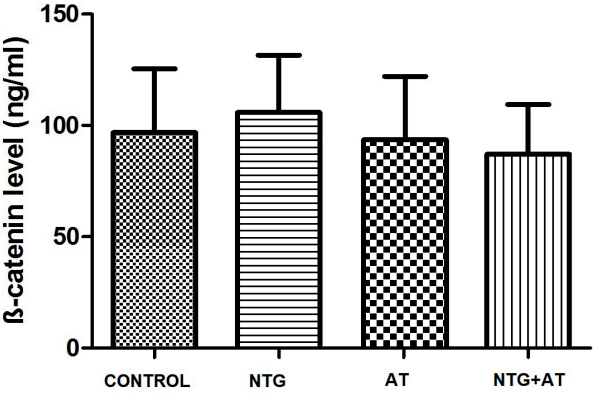

Figure 7. β-catenin levels in brain homogenate samples (NTG: Nitroglycerine, AT: Atorvastatin and NTG+AT: Nitroglycerine+Atorvastatin).

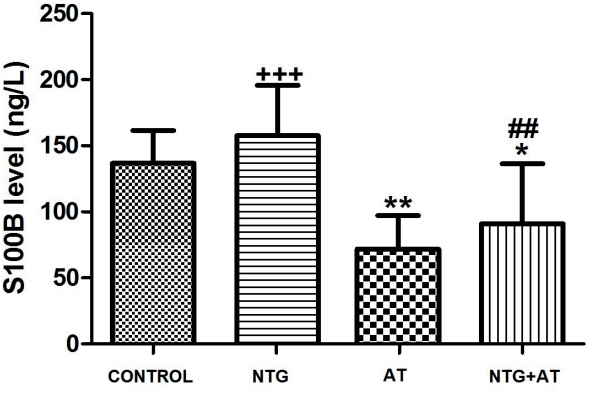

Figure 8. S100B levels in plasma samples. * Statistical significance compared to control group (*$p<0.05$, **$p<0.01$), + Statistical significance compared to atorvastatin group (+++$p<0.001$), # Statistical significance compared to nitroglycerine group (##$p<0.01$) (NTG: Nitroglycerine, AT: Atorvastatin and NTG+AT: Nitroglycerine+Atorvastatin).

group, in NTG group were found higher than in control group but it was not statistically significant (Figure 7).

S100B Levels in Plasma Samples

In the control group plasma S100B levels were (136.870±24.609), in the AT group (71.837±25.420), in the NTG group (157.528±38.172) and in the NTG+AT group (90.924±45.498). Plasma S100B levels were found to be significantly lower after administration of AT ($p<0.01$) and NTG+AT ($p<0.05$). Plasma S100B levels were significantly higher in the NTG group than the AT group ($p<0.001$). Plasma S100B levels were found to be significantly lower after NTG+AT administration than NTG group ($p<0.01$) (Figure 8).

S100B Levels in Brain Homogenate Samples

In the control group brain homogenate S100B level was (1828.581±1370.250), in the AT group (2141.891±777.314), in the NTG group (2510.610±746.165) and in the NTG+AT group (1989.955±865.747). S100B levels in brain homogenate samples were found higher in AT, NTG and NTG+AT groups compared to the control group. In addition, S100B levels in brain homogenate were found to be lower in NTG+AT group compared to the NTG group but it was not statistically significant (Figure 9).

Total Nitrite and Nitrate Levels in Plasma Samples

Plasma total nitrite and nitrate levels were detected in the control group (33.230±3.297), AT group (41.281±7.629), NTG group (59.416±9.364) and NTG+AT group (51.324±11.095). In the plasma samples, NTG and NTG+AT administration significantly increased the total nitrite and nitrate levels grade compared to the control group and also compared to the AT group ($p<0.001$). Total nitrite and nitrate levels in plasma samples was found lower in NTG+AT group than NTG group but there was no statistically significant difference (Figure 10).

Total Nitrite and Nitrate Levels in Brain Homogenate Samples

Brain homogenate total nitrite and nitrate levels were detected in the control group (295.326±68.460), AT group (401.047±90.322), NTG group (676.549±141.254) and NTG+AT group (456.802±96.525). Total nitrite and nitrate levels in NTG ($p<0.001$) and NTG+AT ($p<0.05$) were found significantly higher than the control group. Total nitrite and nitrate levels in NTG group were found to be significantly higher than the AT group ($p<0.01$), whereas in the NTG group (after AT administration) these were found significantly lower ($p<0.01$) (Figure 11).

TAS Levels in Plasma Samples

In the control group plasma TAS level was (1.232±0.095), in the AT group (1.222±0.113), in the NTG group (1.1155±0.047) and in the NTG+AT group (1.107±0.089). TAS levels in plasma samples in NTG+AT group were found to be significantly lower

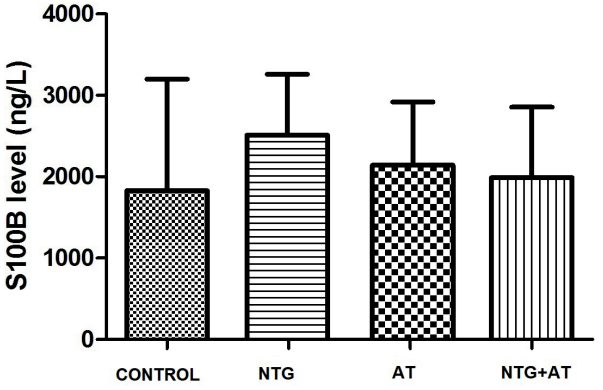

Figure 9. S100B levels in brain homogenate samples (NTG: Nitroglycerine, AT: Atorvastatin and NTG+AT: Nitroglycerine+Atorvastatin).

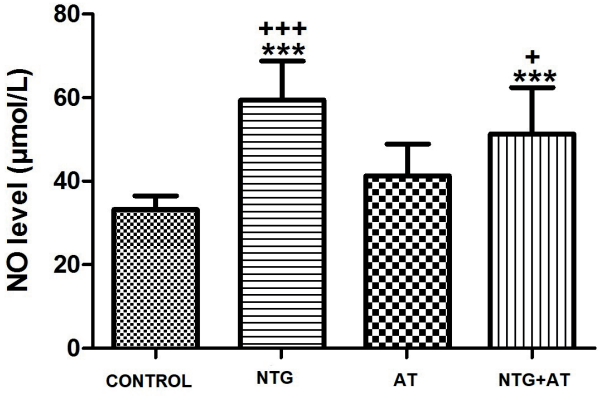

Figure 10. Total nitrite and nitrate levels in plasma samples. * Statistical significance compared to the control group (***$p<0.001$), + statistical significance compared to the atorvastatin group (+$p<0.05$, +++$p<0.001$) (NTG: Nitroglycerine, AT: Atorvastatin and NTG+AT: Nitroglycerine+Atorvastatin).

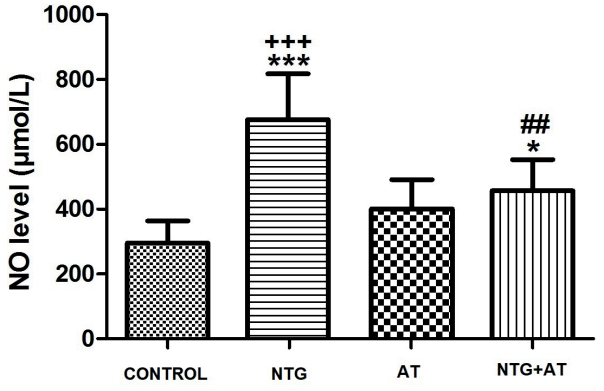

Figure 11. Total nitrite and nitrate levels in brain homogenate samples. * Statistical significance compared to control group (*$p<0.05$, ***$p<0.001$), + Statistical significance according to atorvastatin group (+++$p<0.001$), # Statistical significance compared to the nitroglycerin group (# $p<0.01$) (NTG: Nitroglycerine, AT: Atorvastatin and NTG+AT: Nitroglycerine+Atorvastatin).

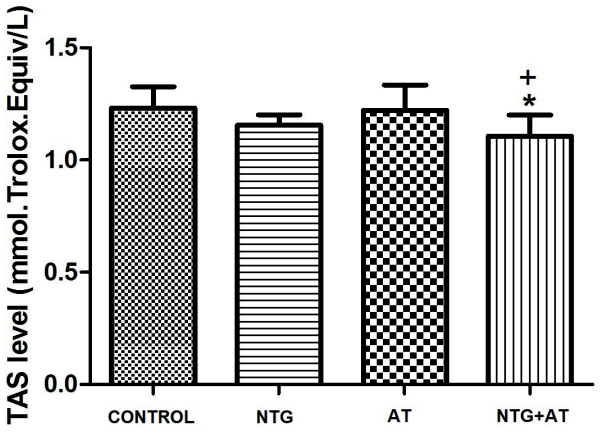

Figure 12. TAS levels in plasma samples. * Statistical significance compared to control group (*p<0.05), +Statistical significance compared to the atorvastatin group (+p<0.05) (NTG: Nitroglycerine, AT: Atorvastatin and NTG+AT: Nitroglycerine+Atorvastatin).

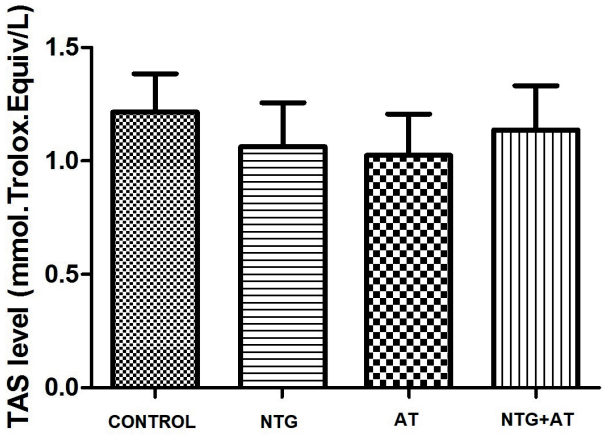

Figure 13. TAS levels in brain homogenate samples (NTG: Nitroglycerine, AT: Atorvastatin and NTG+AT: Nitroglycerine+Atorvastatin).

compared to the control and AT groups (p<0.05), whereas the NTG+AT group was found lower than the NTG group, but there was no statistically significant difference (Figure 12).

TAS Levels in Brain Homogenate Samples

In the control group, brain homogenate TAS levels were (1.215±0.169), in the AT group (1.025±0.182), in the NTG group (1.063±0.194) and in the NTG+AT group (1.137±0.195). TAS levels in brain homogenate samples in AT, NTG and NTG+AT groups were found to be lower than the control. Also TAS levels in brain homogenate samples in NTG+AT was found higher than NTG but there was no statistically significant difference (Figure 13).

TOS Levels in Plasma Samples

In the control group, plasma TOS levels were (12.332±1.619), in the AT group (12.378±1.457), in the NTG group (12.765±1.627) and in the NTG+AT group (11.534±1.873). Plasma TOS levels in

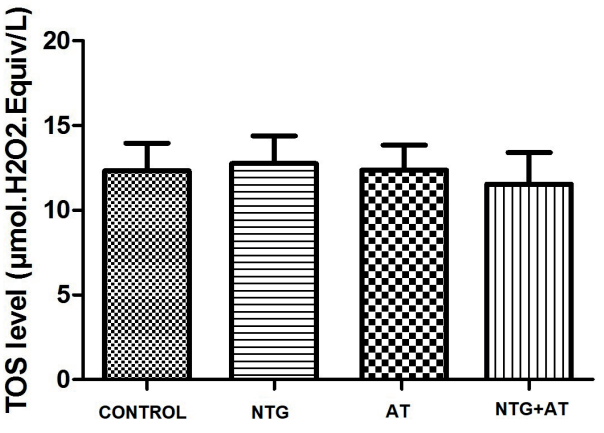

Figure 14. TOS levels in plasma samples (NTG: Nitroglycerine, AT: Atorvastatin and NTG+AT: Nitroglycerine+Atorvastatin).

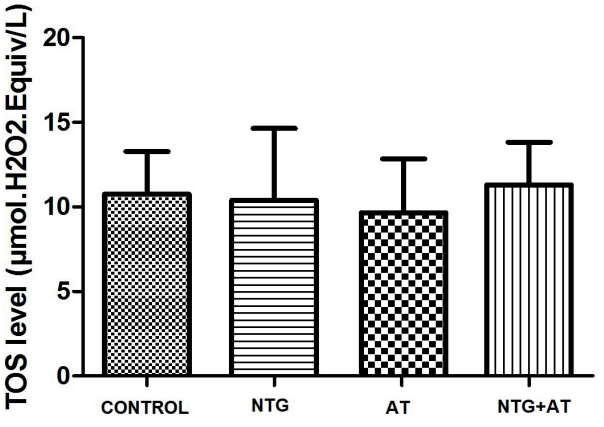

Figure 15. TOS levels in brain homogenate samples (NTG: Nitroglycerine, AT: Atorvastatin and NTG+AT: Nitroglycerine+Atorvastatin).

NTG were higher than the control and AT groups, whereas in NTG+AT, the levels were found lower than NTG, but there was no statistically significant difference (Figure 14).

TOS Levels in Brain Homogenate Samples

In the control group, brain homogenate TOS levels were (10.750±2.525), in the AT group (9.655±3.188), in the NTG group (10.397±4.242) and in the NTG+AT group (11.301±2.518). TOS levels in NTG+AT were found higher than the control, but there was no statistically significant difference (Figure 15).

DISCUSSION

Migraine is a disease characterized by a severe, recurrent headache associated with nausea, vomiting and photophobia. In this study, we investigated whether or not pain developed in the experimental migraine model resulting from NTG administration in ovariectomized female rats and whether there was a protective effect of AT on histological and biochemical changes in rats. To this end, some proteins and NOS levels such

as CGRP and, c-fos which play a role in migraine in both brain homogenates and plasma specimens were investigated. Also, levels of S100B, a marker of brain damage in plasma and total brain homogenates were measured.

Although the mechanism is still not very clear, the chemical vasodilators that trigger migraines are used in the formation of the neuronal activation response and in the observation of vascular changes. It was used to form an experimental migraine, and the NO donor NTG cause neuronal activation at the level of the trigeminal nucleus caudalis and midbrain (45). NTG increases expression of some key molecules such as NF-κB (46), c-fos (28-30), nNOS (47-50) in TNC where most trigeminovascular nociceptive afferents (51, 52). The vasodilator effect of NTG occurs 2-6 hours after administration (53). It has been shown that attacks of a headache in migraine patients are triggered 4 to 6 hours after intravenous administration of NTG. In experimental studies, a dose of NTG (10 mg / kg) used which was higher than the equivalent dose used to induce migraine in humans (1, 12). In our study, ovariectomized female rats were given i.p. administered 10 mg/kg NTG. (40) and in contrast to Erdener and Dalkara (1) we observed pain symptoms immediately after this injection (31). In our study, it was observed that rats hind extremities were held low on the floor, they had difficulty walking, and they searched for a comfortable position. Furthermore, they experienced itching, abnormal sleep, erection of hairs, and an increased sensitivity to sound. These symptoms indicate that NTG causes pain and migraine in rats. NO donors and other potent vasodilators cause an immediate headache and vasodilation of cranial blood vessels in migraine patients and volunteers and animal studies (54, 55). Our findings of pain are parallel to these studies. During migraine, the TNC is warned and remains over active for a long time. Perivascular nociceptive afferents are activated, vasoactive mediators (CGRP, substance P, neurokinin A and pituitary adenylate cyclase-activating polypeptide-PACAP) are released and mast cell degranulation causes neurogenic inflammation in the dura mater (1). Also, CGRP plays an important role in migraine pathophysiology, leading to cranial vasodilatation and facilitation of nociception (56). The key role of CGRP in many regions within the trigeminal system indicates that it participates in migraine pathophysiology (57, 58). In addition, it is suggested that CGRP concentration increases in serum and saliva of patients suffering from acute migraine and headache (58-60). We used ovariectomized female rats in this study and waited 30 days to reset the estrogen (61). Estrogen increases the activity of NOS, NO levels are therefore higher in women. The presence of rich estrogen receptors in the trigeminal system supports that estrogen is directly related to pain. On the other hand, changes in estrogen level have been shown to cause changes in the amount of molecules that are important in the transmission of pain signal such as serotonin and gamma-aminobutyric acid. Recently, changes in depression in the presence of estrogen and progesterone may be related to the frequency of increased migraine aura and to facilitating synaptic transmissions of sexual hormones (62). In a study conducted, it was shown that removal of ovaries reduced CGRP levels (63).However, the neurobiological mechanisms related to the migraine regulatory effects of estrogens are controversial. In our study CGRP statistically insignificantly increased in both plasma and brain homogenate samples in the NTG group compared to the control group which may be due to the absence of ovaries and thus the absence of estrogen.

Studies using monoclonal CGRP or CGRP receptor antagonists in migraine therapy are promising (64). It has been reported that the effect of NO and CGRP on migraine is very important and that NOS inhibition significantly inhibits migraine attacks (65). Plasma nitrite and nitrate, CGRP and c-fos expression have been shown to increase with NTG administration (27). In the same study, it is stated that the effect of Gas-D obtained from Tianma, which has been used for a long time in China for treatment, is shown by decreasing plasma nitrite/nitrate and CGRP level. In our study, a significant ($p < 0.01$) reduction of NO in the brain homogenate samples of the treatment group supports that AT may be useful for migraine pathology. However, we believe that there is a need for more detailed studies on this subject.

By stimulating the trigeminal vasculature, CGRP is released and c-fos expression is increased (31). The activation of the c-fos gene begins within 5 minutes of the stimulation and continues until the end of the stimulation (1). Trigeminal nerve activation was demonstrated by increased expression of ipsilateral c-fos in the caudal trigeminal nucleus of CSD (66). It is indicated that c-fos expression is maximal after 4 hours of NTG injection and animals are perfused after 4 hours from 10 mg/kg NTG injection (67). In our study, there was no significant difference between c-fos levels in either plasma or homogenate samples. Given that CGRP leads to a c-fos increase, the lack of estrogen in our study due to the use of ovariectomized rats can explain the fact that the non-significant CGRP increase is not sufficient enough to increase c-fos levels. Considering that c-fos protein can be detected immunohistochemically in neuronal nuclei for approximately 30 minutes post-stimulation and has a half-life of 2 hours after the end of the stimulation (1), a nonsignificant increase in c-fos levels may be due to the termination of the experiment after 4 hours (68). β-catenin is one of the active paths in brain development and neurodegenerative diseases. β-catenin has been shown to play an important role in the expansion of the precursor pool, in neuronal movement and in cortical lamination (69). In one study, the relationship between the acceleration of seizure induction for the first time after ischemia and the signal pathway of astrocytic β-catenin was demonstrated. Activation or inhibition of the Wnt/β-catenin pathway after pentylenetetrazole administration has been reported to be effective on c-fos expression (70). Prévotat et al. (25) indicated that NO is effective by reducing the amount of β-catenin in colon cancer. In our study, the plasma β-catenin levels were significantly increased in NTG+AT group compared to the control group ($p < 0.05$), whereas in brain homogenates AT and NTG+AT groups were decreased compared to the control group but it was not statistically significant. In recent years, β-catenin effects of anticonvulsants in different brain regions have been studied. Antiepileptic and antidepressant drugs are used in the treatment of migraine (71). In the rats exposed to valproic acid,

an accumulation of β-catenin was shown in the cytoplasm and nucleus (26). We have shown the anticonvulsant effect of AT in our previous studies (9). In this study, unlike valproic acid, AT administration resulted in a nonsignificant decrease in β-catenin levels in brain homogenate, however β-catenin levels significantly increased in plasma. This increase might be the result of the arrival of β-catenin from different sources. These differences may be due to the animal species used, the lack of estrogen, the amount of the substance given, and the duration of the application.

BBB injury is responsible for the pathogenesis of cerebrovascular diseases. In migraine patients an increase in the concentration of serum S100B has been reported (32,33). Brain-specific proteins are involved in many neuropathologies due to intracellular and extracellular functions. The measurement of astroglial protein S100B, which is one of these proteins, especially in serum is essential in showing BBB damage index (34). Yilmaz et al, (2011) showed in their study that patients with migraine-without aura had significantly higher ictal serum levels of S100B and NSE than control subjects. Whereas in the interictal phase, there was a significant increment only in S100B levels compared to controls. These results show that Increased interictal serum levels of S100B might point to an insidious and slow damaging process in a migraine patient (33). The use of drugs used in the treatment of migraine with aura is controversial (72). NTG administration in our study increased the levels of S100B in plasma samples, compared to the control group. In animals treated with NTG+AT, plasma S100B levels were significantly lower compared to NTG group ($p<0.01$). S100B levels in brain tissue homogenate samples in NTG+AT group, compared to NTG group, there was a nonsignificant decrease. Independent of cholesterol-lowering effects of statins in recent years is reported to have neuroprotective and anti-inflammatory effects on neurodegenerative diseases as an antioxidant (7). In our previous study we determined the healing effects of AT on epileptic seizures and memory disorders (9). Our findings showed that atorvastatin could inhibit astroglial activity by its anti-inflammatory effect and showed a protective effect towards BBB and so reduce the risk of possible neurodegenerative diseases in rats with migraine. Our results will provide a significant contribution to studies in this field.

NO is a small gas molecule that plays various roles in the central nervous system (73). Statins are reported to reduce iNOS activity and increase eNOS expression to reduce the inflammatory reaction involving neuropathic pain (74, 75). Also, statins show neuroprotective effects by decreasing microglial and astrocyte activation and cytokine release (76). In our study, we investigated the distributions of eNOS, nNOS and iNOS in sections the brains of control and experimental animals. In this study, we found that while eNOS decreased, iNOS increased in migraine. With the treatment of AT, we found that iNOS decreased and eNOS increased. Also, nNOS has been shown to be functional in many diseases based on migraine, strokes, epilepsy, Huntington's disease and disorders of the nervous system (77). Experimental and clinical studies demonstrated that statins have protective effects in addition to reducing cholesterol. For example statins, control endothelial function through cholesterol-dependent and independent mechanisms or pleiotropic effects and provide eNOS expression (78). These studies support our findings of NOS reactions. In our study, we also measured the total nitrite-nitrate amount in plasma and brain homogenate by ELISA method. Plasma NO levels increased significantly in NTG group compared to the control group but decreased in NTG+AT group compared to NTG. NO levels in brain homogenates increased statistically significantly in NTG group compared to the control group. Brain homogenate NO levels increased in NTG compared to the control group but in NTG+AT group were significantly decreased compared to NTG. Continuous release of cytokines, prostanoids, and NO into the subarachnoid space causes long-term activation of perivascular nociceptive trigeminal afferents in the pia mater (79). As a donor of NO, it is suggested that NTG may affect migraine attacks and may be used to experimentally investigate the mechanisms in migraine pathophysiology (22, 80). These studies support our results. In animal models, it is suggested that drugs that are effective in the treatment of migraine are effective by raising the threshold of initiation of cortical spreading depression (60). Headaches vary depending on ovulation, menstruation, pregnancy, menopause, drugs, reproductive cycle (5, 81). It is suggested that these effects are caused by the rich estrogen receptors on the trigeminal system of estrogen (82). Moreover, it has been observed that estrogen levels may cause changes in molecules such as serotonin and gamma-aminobutyric acid which are involved in pain signal transmission (83). Pro-nociceptive effects of estrogens have been shown.

In recent studies, it has been suggested that estrogen and progesterone increase the frequency of migraine aura by facilitating synaptic transmission (62). In our study, we aimed to remove the possible stimulant effect of estrogen by using ovariectomized rats. Our results showed that the adverse effects of NTG-induced could be improved by AT administration. This conclusion supports the theory that AT may be used as an alternative component in the treatment of migraine.

Migraine is known as a long-lasting neurological disease, but its physiopathology is not clear. Studies support the relationship between migraine and oxidative stress. It was reported that in a study of 151 patients with migraine there was no difference between TAS and TOS between patients and controls (35). It was showed that NO levels elevated in plasma and platelets during migraine (84-86). NO, and its metabolites have been reported to be oxidative stress markers in migraine patients (87). When we compared plasma TAS and TOS levels in our study, we did not find a significant difference. Our results are consistent with the result of other studies. TAS is a method used to detect antioxidant activity. Yilmaz et al. (36), reported that TOS levels increased in migraineurs compared to control, TAS levels did not change. Alp et al. (37) suggest that whileTOS is increased in migraines without aura, TAS is decreased. In another study, antioxidant capacity was found to be lower in patients with chronic migraine. Chronic migraine is associated with mitochondrial dysfunction, and is effective in the balance between oxidative and antioxidant status; it is not

clear how the drugs used in migraine affect the oxidative stress parameters (88,89). In our study, there was an increase in TOS levels in the migraine group compared to the control group but it was not statistically significant. There was no significant change in TAS levels between the groups. Our results are consistent with the literature in this area. We found a significant decrease in antioxidant capacity in the plasma samples of the treatment group. Results of studies related to oxidant and antioxidant system failure in migraine patients are also different from each other. Further detailed studies are needed for a better understanding of what happens during migraine attacks, migraines and follow on work on oxidant and antioxidant balance.

The trigeminovascular system is located outside the BBB and has an important role in migraine pathology. Therefore, the new CGRP drugs that are effective at treating migraines target the trigeminovascular system (38, 90). It has been suggested that vascular MMP-2 breaks down endogenous CGRP and induces vasoconstriction. The results of the same study show that vasoactive regulatory is a new mechanism and the neurohormonal effect of CGRP is regulated by MMP-2 (38). In our study, brain sections were stained with MMP-2 immunohistochemically. The distribution of MMP-2 positive cells was similar in the control, NTG and NTG+AT groups. MMP-2 positive cells were slightly higher in the AT-treated group than in the other groups. In one review, statins are reported to lower cholesterol, increase eNOS, disrupt β-amyloid production and serum apolipoprotein E levels, modulate learning-associated receptors and MMPs, reduce reactive oxygen species, and increase cerebral blood flow (39). In our study, we did not find any significant effect of the NTG and AT on the MMP-2. These results may be due to the short duration of application of the substances we used.

CONCLUSION

In conclusion, histological and biochemical changes in the migraine model developed with nitroglycerin in this study and atorvastatin that we applied on ovariectomized rats for treatment was determined to have positive effects on this damage. Histologically, the expansion of blood vessels in the brains of rats administered with nitroglycerin was seen to be more apparent. Studies on the association between migraine sufferers and the oxidant-antioxidant system failure show disparities. We also compared the level of serum TAS and TOS, we observed no significant difference. We can say that AT generates these effects by increasing eNOS and nNOS levels, reducing the production of iNOS and S100B, and reducing the levels of CGRP and c-fos in sample brain homogenates. Also, our findings showed that AT could inhibit astroglial activity by its anti-inflammatory effect and showed a protective effect towards BBB and so reduce the risk of possible neurodegenerative diseases in rats with migraine. Our results will provide a significant contribution to studies in this field.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of study: K. A. D., A. C. H.; Data Acquisition: K. A. D., A. C. H.; Data Analysis/Interpretation: K. A. D., A. C. H.; Final Approval and Accountability: K. A. D.; Drafting Manuscript: K. A. D.; Critical Revision of Manuscript: K. A. D..

Acknowledgement: We thanks to Facultad Ciencias Exactas Naturales, Universidad Nacional Catamarca (FACEN-UNCA) and Instituto Biodiversidad Neotropical (IBN-UNT) by facilities and other assistances. For the loan of specimens we thank to J. Andreoli Bize (FACEN).

Conflict of Interest: The author has no conflict of interest to declare.

Financial Disclosure: Istanbul University Scientific Research Projects Coordination Unit, 50185.

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RESEARCH ARTICLE

Eur J Biol 2019; 78(1): 23-28

Metal Bioaccumulation of *Mytilaster lineatus* (Gmelin, 1791) Collected from Sinop Coast in the Southern Black Sea

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Please cite this article as: Bat L, Sahin F, Oztekin A. Metal Bioaccumulation of *Mytilaster lineatus* (Gmelin, 1791) Collected from Sinop Coast in the Southern Black Sea. Eur J Biol 2019; 78(1): 23-28.

ABSTRACT

Objective: Metal bioaccumulation of the bivalve *Mytilaster lineatus*, based on different seasons and stations was investigated in Sinop Peninsula of the Black Sea. Soft tissues of mussels from Gazibey Rock and Içliman were taken during the spring and the summer of the year 2016 and were analyzed for Hg, Cd, Pb, Cu and Zn.

Materials and Methods: The soft tissues of the mussels digested with Suprapur® HNO_3 (nitric acid) using a Milestone Systems, Start D 260 microwave digestion system. The accuracy and precision of the method was verified by the simultaneous analysis of the certified reference materials NIST 2976 (mussel tissue) for the samples. The Inductively Coupled Plasma–Mass Spectrometer (ICP-MS), Agilent Technologies, 7700x was used for metal analyses.

Results: Results showed that higher concentrations of almost all metals in Içliman than those in Gazibey Rock. However, no significant differences were detected between seasons except for both Cu and Zn. The metal amounts of *M. lineatus* ranged between 0.024-0.035, 0.054-0.072, 0.13-0.25, 0.64-0.85 and 6-20 mg kg-1 wet wt. for Hg, Cd, Pb, Cu and Zn, respectively.

Conclusion: The measured metals in both sampling areas did not exceed the threshold set by the European Commission and Turkish Food Codex. The results of this study also confirmed the potential of *M. lineatus* to be used as bio-indicators of heavy metal pollution.

Keywords: Heavy metals, bioaccumulation, bio-indicator, *Mytilaster lineatus*, Black Sea

INTRODUCTION

Large amounts of contaminants have been released into the Black Sea over the last several decades (1). Among these contaminants, heavy metals have long been recognized as one of the major problems of the Black Sea (2). Heavy metals are largely loaded to the coastal zone of the Black Sea from rivers, domestic sources, agricultural, fisheries and touristic activities (1). Metal pollution in the Black Sea coasts has been intensively studied in recent years because of the fact that these heavy metals are persistent toxic, prone to bioaccumulation and hazard a risk to people and coastal ecosystems (3).

The comprehension of the bioaccumulation fact of heavy metals in the living biota is of an extreme complexity. This is because of the complex of different parameters which influence this bioaccumulation like the physico-chemical characteristics of surrounding waters of the studied area, the chemical properties of the pollutants and the biologic factors of the organism (4,5).

Marine mussels are a very large group of Mollusca ubiquitous in the seas. They are very important organisms on the rocky and gravel bottoms of the Black Sea and are important prey source for a variety of marine organisms, including fish, crabs and humans and their utilizing contribute to economies (6).

Mytilaster lineatus is a genus of marine mussels from the waters of the Atlantic Ocean and Mediterranean Basin. *M. lineatus* is widely distributed in the Black Sea and is

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Submitted: 05.02.2019 • **Revision Requested:** 26.02.2019 • **Last Revision Received:** 18.03.2019 • **Accepted:** 22.03.2019

one of the most dominant mollusc species of Sinop Peninsula fauna (7). However, data regarding the biology, ecology and evolution of *M. lineatus* is still limited. It inhabits rocky substrata in shelf waters ranging from the shallow to the deeper continental slope. It is known that mussels feed on suspended matter filtered by water. Diatoms (such as *Coscinodiscus* sp., *Chaetoceros* sp., *Nitzscia* sp. and *Pleurosigma* sp.), microscopic algae and detritus are usually found in the contents of their stomach (8). In recent years, therefore, the studies on mussels belonging to the Mollusca phyla have been investigated, with a focus on heavy metal contamination. Heavy metals are accumulated by mollusks with no mobility which tend to be highly vulnerable because they feed on suspended organic particles which is bond of various toxic metals. Thus, they often contain high levels of heavy metals when compared with other marine organisms (9-13).

The main studied tissue for metal content in mussels is the whole soft edible part due to its relevance for human health. There is no pollution source in Gazibey Rock, whereas Içliman receives domestic waste. In addition, fishing activities are active in Içliman (14,15).

Moreover, there is no available data on metal bioaccumulation in *M. lineatus* in the Black Sea. Thus, the present study constitutes using *M. lineatus* as bio-indicator species for the first time, a contribution to investigate the heavy metal accumulation in this species in the Black Sea.

MATERIALS AND METHODS

The area of Sinop Inner Harbour has been chosen as the research area. In the selection of the stations namely Gazibey Rock and Içliman, where the mussels were abundantly identified (Figure 1).

Gazibey Rock is a small island with a maximum depth of 24 meters. While the north facing side descends vertically, the other side slopes and gradually deepens. During the research, the samples were taken from the northern part of the rocks of Gazibey.

The average depth at Içliman station, which is a natural harbour, is 15 meters. The ground has a fairly flat and sandy structure. The floor of the selected area is covered with dead mussel shells, pebbles, algae and marine litter due to the use of port.

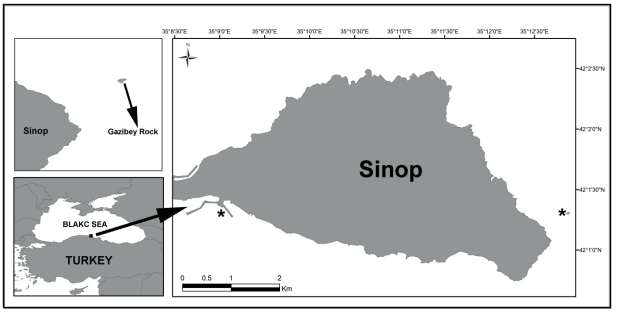

Figure 1. Sampling areas

Preparation of Mussel Samples

M. lineatus has a wide distribution on the Sinop shores and tolerance range for different salinities and temperatures and has sufficient size, sessile life form and is robust in laboratory conditions, making bivalves the prime candidate for studying the bioaccumulation of heavy metals. *M. lineatus* individuals (Figure 2) were collected randomly during each sampling occasion from Gazibey Rock and Içliman by SCUBA diving at the depth of 20 and 10 meters, where the mussel settlements were most dense, respectively. The samples were put in plastic tanks filled with seawater from each station, then immediately transported to the laboratory. About 20 individuals were chosen randomly according to their shell length measurements. There was no significant difference among the shell lengths between different samplings (p>0.05, one-way ANOVA). For each sampling, specimens were pooled together after a 48 h depuration of their gut contents in filtered clean seawater (11-13). The shells were opened and soft tissues were carefully taken (16). The soft parts of the specimens were put in sterilized petri dishes, weighed and stored at a temperature of -21°C until metal analysis (17,18).

Figure 2. *Mytilaster lineatus*

Determination of Metals

The soft tissues of the mussels digested with Suprapur® HNO_3 (nitric acid) using a Milestone System, Start D 260 microwave digestion system. The accuracy and precision of the method was verified by the simultaneous analysis of the certified reference materials NIST 2976 (mussel tissue) for the samples. The certified reference values were 61.0 ± 3.6 for Hg, 0.82 ± 0.16 for Cd, 1.19 ± 0.18 for Pb, 4.02 ± 0.33 for Cu and 137 ± 13 for Zn mg/kg. The results of the elements for the standard reference material were found as 63.2 ± 3.8 mg/kg (104%) for Hg, 0.79 ± 0.12 mg/kg (96%) for Cd, 1.12 ± 0.13 mg/kg (94%) for Pb, 3.92 ± 0.28 mg/kg (98%) for Cu and 129 ± 8.0 mg/kg (94%) for Zn. The results indicated good agreement and all analyses were considered satisfactory, with the Relative Standard Deviation (RSD) percentage which was between 94 and 104 % for the metals. The ICP-MS, Agilent Technologies, 7700x was used for metal analyses.

In addition, some physical and chemical parameters (salinity, pH, conductivity, total dissolved solids, temperature and dissolved oxygen) of sea water were measured at the same time.

Statistical Analysis

In order to distinguish if the observed differences among datasets were statistically significant, analysis of variance (ANOVA) using SPSS 21.0 statistical software was used to investigate the effects of seasons of collection and sampling site on the variations in metal concentrations in the bivalves. The Tukey's (HSD) test was used as post hoc test to indicate the pairs of data which have significant differences (19). The results are expressed in mg kg^{-1} of wet weight.

RESULTS

Sea Water Analysis

Sea water samples were taken from the surface of the stations at the time of sampling and the parameters determined as a result of analysis (salinity, pH, conductivity, total dissolved solids, temperature and dissolved oxygen) are given in Table 1 according to the spring and summer.

Table 1. Physico-chemical parameters of sea water samples

Parameters	Gazibey Rock		Içliman	
	spring	summer	spring	summer
Salinity (‰)	16.8±0.2	17.3±0.2	17.1±0.1	17.2±0.2
pH	7.91±0.3	8.11±0.3	8.18±0.1	8.21±0.2
Conductivity (uS/cm)	27426±14	281432±13	27382±22	28876±31
TDS (g/L)	18.25±0.1	18.1±0.1	18.38±0.1	18.91±0.1
Temperature (°C)	21.1±0.2	21.7±0.1	22.2±0.1	23.4±0.2
Dissolved oxygen (mg/L)	4.91±0.1	4.72±0.1	4.84±0.1	4.65±0.1

Heavy Metals in *Mytilaster lineatus*

Mussels tolerate salinity changes from 5 up to 40‰. There is not much growth in low and extreme salinity values in surrounding waters. Water temperature is an important factor that affects the general condition and distribution of the mussel. Generally suitable for feeding, and growing of the mussels that can tolerate temperature changes up to 2-30°C, the optimum temperature is 8-26°C (6). The results from Table 1 indicated that physico-chemical parameters of sea water were considered suitable for mussel life.

The concentrations of heavy metals in the soft tissues of the mussels collected from the Sinop shores of the Black Sea during the spring and the summer of the year 2016 are shown in Figures 3-7.

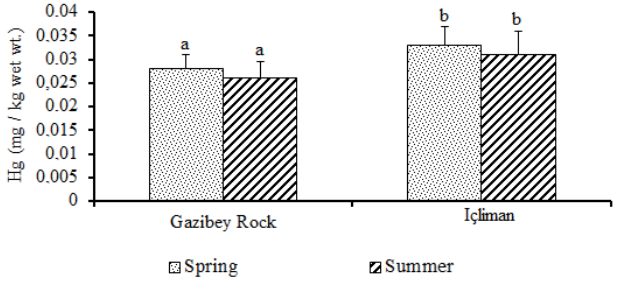

Figure 3. The means with standard errors (vertical line) of Hg amounts (mg/kg wet wt.) in the soft tissues of *Mytilaster lineatus* collected from Sinop shores of the Black Sea during the spring and the summer of the year 2016. a, b = The same letters beside the vertical bars in graph indicate the values are not significantly different ($p > 0.05$).

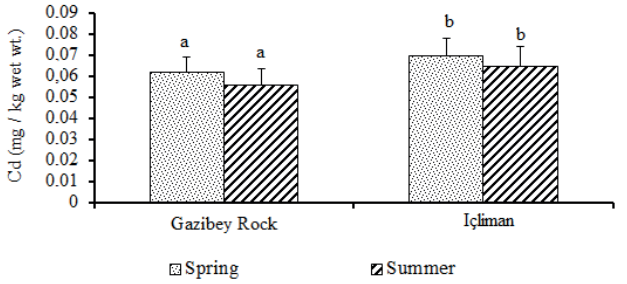

Figure 4. The means with standard errors (vertical line) of Cd amounts (mg/kg wet wt.) in the soft tissues of *Mytilaster lineatus* collected from Sinop shores of the Black Sea during the spring and the summer of the year 2016. a, b = The same letters beside the vertical bars in graph indicate the values are not significantly different ($p > 0.05$).

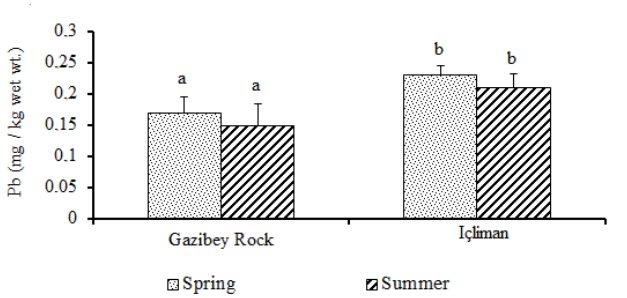

Figure 5. The means with standard errors (vertical line) of Pb amounts (mg/kg wet wt.) in the soft tissues of *Mytilaster lineatus* collected from Sinop shores of the Black Sea during the spring and the summer of the year 2016. a, b = The same letters beside the vertical bars in graph indicate the values are not significantly different ($p > 0.05$).

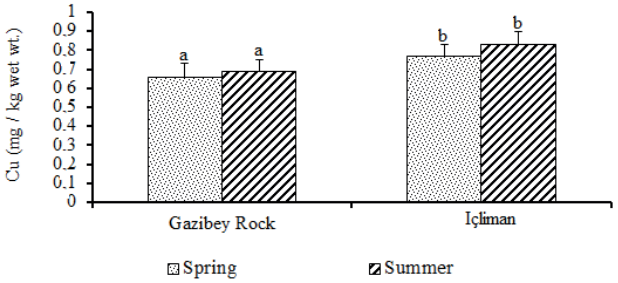

Figure 6. The means with standard errors (vertical line) of Cu amounts (mg/kg wet wt.) in the soft tissues of *Mytilaster lineatus* collected from Sinop shores of the Black Sea during the spring and the summer of the year 2016. a, b = The same letters beside the vertical bars in graph indicate the values are not significantly different ($p > 0.05$).

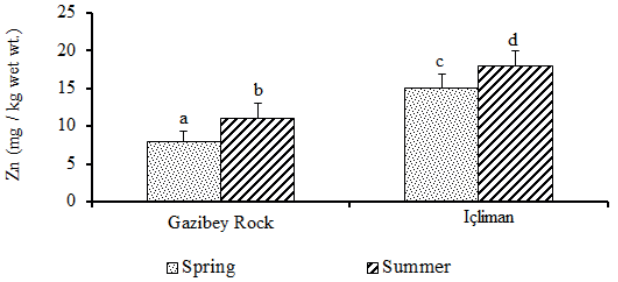

Figure 7. The means with standard errors (vertical line) of Zn amounts (mg/kg wet wt.) in the soft tissues of *Mytilaster lineatus* collected from Sinop shores of the Black Sea during the spring and the summer of the year 2016. a, b, c, d = The same letters beside the vertical bars in graph indicate the values are not significantly different ($p > 0.05$).

Results showed that the levels of metals in the mussels from Içliman were higher than those in the mussels from Gazibey Rock ($p<0.05$). Essential metal levels were higher than non-essential metals in both sampling areas. Concerning the effect of seasons on Hg, Cd and Pb bioaccumulations slightly higher levels of the heavy metals in spring was observed than those in summer. This was vice versa for both Cu and Zn. However, the differences in metal levels between spring and summer were not significant in each sampling area except for Zn ($p<0.05$).

The concentrations of heavy metals in soft tissues of the mussels ranged between 0.024-0.035, 0.054-0.072, 0.13-0.25, 0.64-0.85 and 6-20 mg kg^{-1} wet wt. for Hg, Cd, Pb, Cu and Zn, respectively.

DISCUSSION

The comparison of results with those of EU Guideline and TFC (Table 2) shows that the concentrations of all metals in both sampling areas did not exceed the threshold set by the guideline categories.

Table 2. The tolerable values of measured metals in Mollusca (mg/kg wet wt.)

Standards	**Hg**	**Cd**	**Pb**	**Cu**	**Zn**
MAFF, The Food Safety (20)	--	<0.2	10	20	50
Turkish Food Codex (21)	0.5	1.0	1.5	20	50
Council of Europe (22)	0.5	1.0	1.5	--	--
Commission Regulation (23)	0.5	1.0	1.5	--	--
Turkish Food Codex (24)	0.5	1.0	1.5	--	--

Bat et al. (11) investigated seven heavy metal (Fe, Zn, Mn, Cu, Pb, Cd and Hg) concentrations in *Mytilus galloprovincialis* samples for evaluating marine pollution. In their study high level of these metals found in mussel populations located close to major cities and industrialized areas (11). It was suggested that consumption of about 1 serving of mussels from clean coastal waters per week is enough (11). They also found that there is no health risk for mussel consumers living on the Sinop shores of the Black Sea as we found for *M. lineatus*.

The seasonal variability may result from either internal biological cycles of the aquatic organisms or from changes in the variability of metals in the ecosystem. The seasons of spring and summer are with high biological activities which cause an increment in the metabolic rate and eventually increase the oxygen use and uptake of dissolved metals via the gills. Additionally these seasons could be related to the reproductive period which could lead to considerable energy consumption, decline the detoxification ability and increase the metal bioaccumulation (25,26).

Unfortunately, there is no study with *M. lineatus*. For this reason, we compared the data obtained in our study with other Mollusca species especially *Mytilus galloprovincialis* from other coasts of the Black Sea. Large differences in heavy metal amounts in Bivalve species were found between different localities of the Black Sea.

In Turkish coasts of the Black Sea, the highest values of Zn and Pb in Trabzon and Cu in Artvin were found. The order of the metal values obtained in all studies conducted on the Turkish coast of the Black Sea are listed as Zn> Cu> Pb> Cd> Hg (11). The highest values of Zn, Cu and Pb in mussels were found on the shores of Giresun, Trabzon, Artvin and Rize coasts of the Turkish Black Sea were determined by Baltas et al. (27).

On the Russian coasts of the Black Sea Cu (8.83±2.71 mg/kg dry wt.) and Pb (3.07±0.85 mg/kg dry wt.) values were highest in Blue Bay (28). Zn (106-196 mg/kg dry wt.) and Cd (1.9-3.4 mg/kg dry wt.) values were highest in Inal Bay (29) and Hg (3-83 mg/kg wet wt.) values were observed on the Crimean coasts (30). When the maximum values of the metals are taken into consideration, the order of the metal values obtained in all studies conducted on the Russian coasts of the Black Sea is Zn>Cu> Pb> Cd> Hg.

Cu, Cd and Pb amounts studied on the border between Ukraine and Romania were 0.57-1.31, 0.07-0.23 and 0.07-0.163-83 mg/kg wet wt., respectively (31).

In Romania the highest values of Cu (6.5 mg/kg wet wt.), Pb (11.02 mg/kg wet wt.) and Cd (3.3 mg/kg wet wt.), were observed on Navodari, South Constanta and Vama Veche coasts of the Black Sea, respectively (32). However, Zn (190±18 mg/kg dry wt.) and Hg (33±2 mg/kg dry wt.) values on the highest North Efoire coasts of the Black Sea (33). When the highest amounts of heavy metals are taken into consideration, the order of the metal values on the Romanian coast of the Black Sea is as follows: Zn> Cd> Cu> Pb> Hg.

The highest values of Zn (104.4-239.2 mg/kg dry wt.) and Cd (0.98-2.24 mg/kg dry wt.) were found in the Gulf of Varna (34). In Cape Galata, the highest of both Cu (24.2±2.2 mg/kg dry wt.) and Pb (59.1±6.2 mg/kg dry wt.) were recorded by Gorinstein et al. (35) and Gorinstein et al. (36). The order of the metal values obtained in the studies carried out on the Bulgarian coast is found as Zn> Cu> Cd> Pb.

In general, the amounts of studied heavy metals found in *Mytilaster lineatus* were lower than that of *Mytilus galloprovincialis* in the Black Sea coasts.

Molluscs are known to take up and accumulate heavy metals, both essential and nonessential, from the surrounding water and suspended particles. Cu and Zn are essential metals in the organisms. Zn is used as an active centre for metal enzymes and activators of other enzymes systems, while Cu is an integral part of the respiratory pigment haemocyanin. These metals are introduced into the marine coastal environment through different human activities especially urban.

Indicated in many studies (3), bivalves accumulate Cd mostly in hepatopancreas and in kidney in the form of stores in lysosomes and accumulate Cu mostly in hepatopancreas, gonads and gills. In fact, in Mollusca, the blood carries a respiratory pigment depend on Cu, the hemocyanin, which clarifies its circulation in these largely vascularized organs.

CONCLUSION

M. lineatus collected from Sinop shores of the Black Sea during the spring and the summer of the year 2016 have been found to be contaminated with Hg, Cd, Pb, Cu and Zn. Although *M. lineatus* was not preferred as food such as *Mytilus galloprovincialis*, in the current study these measured metals were well under the permissible levels. The results of this study also confirmed the potential of *M. lineatus* to be used as bio-indicators of heavy metal pollution.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of study: L.B.; Data Acquisition: F.S., A.O.; Data Analysis/Interpretation: L.B., F.S., A.O; Final Approval and Accountability: L.B., F.S., A.O.; Drafting Manuscript: L.B; Critical Revision of Manuscript: L.B., F.S., A.O.; Technical or Material Support: L.B.; Supervision: L.B., F.S, A.O.

Conflict of Interest: The author has no conflict of interest to declare.

Financial Disclosure: There are no funders to report for this submission

Acknowledgements: The authors wish to acknowledge the Department of Hydrobiology, Fisheries Faculty, University of Sinop for providing laboratory facilities during the study.

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ISTANBUL UNIVERSITY PRESS

RESEARCH ARTICLE

Eur J Biol 2019; 78(1): 29-39

The Effect of Niclosamide on Certain Biological and Biochemical Properties of *Drosophila melanogaster*

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Please cite this article as: Ustundag G, Buyukguzel K, Buyukguzel E. The Effect of Niclosamide on Certain Biological and Biochemical Properties of *Drosophila melanogaster*. Eur J Biol 2019; 78(1): 29-39.

ABSTRACT

Objective: Laboratory research on management strategies such as biological and chemical control usually demands rearing high numbers of high-quality insects. For this reason, there is a need for detailed information about which antimicrobial agents should be added to their diets in order to obtain high quality insects.

Materials and Methods: Niclosamide, which is an anthelmintic derivative of salisilanilid, was added in amounts of 100, 200, 400 and 800 mg/L into artificial diets of *Drosophila melanogaster* (Meigen). The effects on survival rate, developmental time and adult longevity of the insects were examined. Furthermore, the effect of this anthelmintic antibiotic on important oxidative stress indicators; lipid peroxidation product, malondialdehyde (MDA) and protein oxidation products; protein carbonyl (PCO) contents and a detoxification enzyme, glutathione S-transferase (GST) activity in 3rd instar larvae, pupae and adult stage of the insect were also investigated.

Results: Compared with those insects on the control diet, the tested concentrations of niclosamide significantly decreased survival rate in all developmental stages of the insect. The control diet produced 94.0 ± 1.0 % of 3rd stage larvae whereas in the highest concentration (800 mg/L) this ratio decreased to 14.00 % ± 1.73. While 42.08 ± 0.50 % of the adults survived in the control diet, longevity decreased to 2.30 ± 0.15 days in the highest concentration. All concentrations of niclosamide resulted in increased MDA contents in the last larval stage of *D. melanogaster* and concentrations of 100, 200 and 800 mg/L niclosamide increased pupal MDA content in comparison to the control group. At low concentrations of niclosamide, PCO decreased in the last stage of larvae. The diet with 400 mg/L niclosamide concentration significiantly increased GST activity in pupal stage.

Conclusion: The results of this work indicated that the negative effects of niclosamide on biological characteristics of *D. melanogaster* are due to an increase in the oxidative stress and crippled detoxification capacity of the insect.

Keywords: *Drosophila melanogaster*, oxidative stress, niclosamide, survival, development

INTRODUCTION

Agricultural pesticides that reduce the growth and yield of plants of nutritional significance are widely used today. Although these pesticides are effective methods for controlling insect populations in agricultural fields the improper use of these pesticides can cause several pest species to develop resistance against them (1).

Recent studies have led to the use of clinically important antimicrobial antibiotics as pesticides and significant results were obtained in these studies. In previous studies, antibiotics were used to prevent microbial contamination, to stimulate food intake, and to determine their effects on pests by administering them into the culture medium where pests were propagated (2-8). The most recent studies have demonstrated that these antibiotics cause oxidative effects on pests in various biochemical assays. Büyükgüzel and Kalender (9-11) reported that changes in the activities of alanine aminotransferase and aspartate aminotransferase enzymes are indicators of the severity of antibiotic

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Submitted: 05.04.2019 • **Revision Requested:** 28.04.2019 • **Last Revision Received:** 02.05.2019 • **Accepted:** 09.05.2019

toxicity in pests. In many studies, it has been shown that a low concentration of antibiotics can be used to protect the synthetic diets from contamination and also that high concentrations of antibiotics can be used in insect management (8-15).

Furthermore, it is also known that certain insects, including *Galleria mellonella* (Linnaeus), *Drosophila melanogaster* (Meigen) are used as a models to test the action of clinical antiparasitic, antibacterial and antifungal drugs (16). Several species in the *Drosophila* genus became model organisms used in genetic and biochemical studies due to their easy culture in a laboratory environment, short life cycle, and the ability to produce a large number of offspring in one generation (17). Thus, it is a suitable experimental animal to test the biological effects of certain commonly used chemicals (18-20).

Niclosamide is among the active ingredients of drugs used in the treatment of infections induced by cestodes in humans and animals (21,22). Niclosamides act by inhibiting oxidative phosphorylation in parasite mitochondria. Furthermore, they can inhibit the anaerobic metabolism in several helminthic parasites. Anthelmintic drugs interfere with neuromuscular transmission by disrupting the main energy metabolism pathways in parasites. By affecting glucose absorption or transportation, they disrupt the glycogen metabolism, preventing glycolysis. They inhibit nucleic acid synthesis and eventually reproduction. Niclosamide is not significantly absorbed in the gastrointestinal tract and excreted in the stool. The absorbed part is converted to an active metabolite, aminoniclosamide (23).

Various xenobiotics stimulate production of free radicals (24) which lead to oxidative damage in all cellular compounds (carbohydrate, lipid, protein, DNA, RNA), especially to membrane phospholipids. As a result, the membranes become depolarized, the activity of the detoxifying enzymes increases, and the permeability of the cell membrane and the electrical charge balance changes (25). Antioxidants are vital biomolecules that can dissolve in water or oil, providing defense against free radicals and oxidative stress that are harmful to the body (26). Antioxidant enzymes are capable of minimizing the damage caused by free radicals, or eliminating it. These enzymes are catalase (CAT), glutathione-S-transferases (GST), peroxidase, and superoxide dismutase (SOD). GSTs are detoxifying enzymes found in vertebrates, plants, insects, yeasts, and aerobic bacteria (27). The phase II biotransformation enzyme GST catalyzes the conjugation of glutathione to a wide variety of xenobiotics with an electrophilic site, yielding more water-soluble xenobiotics and facilitating their excretion. Therefore, we decided to examine if niclosamide can alter lipid peroxidation, measured as malondialdehyde (MDA) concentration, as well as the activity of the detoxification enzyme GST.

In the present study, *D. melanogaster* was used as a model organism. The effects of niclosamide (a salicylanilide group anthelminthic substance) on the biological properties of *D. melanagaster*, lipid peroxidation and protein oxidation levels in the 3rd stage larval, pupal and adult stages of the insect and detoxification enzyme GST activity and the biochemical and physiological bases of the correlation between the oxidative stress and the mechanism of action of certain concentrations of niclosamide on biological properties of the insect were investigated.

MATERIALS AND METHODS

Drosophila melanogaster Culture and Experimental Medium

W^{1118} strain adult *D. melanogaster* (Diptera: Drosophilidae) individuals were procured from the University of Masaryk, Czech Republic, and brought to our laboratory. Stock culture was produced in the Zonguldak Bülent Ecevit University, Molecular Biology and Genetics Department, Biochemistry and Physiology research laboratory in glass bottles with artificial diets. The egg-laying of adult individuals, larval stage, pupal stage and adult stage were sustained on this artificial diet including potatoes and sucrose (28,29) and the adult individuals were sustained with a new culture. The stock culture and experimental setup were conducted in a laboratory environment with a relative humidity of 60-70%, 12 hours of light, 12 hours of darkness and 25 ± 2 °C. The diet used to sustain the culture was also used as a control diet in culture tests conducted to examine the effect of niclosamide on *D. melanogaster*.

Niclosamide Use in the Experiments

Niclosamide (2',5-Dichloro-4'-nitrosalicylanilide white yellow powder, 98%) was obtained from Sigma-Aldrich (St. Louis, Mo., USA). Pre-nutritional tests were conducted with niclosamide to determine the range of test concentrations. The concentration range where *D. melanogaster* could complete the development stages until the adult stage was determined. In addition to the control group, 100, 200, 400 and 800 mg niclosamide doses were added to 1000 mL diet in tests conducted with a niclosamide supplement. Niclosamide was added when the temperature of the food prepared for the experiment reaches room temperature. Niclosamide was mixed with the diet until it was homogenously mixed. In control experiments, only diets without niclosamide were used.

Niclosamide Tests on Survival Rate and Developmental Time of *D. melanogaster*

In tests conducted to determine the survival rate and developmental time of *D. melanogaster*, an artificial culture medium was prepared and the tested concentrations of niclosamide were added to the culture media. Cultures were distributed in equal amounts into 15 ml glass bottles. Twenty-five 1st stage larvae were placed in each control media without niclosamide and the media containing 100, 200, 400 and 800 mg/L niclosamide using a soft-tipped brush and then covered with a cotton wad. The rates of the larvae that reached to the 3rd stage, pupa and adult stages from the 1st stage were calculated. The time it took the larvae to reach the 3rd stage, pupa and adult stages was determined in days. All experiments were conducted in 4 replicates at 25 ± 2 ºC and 60-70% relative humidity, 12 hours light, 12 hours dark in the laboratory.

Niclosamide Tests on the Adult Longevity of *D. melanogaster*
A 5 ml control group culture media without niclosamide and 100, 200, 400 and 800 mg/L niclosamide administered groups were added in twenty five 15 ml glass bottles. Using a soft-tipped brush, the first stage larvae were placed in the culture media. The 1st stage larvae were cultured until the adult stage in this way. Until the adult stage, individuals were transferred daily to bottles containing 5 ml evenly distributed culture media. The adults in the control group and the study groups with different niclosamide concentrations were checked every day at a certain time. The adult longevity of each adult was determined until the last individual expired. This process was conducted under the environmental conditions where the stock insects were cultured.

Biochemical Analysis
Twenty-five larvae, pupa and adults were collected from the *D. melanogaster* eggs released into the culture media in the control and each of the above-mentioned niclosamide concentration groups. The process was repeated four times. MDA and protein carbonyl (PCO) amounts and GST activity were determined for the collected larvae, pupae and adults. The individuals harvested at each developmental stage were extracted in a homogenization buffer using an ultrasonic homogenizer (10 sec, 30 W) (Bandelin Sonoplus, HD2070, Berlin, Germany) and conducted at +4 ºC for 15 seconds. The obtained homogenate was centrifuged at 16000 x g for 20 min for the GST assay. In the MDA quantity assays, the samples were passed through an ultrasonic homogenizer and the homogenate was centrifuged at 2000 x g for 15 min at 4 ° C. For the determination of PCO, the supernatant was obtained by centrifuging the homogenized samples at 1000 x g for 10 min at +4 ºC. In this stage of the study, MDA, PCO levels and GST activity were determined for the larvae, pupa and adult individuals cultured in 100, 200, 400 and 800 mg/L niclosamide concentrations.

Measurement of MDA Level
An ultrasonic homogenizer was used for the disintegration of the *D. melanogaster* samples (15 sec, 30 W) (Bandelin Sonoplus, HD2070, Berlin, Germany). The MDA level, which is the final product of lipid peroxidation with TBA, was calculated by reading the absorbance at 532 nm. Plastic 1.5 cm cuvettes were used in MDA analysis. The MDA level was expressed as nmol/mg protein (30) using 1.56 x 105 $M^{-1}cm^{-1}$ coefficient.

Measurement of PCO Level
Protein carbonyl determination was conducted based on the method developed by Levine et al. (31) with certain modifications (32) where with carbonyl groups in the protein formed a stable 2,4-dinitrophenyl hydrosol (DNP) with 2,4-dinitrophenyl hydrazine (DNPH) in strong acidic environment (2 M HCl) and the absorbance of the products was measured at 370 nm.

Measurement of GST Activity
The method developed by Habig et al. (33) was used to measure the GST (EC 2.5.1.18) activity. Enzyme activity was measured as the amount of thioether generated at 1 minute per 1 mg total protein in the supernatant at 340 nm (ε_{340}: 0.0096 $\mu M.cm^{-1}$). The specific activity of the enzyme is μmol/mg protein/min.

Measurement of Total Protein Content
Total protein amounts of the samples were used to calculate MDA levels and GST activity. The absorbances of the samples were measured at 600 nm. Different concentrations of BSA (Bovine serum albumin) solutions were prepared for the protein assay and a standard graph was obtained. Total protein content was calculated using the above-mentioned standard graph (34). Total protein assay was conducted to calculate the PCO level that occurred as a result of protein oxidation. The absorbance of the samples was measured spectrophotometrically at 280 nm. BSA standard solutions were prepared with 6 M guanidine hydrochloride. The total protein amount was calculated by plotting a standard graph (35).

Statistical Analysis
One-way analysis of variance (ANOVA) was used to analyze the data on the developmental time, adult longevity, MDA, PCO levels, GST activities (36), the "LSD Test" (37) was used to determine the significance of the difference between the means, and the "χ2 (Chi square) test" was used to analyze the survival rate (36). The significance of the mean values was determined at a 0.05 significance level. Correlation analysis was also performed between the tested concentrations and our data (37).

RESULTS

The Effects of Niclosamide on Survival Rate and Developmental Time of *D. melanogaster* Larvae
The survival rate of *D. melanogaster* indicated that all tested niclosamide concentrations significantly reduced the survival rate of 3rd stage larvae, pupae and adults when compared to the niclosamide-free control diet (Figures 1-3). We found a significant negative correlation between the niclosamide concentration and survival rate of 3rd stage larvae ($R^2 = 0.97$, $P \leq 0.05$), pupae ($R^2 = 0.97$, $P \leq 0.05$) and adults ($R^2 = 0.97$, $P \leq 0.05$).

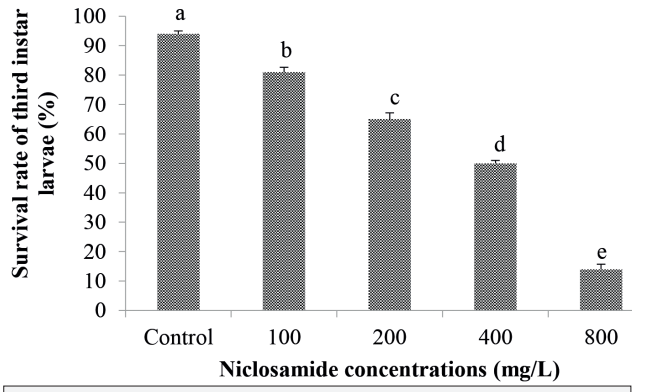

Figure 1. The effects of niclosamide on the survival rate of D. *melanogaster* 3rd larval stage. Each columns represents the mean of four treatment groups, 25 insects in this developmental stages were used for each groups. The columns containing the same letter are not different from each other, P≤ 0.05 (X^2 test).

In the control culture, 94.00 ± 1.00% of the larvae reached the third stage and this rate decreased to 14.00 ± 1.73% at the highest niclosamide concentration of 800 mg/L. Similarly, the survival rates decreased to 81.00 ± 1.65, 65.00 ± 2.17 and 50.00 ± 1.00 at 100, 200 and 400 mg/L niclosamide concentrations, respectively (Figure 1).

The rate of reaching the pupal stage in the control culture was 94.00 ± 1.00, while the rate of reaching the pupal stage decreased 81.00 ± 1.65, 63.00 ± 3.57, 50.00 ± 1.00, 14.00 ± 1.73 in niclosamide concentrations (100, 200, 400, 800 mg/L), respectively (Figure 2).

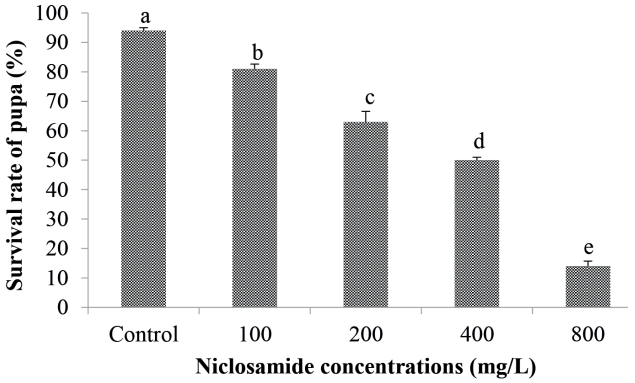

Figure 2. The effects of niclosamide on the survival rate of *D. melanogaster* pupa. Each columns represents the mean of four treatment groups, 25 insects in this developmental stages were used for each groups. The columns containing the same letter are not different from each other, P≤ 0.05 (X^2 test).

Similar to the larval and pupal stage survival rates, the survival rate of the adult individuals decreased significantly when compared to the control culture. The survival rate significantly decreased at 800 mg/L niclosamide concentration, the highest concentration tested, to 11.00 ± 0.86, while in control culture, the survival rate was 94.00 ± 1.00 (Figure 3).

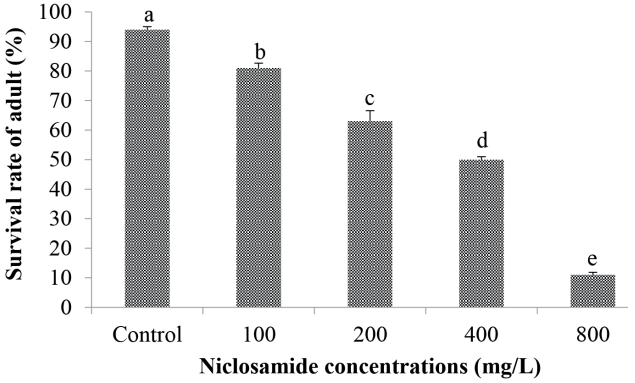

Figure 3. The effects of niclosamide on the survival rate of *D. melanogaster* adult. Each columns represents the mean of four treatment groups, 25 insects in this developmental stages were used for each groups. The columns containing the same letter are not different from each other, P≤ 0.05 (X^2 test).

First three niclosamide concentrations (100, 200, 400 mg/L) did not have a statistically significant effect on the developmental time of *D. melanogaster* to reach the last larval stage, pupal and adult stages (Figures 4-6).

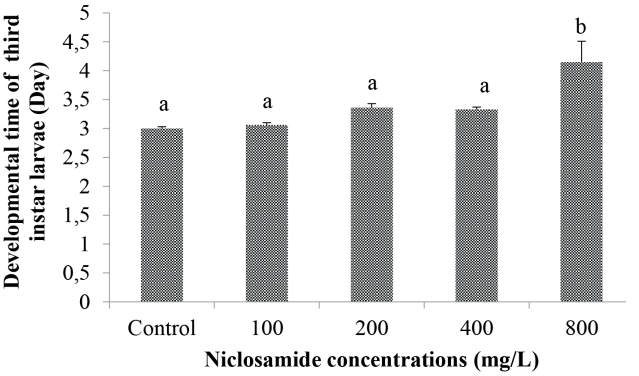

Figure 4. The effects of niclosamide on the developmental time of *D. melanogaster* 3rd larval stage. Each columns represents the mean of four treatment groups, 25 insects in this developmental stages were used for each groups. The columns containing the same letter are not different from each other, P≤ 0.05 (LSD test).

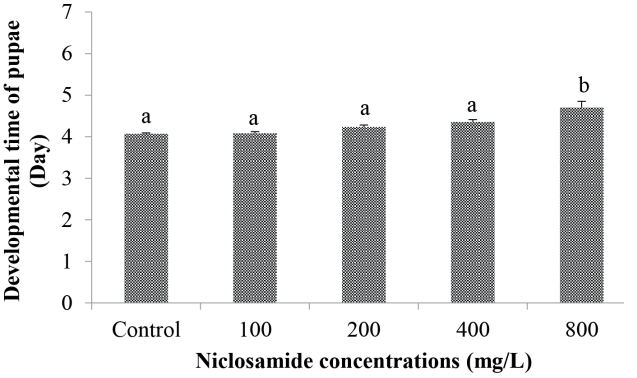

Figure 5. The effects of niclosamide on the developmental time of *D. melanogaster* pupa. Each columns represents the mean of four treatment groups, 25 insects in this developmental stages were used for each groups. The columns containing the same letter are not different from each other, P≤ 0.05 (LSD test).

When the control culture was compared with the culture that contained the highest niclosamide concentration (800 mg/L), it was found that the highest tested niclosamide concentration prolonged the time required to reach the 3rd larval to the pupal stages by 1 day and the time required to reach the adult stage was determined as 3 days. In the present study, we obtained a positive correlation between the tested concentrations and developmental time of larval (R^2 = 0.88, P ≤ 0.05), pupal (R^2 = 0.93, P ≤ 0.05), adult (R^2 = 0.76 P ≤ 0.05) stage.The developmental effects of niclosamide on 3rd larval, pupal and adult individuals are presented in Figures 4-6.

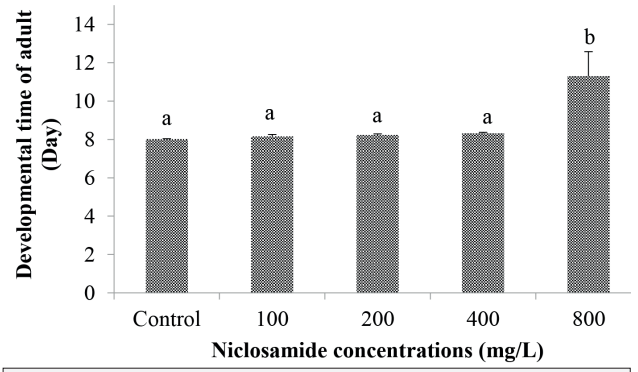

Figure 6. The effects of niclosamide on the developmental time of *D. melanogaster* adult. Each columns represents the mean of four treatment groups, 25 insects in this developmental stages were used for each groups. The columns containing the same letter are not different from each other, P≤ 0.05 (LSD test).

The Effects of Niclosamide on *D. melanogaster* Adult Longevity

The effect of niclosamide on the adult longevity of adult *D. melanogaster* individuals is presented in Figure 7. The mean adult longevity was 42.08 ± 0.50 days in the control group, while it was 22.89 ± 0.52 days in 100 mg/L anthelmintic concentration, 16.35 ± 0.33 days in 200 mg/L concentration, 8.41 ± 0.51 days in 400 mg/L concentration, and 2.30 ± 0.15 days in 800 mg/L concentration. It was determined that the adult longevity decreased as the niclosamide concentration in the culture increased. These result was showed that a negative correlation was found between the tested niclosamide concentration and adult longevity of *D. melanogaster* ($R^2 = 0.096$, $P \leq 0.05$)

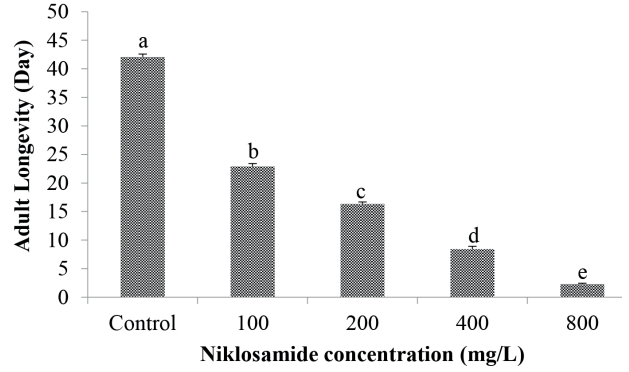

Figure 7. The effects of niclosamide on the adult longevity of *D. melanogaster* adult. Each columns represents the mean of four treatment groups, 25 insects in this developmental stages were used for each groups. The columns containing the same letter are not different from each other, P≤ 0.05 (LSD test).

The Effects of Niclosamide on MDA, PCO, and GST Activity in Different Development Stages of *D. melanogaster*

The MDA levels in the 3rd stage *D. melanogaster* larvae fed with artificial diets that included the tested niclosamide concentrations decreased when compared to the control

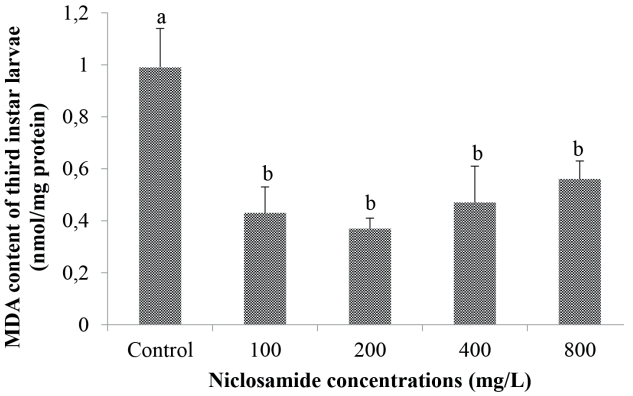

Figure 8. The effects of niclosamide on MDA levels in *D. melanogaster* 3rd larval stage. Each columns represents the mean of four treatment groups, 25 insects in this developmental stages were used for each groups. The columns containing the same letter are not different from each other, P≤ 0.05 (LSD test).

group. In the control group, the MDA level was 0.99 ± 0.15 nmol/mg protein, while the MDA levels in all tested niclosamide concentrations (100, 200, 400, 800 mg/L) were 0.43 ± 0.10, 0.37 ± 0.04, 0.47 ± 0.14, 0.56 ± 0.07 nmol/mg protein, respectively (Figure 8). The MDA levels in the pupal stage insect cultured with different niclosamide concentrations decreased in 100, 200 and 800 mg/L concentrations when compared to the control group. The control group MDA level was 1.50 ± 0.42 nmol/mg protein, while the MDA levels at all tested concentrations (100, 200, 800 mg/L) were 0.58 ± 0.14, 0.59 ± 0.09,1.67 ± 0.06 and 0.71 ± 0.04 nmol/mg protein, respectively (Figure 9). Although we observed increasing MDA contents in adult stage insects, there was no statistically significant effect (Figure 10).

It was determined that the PCO content was 341.13 ± 92.5 nmol/mg protein in the niclosamide-free control group 3rd stage larvae. The PCO levels in the third stage *D. melanogaster*

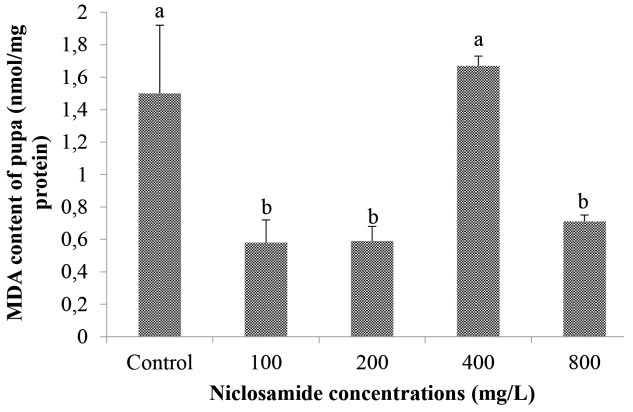

Figure 9. The effects of niclosamide on MDA levels in *D. melanogaster* pupa. Each columns represents the mean of four treatment groups, 25 insects in this developmental stages were used for each groups. The columns containing the same letter are not different from each other, P≤ 0.05 (LSD test).

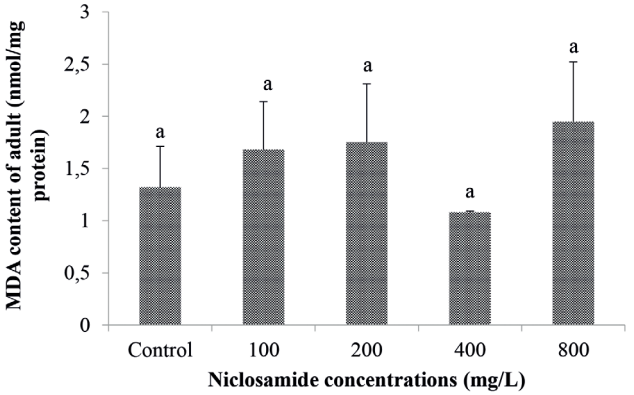

Figure 10. The effects of niclosamide on the MDA levels in *D. melanogaster* adult. Each columns represents the mean of four treatment groups, 25 insects in this developmental stages were used for each groups. The columns containing the same letter are not different from each other, P≤ 0.05 (LSD test).

larvae in the groups cultured with artificial diets containing 100, 200 and 400 mg/L niclosamide concentrations were 97.68

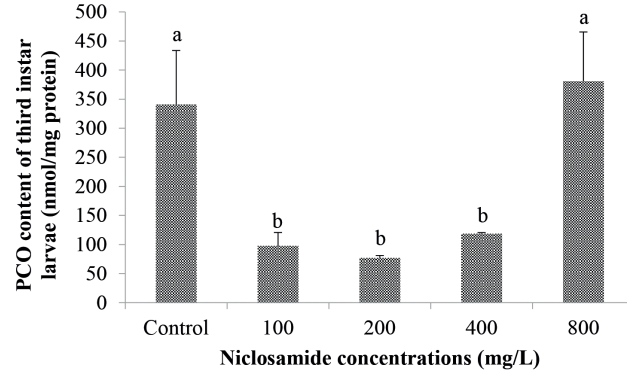

Figure 11. The effects of niclosamide on PCO levels in *D. melanogaster* 3rd stage larvae. Each columns represents the mean of four treatment groups, 25 insects in this developmental stages were used for each groups. The columns containing the same letter are not different from each other, P≤ 0.05 (LSD test).

± 23.0, 76.89 ± 4.2, 118.87 ± 1.6 nmol/mg protein, respectively, and this decrease was statistically significant (Figure 11). Compared with the control group, there was no statistically effect in all the niclosamide groups, but our data showed that there was a significant effect between the 400mg/L and 800 mg/L niclosamide concentrations (Figure 12). In the control group, it was found that the PCO level in adult *D. melanogaster* individuals was 158.63 ± 34.1 nmol/mg protein. Although the PCO level was 95.35 ± 12.4 nmol/mg protein in the highest tested niclosamide concentration of 800 mg/L, the difference was not statistically significant when compared to the control group. However, there was a significant effect difference between the highest niclosamide concentration and 200 mg/L niclosamide concentration (Figure 13).

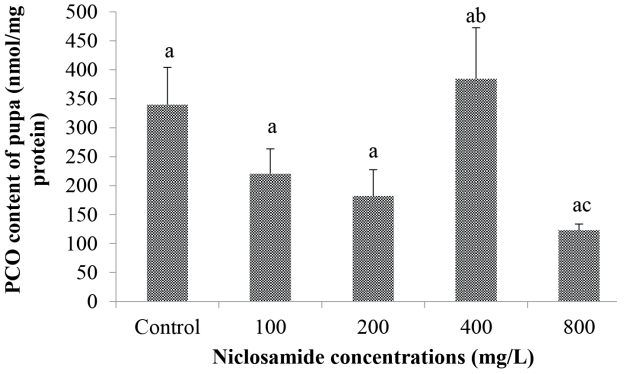

Figure 12. The effects of niclosamide on PCO levels in *D. melanogaster* pupa. Each columns represents the mean of four treatment groups, 25 insects in this developmental stages were used for each groups. The columns containing the same letter are not different from each other, P≤ 0.05 (LSD test).

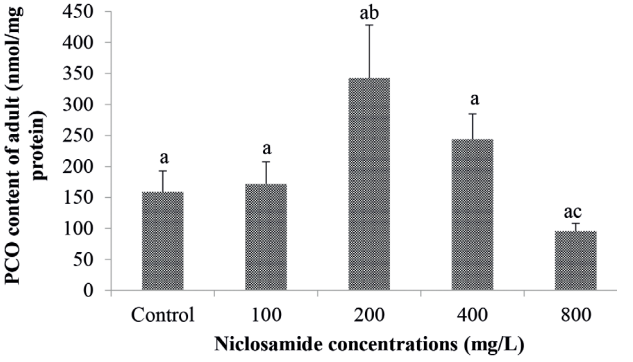

Figure 13. The effects of niclosamide on PCO levels in *D. melanogaster* adult. Each columns represents the mean of four treatment groups, 25 insects in this developmental stages were used for each groups. The columns containing the same letter are not different from each other, P≤ 0.05 (LSD test).

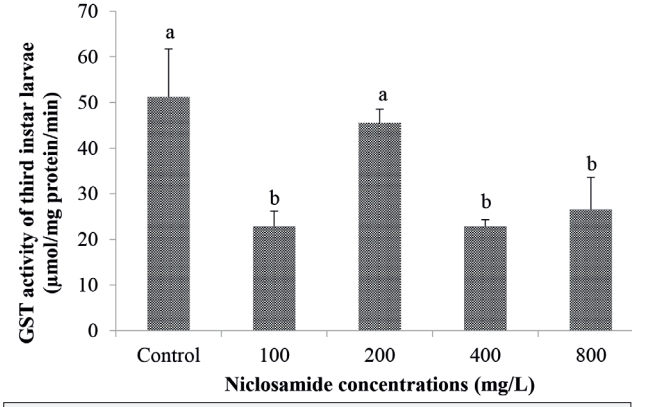

Figure 14. The effects of niclosamide on GST activity in *D. melanogaster* 3rd stage larvae. Each columns represents the mean of four treatment groups, 25 insects in this developmental stages were used for each groups. The columns containing the same letter are not different from each other, P≤ 0.05 (LSD test).

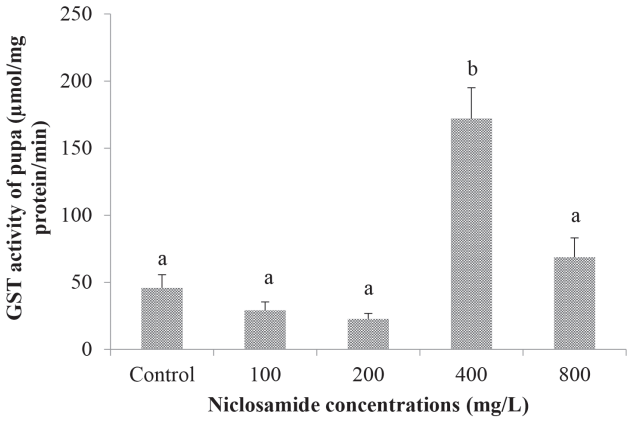

Figure 15. The effects of niclosamide on GST activity in *D. melanogaster* pupa. Each columns represents the mean of four treatment groups, 25 insects in this developmental stages were used for each groups. The columns containing the same letter are not different from each other, P≤ 0.05 (LSD test).

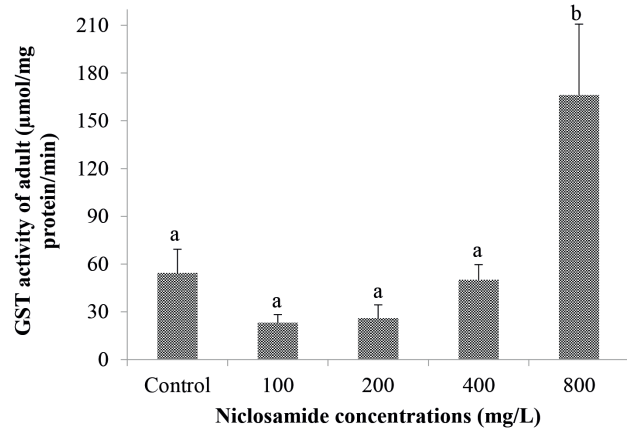

Figure 16. The effects of niclosamide on GST activity in *D. melanogaster* adult. Each columns represents the mean of four treatment groups, 25 insects in this developmental stages were used for each groups. The columns containing the same letter are not different from each other, P≤ 0.05 (LSD test).

The effects of niclosamide on the GST activity in *D. melonogaster* larvae, pupae and adults were investigated in the present study conducted with the anthelmintic substance niclosamide, and it was observed that GST activity decreased at higher concentrations of niclosamide when compared to the control group results (Figures 14-16). Analysis of the impact of niclosamide on the GST activity in *D. melanogaster* pupa demonstrated that control group GST activity was 45.82 ± 9.8 µmol/mg protein/min and GST activity significantly increased to 171.99 ± 23.0 µmol/mg protein/min at 400 mg/L niclosamide concentration. It was observed that GST activity increased three-fold when the GST activity in the group with the highest niclosamide concentration (800 mg/L) was compared to that of the control group. The GST activity determined in the control group was 54.31 ± 15.0 µmol/mg protein/min (Figure 15).

DISCUSSION

In the present study, the *D. melanogaster* model was used to research the effects of niclosamide, a salycilanide group anthelmintic substance, on vital parameters and antioxidant and oxidant levels of the insect. We investigated the effect of the niclosamide supplement to the semi-synthetic diet on the survival and development rates until adult stage and adult longevity of the newly hatched larvae of the model organism *D. melanogaster*. In the next stage, lipid peroxidation, protein oxidation and the antioxidant enzyme GST activity in different development stages of the insect (larval, pupal and adult stage) were investigated.

It was determined that all niclosamide concentrations significantly reduced the survival rate until the 3rd larval, pupal and adult stages. In a study conducted by Sak and Uçkan (38) on the effects of cypermethrin on pupation and mortality rates of *Galleria mellonella*, and similar to our findings, it was determined that as the cypermethrin dose increased, larval development and pupation time were delayed, and, pupation ratio decreased. In certain studies, it was observed that administration of boron-derivative chemicals such as boric acid and sodium tetraborate to various diets as pesticides delayed the development at high concentrations, decreased egg production and hatching, increased mortality in larval and pupal stages and adult longevity (39-43). In our study, the highest concentration delayed the development stage of the larvae, pupae, and adult stages of *D. melanogaster* and was also statistically significant. In the present study, a statistically significant decrease was observed in adult longevity with the increase in niclosamide concentration. It was determined that catalase (CAT) activity increased in *Zaprionus paravittiger* (Godbole & Vaidya) fed with a diet that included antioxidant sodium hypophosphite, and there was a positive correlation between adult longevity and CAT activity (44). Similarly, the adult longevity of *Z. paravittiger*, fed with low concentrations of butylated hydroxyl anisole as an antioxidant, increased, its development was delayed, and egg production was decreased (45). The findings of the present study demonstrated that the adult longevity significantly decreased in the highest concentration of niclosamide when compared to the control culture, and the antioxidant enzyme GST activity increased significantly at the same concentration in the adult stage.

The highest niclosamide concentration (800 mg/L) led to a significant delay in all development stages, while other tested niclosamide concentrations did not have a significant impact on the developmental time. In a previous study by Büyükgüzel and Kayaoğlu (46), the *in vivo* insecticidal effect of niclosamide was investigated on *G. mellonella* larvae and it was determined that niclosamide significantly reduced the survival rates of 7th stage larvae, pupae and adults, and similar to the present findings, the highest niclosamide concentration (1.0%) significantly prolonged the adult developmental time. The highest niclosamide concentration delayed larval development by 5 days, pupal development by 4 days and adult stage by 6 days. In

a similar study, excessive production of the peptides (peptide-S-methionine sulfoxide reductase) that contain methionine, an amino acid that plays a role in the treatment of oxidative damage, in *D. melanogaster* increased the maximum adult longevity, excessive production of glutamate-cysteine-ligase increased the adult longevity by 24%, excessive production of apolipoprotein D was effective in the resistance against hydrogen peroxide (H_2O_2) and prolonged the adult longevity of *D. melanogaster* by 26% (47). In a study conducted by Cruz et al. (48), boric acid that was added to the *Apis mellifera* (L.) culture at the rates of 2.5 and 7.5 (mg/g diet) increased the mortality rate in worker larvae compared to the control group and 2.5 mg boric acid administration killed 100% of the larvae on the 5th and 6th days, while the mortality rate with the 7.5 mg dose was 100% on the 4th day.

It is known that several biomolecules play a role in the regulation of various enzyme activities and preservation of the cell membrane integrity. Thus, the diet intake during the larval time has a significant impact on the development of the insect (49). The present study determined that niclosamide had a negative impact on the survival and development parameters of the insect and significant changes were observed in MDA, PCO levels and GST activity in all development stages. A similar effect was observed with the significantly increased MDA levels in the midgut of *G. mellonella* larvae that were cultured with streptomycin (11). It was determined that MDA and hence, GST activities significantly decreased in 100, 200, 400 and 800 mg/L niclosamide supplementation to the insect larvae culture when compared to the control group. It was observed that niclosamide did not alter the structure of the culture medium where the insect was cultured, and even had a positive effect on the existing artificial diet components. Microbial contamination is the most common problem in insect species artificially cultured with artificial diets in a laboratory environment (10). Thus, various antimicrobial agents have been used in culture studies to prevent contaminations due to artificial culture media and to culture high quality adult individuals (12-14). Based on the present study findings, it is suggested that the tested niclosamide concentrations had a positive impact on the MDA, PCO levels and GST activities in the larval stage when the diet quality was increased by increasing the diet quality, and that a decrease in MDA levels during larval and pupal stages could be due to an adaptation developed by the insect. It can also be suggested that the reduction in PCO and MDA levels could be due to the proper functioning of the insect antioxidant defense system enzymes. Furthermore, it is known that the same insect species could require different physiological conditions and diets in different development stages (37). Thus, the changes in the effects of the tested niclosamide in the diet mixture on the life parameters and biochemical analyzes conducted in different stages were expected results.

It is known that xenobiotics lead to an increase in MDA and PCO levels due to the oxidative stress in tissues (50-53). It was determined that a 400 mg/L niclosamide concentration in the pupal stage and 200 mg/L concentration in the adult stage of *D. melanogaster* significantly increased PCO levels, one of the important oxidative stress markers. These findings demonstrated that the effects of niclosamide on the biological properties of insects were related to oxidative stress, and it also showed that these effects varied based on niclosamide concentration and the developmental stage of the insects. It was determined that GST activity significantly increased with 400 mg/L niclosamide concentration in pupal stage and with the highest concentration of 800 mg/L in the adult stage. It was observed that this increase was parallel to the increase in the PCO levels. The findings suggested that niclosamide could affect life parameters and other biochemical functions in insects through a second activity mechanism, namely the oxidative stress it causes, in addition to its primary activity. A similar effect was reported by Agianian et al. (54). They reported that two major forms of GST were found in *D. melanogaster*; GST-Delta (GST-1) and GST 2, and a third form of GST was found in *Drosophila simulans* (*Sturtevant*) (GST-Epsilon). Hunaiti et al. (55) found that the GST-Delta and the third form increased resistance to insecticides in *Drosophila*, while GST 2 was found in the flight muscles of the insect. In another study, it was demonstrated that 620 ppm and higher (1250 and 2500 ppm) concentrations of boric acid decreased the survival rate of *G. mellonella*, shortened the developmental time and extended the adult life span, and the same concentrations increased the MDA levels in the hemolymph and fat tissues of newborn individuals and significantly altered the antioxidant enzymes superoxide dismutase (SOD), CAT, GST and glutathione peroxidase (GPx) activities in final stage larvae and new pupae (43). A previous study investigated the effect of endosulfan on GST activity in different stages of *Helicoverpa armigera* (Hübner), and it was found that the activity was the lowest in insect eggs, pupae and adults, and it was suggested that this decrease was the resistance of the insect against the chemical exposure (56).

Several studies on certain insect species demonstrated that xenobiotics led to oxidative stress in various insect tissues and alterations were observed in antioxidative defense mechanisms against this stress (32, 57-60). The increase in GST activity in the pupal and adult stages of *D. melanogaster* in high niclosamide concentrations (400-800 mg/L) could be due to the prooxidant effect of niclosamide supplement in the culture. In a study conducted on the effects of different concentrations of diazinon, an organophosphate insecticide, on SOD and CAT enzyme activities in *Pimpla turionellae* (Linnaeus), it was reported that SOD activity significantly increased when compared to the control group, and as a result, CAT activity increased as well (61). The effects of pyriproxyfen, a juvenile hormone analog, on SOD and CAT activities in *G. mellonella* larvae was investigated and 0.0001, 0.0005, 0.001 and 0.005 mg/ml pyriproxyfen doses increased the SOD and CAT antioxidant enzyme activities after 24, 48 and 72 hours of application (62). Another study demonstrated that organophosphate insecticide fenitrothion induced changes in fatty tissue weight and SOD and CAT antioxidant enzyme activities in cotton moth *Spodoptera exigua* L. and flour pest *Tenebrio molitor* L. (63) Cadmium administration increased

MDA levels, a lipid peroxidation product, in *Oncopeltus fasciatus* (Dallas), a heteropteran species, and reduced the activities of certain antioxidant enzymes (CAT, GR, GST) (64).

CONCLUSION

We believe that this study contributes to science in three ways. Firstly, there is no information regarding the effect of niclosamide on insects when added to the Drosophila diet under laboratory conditions. Secondly, our data showed that the low concentrations of niclosamide may be applied to prevent diet contamination in mass production of insects in laboratory conditions. Thirdly, the high concentrations of this chemical can negatively affect survival, development and certain physiological parameters of the insect. In this context, with the results obtained from this study the significance of *D. melanogaster*, which is an important model organism, in toxicology studies is revealed. Due to the above mentioned reasons, it can be concluded that this is a pioneering study in its evaluate as an alternative chemical substance that may be used in against to harmful insects. While various studies were conducted with antibiotics with different mechanisms of action and chemical structures on various insect groups, further detailed studies on a wider group of insects should also be conducted.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of study: E.B.; Data Acquisition: G.Ü.; Data Analysis/Interpretation: E.B., G.Ü.; Final Approval and Accountability: E.B., K.B., G.Ü.; Drafting Manuscript: E.B., G.Ü.; Critical Revision of Manuscript: E.B., G.Ü.; Technical or Material Support: E.B., K.B., G.Ü.; Supervision: E.B.

Conflict of Interest: The author has no conflict of interest to declare.

Financial Disclosure: There are no funders to report for this submission

Acknowlegements: This study was supported by Research Fund of Zonguldak Bülent Ecevit University, Project No: 2015-50737594-01.

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RESEARCH ARTICLE

Eur J Biol 2019; 78(1): 40-50

Record of Myxomycetes on Monumental Trees in Bursa City Center – Turkey

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Please cite this article as: Touray F, Ergul CC. Record of Myxomycetes on Monumental Trees in Bursa City Center – Turkey. Eur J Biol 2019; 78(1): 40-50.

ABSTRACT

Objective: Urbanization has been reported to affect the biodiversity of myxomycetes. There is no data in the Bursa city on the diversity of myxomycete in the urban area. Therefore, we aim to determine the presence of corticolous myxomycetes on monumental trees that are located in the most populated province of Bursa city (Osmangazi and Yıldırım).

Materials and Methods: In August and October 2018, 100 barks of monumental trees located in the Osmangazi and Yıldırım) county of Bursa was collected. Moist chamber culture technique was used for the isolation and identification of the species.

Results: 16 species within 9 genera were identified. The species identified are listed, and six new species *Didymium bahiense* (Gottsberger), *Didymium difforme* (Pers.) S. F. Gray), *Macbrideola martinii* (Alexop. & Beneke), *Macbrideola oblonga* (Pando, Lado), *Physarum gyrosum* (Rost.), *Physarum notabile* (Macbr.) were recorded for Bursa city.

Conclusion: This is the first report of corticolous myxomycetes on monumental trees in Bursa city center. It also added data and 6 new records of myxomycetes on the myxomycete biodiversity of Bursa.

Keywords: Bursa, corticolous, moist chamber, monumental, myxomycetes, turkey

INTRODUCTION

The myxomycetes, also refer to as slime molds or myxogastrid are a group of a fungus-like organism often found and occasionally common in the terrestrial ecosystem (1, 2). They are heterotrophic motile organisms and produce spores as a mechanism for reproduction and dispersal. Although most are tiny and need the aid of a microscope to see them, some of the myxomycetes can produce macroscopic fruiting bodies that can be easily collected (3). They feed on bacteria, and fungal spores on plant parts and decaying plant materials (decaying logs, dead leaves etc. (2), algae (4) and soil (5). Their role in the ecosystem is not well understood but since they feed on bacteria, and fungal spore they are believed to function as modulators of decomposition (1). There are about 1000 known species of myxomycetes recorded worldwide (6). The myxomycetes spore germination and successive phases, together with parts of fruiting body formation were first demonstrated by Heinrich Anton de Bary who is usually refered to as the father of mycology (7).

Little research has been done on the myxomycetes of Turkey (8, 9). The first report of myxomycetes in Turkey was by Lohwag (10) and Härkonen & Uotila (11) since then, some Turkish researchers began to study myxomycetes intensively especially in recent years. In 1993 C. Ergül (12) reported 61 taxon were reported in the Marmara region in the first doctorate thesis in Turkey on myxomycetes. The number of myxomycetes species reported in Turkey so far is 252 reported in different parts of the Country (13).

This study was presented at the 4th International Scientific Research Congress with the Title "Distribution of Corticolous Myxomyctes in Bursa City Center" held on 14-17 February 2019, Yalova, Turkey.

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Submitted: 05.04.2019 • **Revision Requested:** 08.04.2019 • **Last Revision Received:** 06.05.2019 • **Accepted:** 07.05.2019

Hosokowa et al. (1) state that not many studies have been done on the presence and diversity of myxomycetes in the urban or city areas. Most of the work done was based on macro– organisms like birds etc. A change in biodiversity is due to the urbanization and changing of agricultural setting to the urban environment can lead to the low level of species within these areas (1). Some studies have shown that environmental pollutants like acid can affect the biodiversity of myxomycetes (14).

There have been some studies done in Bursa province on myxomycetes, but they mostly concentrate on the Uludağ region and region outside of the city (15). In other to better understand myxobiota of Bursa city, we aim to determine the diversity of corticolous myxomycetes on monumental trees that are located in the most populated province of Bursa city (Osmangazi and Yıldırım). This study provides the first report of myxomycetes in monumental trees in city center area and also records new species for the Bursa city.

MATERIALS AND METHODS

Study Area

This study was carried out in the Bursa province of Turkey, situated between 40° 11′ and 44° 12′ N latitude and 29° 03′ and 36° 47′ E longitude (16). Bursa is 155 m above sea level and has a total surface area of 10,819 km^2 of which the plains covered 17% of the land, and 35% is made of mountains which are chains of mountains running from east to west (17).

Bursa is found in the Marmara region, and lies under the slope of mount Uludağ which is 2543 m above sea level. The Marmara region has a Mediterranean climate in its western part and a continental climate in its northern and eastern part (18). Although Bursa has the characteristics of a Mediterranean climate, it has a high total yearly precipitation and an average low temperature. The coldest months of the city is usually from January to March, and the warmest from July to September with an average annual humidity is 69%, and the mean annual rainfall is 691.9 mm (19). Osmangazi and Yıldırım are districts of Bursa city. They are the most populated and the second most populated districts in Bursa with a population of 856,770 (Osmangazi) and 647,520 (Yıldırım). Osmangazi, the largest district in the center of Bursa, starts from the foot of Uludağ in the east with the Gökdere Valley, it reaches the Samanlı Mountain in the north with Nilufer creek and Mudanya road to the west. Yıldırım is located at the foothills of Uludağ surrounded by Gürsu and Kestel in the east, Osmangazi in the west and south, Osmangazi and Gürsu in the north. These two regions were the early settlements of the Ottomans, and there you can find a lot of historical sites. These regions are also very rich in vegetation and home to a lot of monumental trees some of which are as old as 500 years like the historical İnkaya Plane Tree (20).

These monumental trees consist of mainly *Platanus orientalis, Pinus pinea, Cedrus atlantica, Cupressus sempervirens, Quercus robur, Platanus occidentalis*. From the Bursa municipality report 2014 (1), there are 1219 documented monumental trees of which 65% are *Platanus orientalis*. About 60% of the total monumental trees are found in the two study areas, of this, 566 (47.25%) almost 50% of the documented monumental trees are found in Osmangazi districts only 177 (14.5%) are found in Yıldırım) district (21).

Specimen Collection

In August and October 2018, this period falls under the dry month which is a suitable time to collect bark specimens for moist chamber culture and also to prevent the existence of filamentous bacteria and fungi from over growing on the specimens during culture. We collected samples form bark of 100 monumental trees in the Osmangazi district (40° 11′ 22″ N latitude and 29° 01′ 52″ E longitude) and Yıldırım district (40° 12′ 10″ N latitude and 29° 01′ 52″ E longitude) of Bursa city as showed in Figure 1. Ninety of the trees the samples were collected from were located within the city center and ten from outside the city center. The collected specimens (bark) were then put in a paper bag and transported to the laboratory for the preparation of moist chamber culture as stated in Stephenson and Stempen (22). The location, tree species, date, age, and the number given to the tree by the municipality, the coordinator were recorded on each of the paper bags for each of the samples. The samples are given collection number FT.

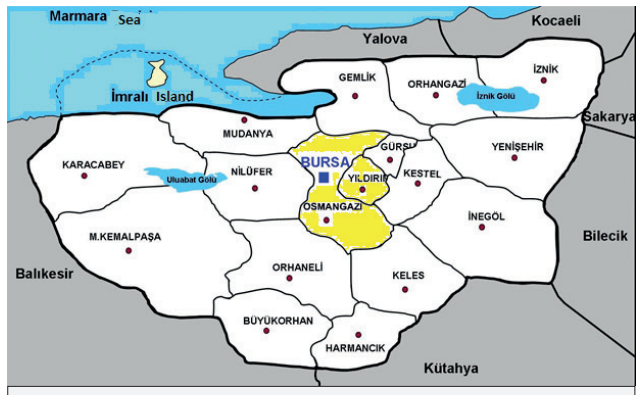

Figure 1. Map of Bursa, the Study Area indicated by the yellow color

Moist Chamber Culture

In moist chamber culture technique, a 9 cm diameter filter paper is placed in a petri dish; collected samples are placed on the filter. The bark is immersed in distilled water for 24 – 48 hrs, and then unabsorbed water is poured out. The dish is stored at room temperature (20° C to 25° C). Using a stereomicroscopic (Nikon SM 800), the samples are examined daily for about a month for detection of any growth. Observations from the culture are recorded every day until the fruiting bodies are fully matured. A microscope mount is then prepared by collecting the matured fruiting bodies and putting in it a drop of Hoyer's Medium. In order to observe the microscopic characterization of the specimens, the light photomicrographs were obtained by using a Nikon DS–Fi 1 microscope equipped with a Nikon ECLIPSE 50i camera system. This helped us observed the characteristics of the capillitium, peridium, spore shapes and sizes, stalk, color,

etc. These are the features that help in taxonomic identification or classification of the specimens. The identification of the specimens was done by using a key provided by (2, 3, 23-29). The samples were stored in Bursa Uludağ University Biology Department, Mycobiology laboratory.

RESULTS

30 of the 100 samples cultured came out positive for one or more corticolous myxomycetes, 27 of which is *Platanus orientalis*, 2 *Quercus robur*, and 1 *Platanus occidentalis*. 16 species within 9 genera were identified. The distribution of species in the different orders were Physarales (38%), Stemonitales (31%), Trichiales (19%), Liceales (1%) and Echinosteliales (1%) as shown in Figure 2. The species distribution of the 16 species identified is shown in is as follows; *Cribraria violacea* 12, *Perichaena corticalis* 10, *Macbrideola cornea* 7, *Macbrideola martinii* 2, *Physarum notabile* 2, *Enerthenema papillatum* 2, *Arcyria cinerea* 2, *Arcyria insignis* 1, *Badhamia affinis* 1, *Didymium bahiense* 1, *Didymium difforme* 1, *Echinostelium minutum* 1, *Macbrideola decapillata* 1, *Macbrideola oblonga* 1, *Physarum gyrosum* 1 *and Physarum serpula* 1. Figure 4 shows the percentages of the 9 genera identified with the most isolated being *Macbrideola* (25 %), *Physarum* (19 %), *Arcyria* and *Didymium* (13 %) and the rest

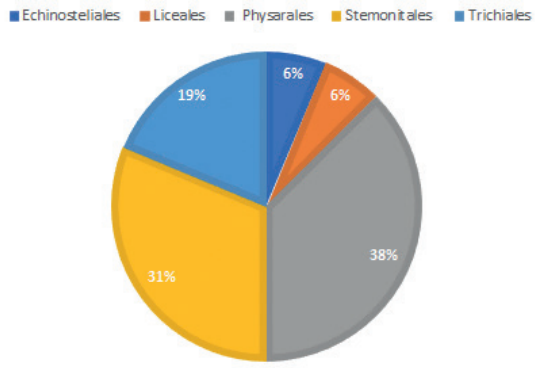

Figure 2. Distribution of orders

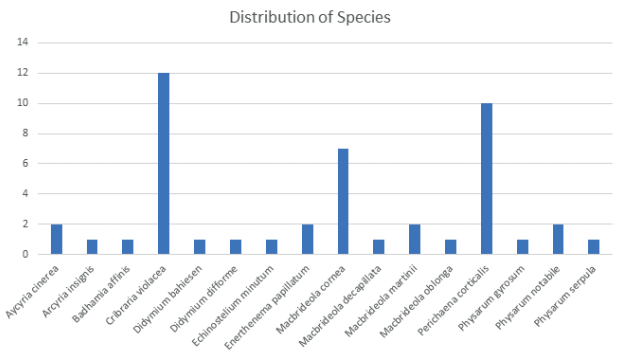

Figure 3. Distribution of species isolate

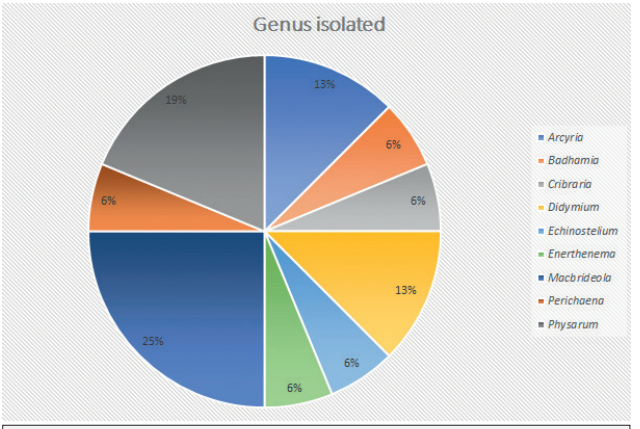

Figure 4. Distribution of corticolous myxomycetes genus

were 6%. All the recorded taxa in this study are alphabetically listed below according to orders and then by species.

List of Species Isolated

Echinosteliales

Echinostelium minutum de Bary, in Rost., Mon. 215 1874 (Figure 5)

Description–The sporangia are scattered, globose, white or pale pink, 40–70 µm in diameter, often nodding. Stalk longer in other species, to 500 µm high, colorless or pale yellow in transmitted light. Columella is 4–20 µm long. The capillitium thread forms a close, lax net around the sporangium. Spores in mass concolorous with the sporangium, colorless in transmitted light and it is about 9–12 µm in diameter. Protoplasmodium is colorless.

Specimen examined–Turkey: Bursa/ Yıldırım; Eşrefiler street near the bridge, on the bark of *Platanus orientalis*, 06.10.2018, FT15.

Echinostelium minutum is the most common species of Echinostelium that tends to appear on the bark than other species of the same genus. It is a cosmopolitan species commonly found in the British Isles. It can be found on bark of trees, wood

Figure 5. Sporangia, capillitium and spore of *Echinostelium minutum*

and litters (23). It has been previously reported in several studies conducted in Turkey. This species was isolated from snowy twigs in a study done at the Uludağ National Park in Bursa province (15). In Kütahya province, it was isolated from the bark of living *Prunus domestica* (9).

Liceales

Cribraria violacea Rex. Proc. Aca. Phila. 43:393 1891 (Figure 6)

Description–The sporangia are normally deep purple to purplish bronze in color in groups (gregarious), spherical and about 0.1–0.3 mm in diameter, it is erect and about 0.5–1.5 mm. The hypothallus is a small disc at the base of the stalk. The stalk is usually two 3–5 times the diameter of the sporangium, slender, tapering upwards, dark violet and under a transmitted light it appears to be red-brown inclusion in the basal part and violet. The peridium is a violet, deep cup that surrounds about 2/3 of the sporangium, it is smooth or weekly pilicate, with a smooth rim merging into the flat nodes and threads of the reticulum, the nodes and meshes are usually large and irregular, and there are very dark violet lime nodes inside the cup with a diameter of 1.0–1.5 µm. It has a spore mass of 7 µm in diameter densely minutely warted and violet or lilac under transmitted light. The plasmodium is purplish black.

Figure 6. Sporangium, spores, capillitial nodes, peridium, hypothallus and stalk of *Cribraria violacea*

Specimen examined–Turkey: Bursa/Osmangazi; Behind Pınarbaşı cemetery, on the bark of *Platanus orientalis,* 06.10.2018, FT25. Pınarbaşı Park opposite war cemetery, on the bark of *Platanus orientalis*, 06.10.2018, FT42. In front of Orhangazi tomb, on bark of *Platanus orientalis*, 05.08.2018, FT07. Opposite Sarayönü tea garden, on the bark of *Platanus orientalis,* 05.08.2018, FT12. In the garden in front of Muradiye Medresesi, on the bark of *Platanus orientalis*, 05.08.2018, FT60. Hamzabey road, on the bark of *Platanus orientalis*, 05.08.2018, FT74. Next to Çekirge Police center, on bark of *Platanus orientalis*, 05.08.2018, FT23. I. Murat road, behind Mutlu hotel, on the bark of *Platanus orientalis,* 05.08.2018, FT03. Çukurca village between the intersection of Çalı street and Ocak street, on the bark of *Quercus robur*, 06.10.2018, FT34. Sultan Palace (Hünkar Köşkü) road, on the bark of *Platanus orientalis*, 06.10.2018, FT48. Sultan Palace road, on the bark of *Platanus orientalis*, 06.10.2018, FT22.

Cribraria violacea is found on dead wood, the bark of dead and living trees, and mosses. Its bright violet color makes its identification easy. This species is cosmopolitan and widely distributed in North America and Switzerland (23). First reported in Turkey by, (11). It has now been reported in several parts of Turkey as explained in checklist myxomycetes in Turkey (13). It was first reported in Bursa by Ergül (12) on the bark of *Platanus orientalis*. In the Hatay region of Turkey, *Cribraria violacea* was isolated from the bark of coniferous woods at a university campus, using the moist chamber technique (30), It was also reported from the bark of *Pinus brutia* from the İzmir region (31) also isolated in Konya on the log of *Ficus* sp.*, Pinus nigra and bark of Malus* sp. (32). This species was isolated from 11 samples in our study all of which happen to be *Platanus orientalis.*

Physarales

Badhamia affinis Rostaf., Mon. 143. 1874. (Figure 7)

Description–Sporangia short of plasmodiocarps, gregarious, sessile or short-stalked, subspherical to discoid, often depressed in the center, 0.5–1.0 mm wide, 2.0 mm long, pale grey to whitish, usually with a darker base. Hypothallus sometimes black from absorbed dirty particles. Stalk if present, short, grooved and black. Peridium single, colorless above, brown below, with flake-like, scanty or abundant incrustations. Capillitium tube filled with white lime, poorly branched and hardly or no interconnected. Spores in mass brown, pale lilac-brown in transmitted light, spherical, 12–15 µm in diameter, densely covered with pale warts. Plasmodium creamy.

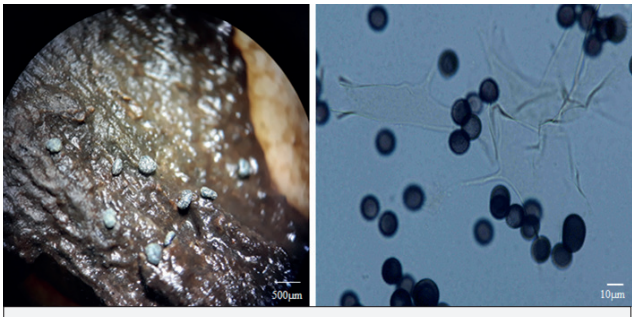

Figure 7. Capillitium, spores and plasmodiocarps of *Badhamia affinis*

Specimen examined – Turkey: Bursa/ Osmangazi; Timurtaşpaşa park, on bark of *Platanus orientalis*, 05.08.2018, FT05.

Found in Australia, Japan and in most parts of Europe and especially in the mountains (23), *Badhamia affinis* is a rare species of myxomycetes in Turkey. It was first reported in Turkey by, (11). This species is mostly found on the bark of living and dead trees, found around Great Britain, Greece, Canada and widely distributed in America (2). In our study, it was isolated from the bark of *Platanus orientalis* only one sample.

Didymium bahiense Gottsberger, Nova Hedw. 16: 365. 1968, em. Nann–Brem., Proc. K. Ned. Akad. Wet. C. 75: 360. 1972 (Figure 8)

Description–Sporangia in groups, stalked, 0.8–2.0 mm tall, discoid but reflexed around the stalk forming a deep umbilicus and appearing sub-globose, 0.2–0.7 mm in diameter, white or pale grey. Hypothallus small discoid, dull and almost black. Stalk 2–3 times longer than the width of the sporangium, tapering but keeping somewhat erect dark at the base and paler above, in transmitted light it is translucent red-brown, limeless. Peridium mainly thin, colorless or pale-yellow with a thickened basal plate on which crystalline lime in the form of a pseudocollumela is present. Capillitium tubules abundant, dichotomously branched, dark or pale brown with the exception of pale ends and small dark swellings. Spores are dark brown in mass, pale brown to dark lilac-grey in transmitted light (9) 10–12 (14.5) µm in diameter, covered with warts.

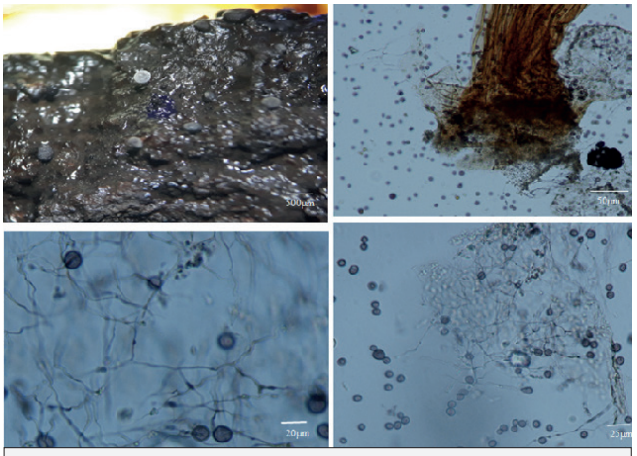

Figure 8. Capillitium tubules, spore, sporangia of *Didymium bahiense*

Specimen examined–Turkey: Bursa/Osmangazi; In front of Çekirge police center, on the bark of *Platanus orientalis*, 05.08.2018, FT28.

Didymium bahiense is cosmopolitan and commonly found on dead leaves, and plants refuse (23). First reported in Turkey Kastamonu province, on the bark of *Creatagus* sp. (33). In our study it was isolated on the bark of *Platanus orientalis*. This species has only been reported a few times in Turkey (13).

Didymium difforme (Pers.) S. F. Gray, Nat. Arr. Brit. Pl. 1: 571. 1821 (Figure 9)

Description–Sporangia sessile gregarious, 0.3–1.0 mm wide, 0.3–1.0 mm tall, almost round, oval or oblong seen from above, white or ochraceous. Hypothallus is inconspicuous. Peridium double, layers usually distant, a smooth outer crust of densely packed small lime crystals, the inner membranous, colorless, pale purple, shining. Columella absent. Capillitium tubules sparse, stiff, thick, sparsely dichotomously branched,

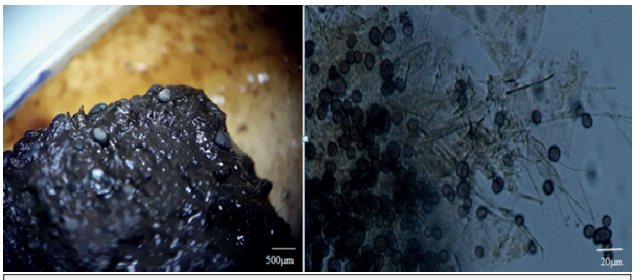

Figure 9. Capillitium tubules, sporangia, spore of *Didymium difforme*

exceptionally a lax net is formed lilac–brown or almost colorless, often with bread like swellings, sometimes with large vesicle-like partitioned swellings in which lime is occasionally present, weakly connected to the peridium above, sometimes connected somewhat longer to the base. Spores in mass deep black, dark purple-brown in transmitted light, paler on one side, roughly spherical, rarely ovoid, 11–14 µm in diameter, densely covered with fine warts. Plasmodium colorless or grey.

Specimen examined–Turkey: Bursa/Osmangazi; In front of Çekirge police center, on the bark of *Platanus orientalis*, 05.08.2018, FT28.

It could be found on rotting plant refused and dead leaves occasionally in bark culture and one of the commonest myxomycetes that can be found in Europe (29). *Didymium difforme* has been reported in Turkey before in several studies (13). We isolate it on the bark of *Platanus orientalis*. Although similar to a few other species of *Didymium* like *D. commatum, D. tubulatum, D. trachysporum*, in our study the characteristics of our sample is that of *Didymium difforme*. A similar study (31), reported *D. difforme* in leaves of *Pinus brutia* in İzmir province using moist chamber and also reported in the Konya province on decaying wood of *Pinus nigra* (9).

Physarum gyrosum Rost., Mon. 111. 1874 (Figure 10)

Description–Plasmodiocarps usually in crowded rosette-like or reticulate groups, sessile about 1.0 mm tall, laterally compressed, usually curved sometimes ring-shaped, 0.2–0.4 mm wide and up to few mm long, reddish, rosy–grey or ash-colored. Hypothallus translucently red-brown, sometimes curving up under a plasmodiocarp as a tiny stalk. Peridium single, encrusted with rosy or red lime, crumbling away at the apex. Capillitium a dense, elastic, network of delicate hyaline threads with numerous large, spike-like, transverse, white nodes and smaller fusiform nodes. Spores dark brown in mass, pale violaceous brown by transmitted light, minutely spinulose, 7–10 µm in diameter. Plasmodium white changing to yellow upon exposure to light.

Specimen examined–Turkey: Bursa/Osmangazi; In front of Çekirge police center, on the bark of *Platanus orientalis*, 05.08.2018, FT28.

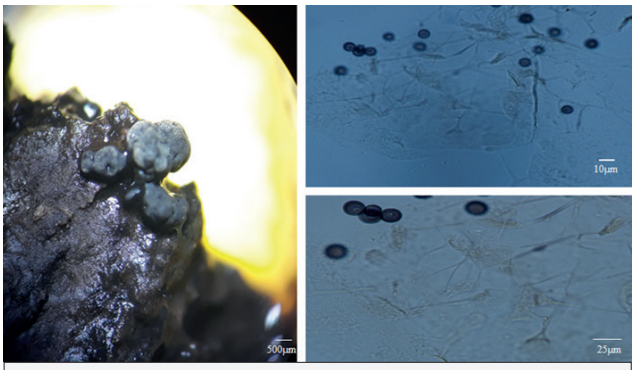

Figure 10. Plasmodiocarp, hyaline threads, and spore of *Physarum gyrosum*

Mostly found in Germany (29), *Physarum gyrosum* is not distributed in Turkey, it has only been reported in recent years first by Demirel & Kaşık (34) and then later in the Konya province on the decaying wood of *Pinus nigra* and *Juniperus* sp. (33). This species is said to be a transitional form between Fuligo and Pysarum by Lister, Macbride and Martin stated that it resembles a large sessile form of *Physarum polycephalum* a species usually used in the laboratories (29).

Physarum notabile Macbr., N. Am. Slime–Moulds ed. 2. 80. 1922 (Figure 11)

Description–Stalked sporangia, occasionally sessile or forming short plasmodiocarps, gregarious often in large developments, globose to kidney-shaped, usually with 2–10 sporangia joined by a stalk 0.3–1.0 µm in diameter. Hypothallus continuous under the group, white. Stalk if present often misshaped, deeply plicate – furrowed, opaque, dark or covered with white calcareous granules. Capillitium is abundant with large angular white nodes connected by long colorless threads. Spores are black in mass, olivaceous brown in transmitted light, 10–11.5 µm in diameter, minutely warted. Plasmodium is white or pale grey.

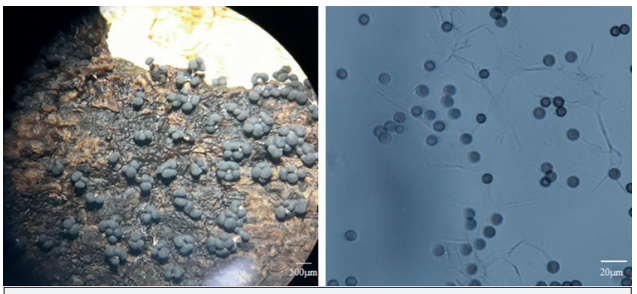

Figure 11. Sporangia, capillitium with nodes, and spore of *Physarum notabile*

Specimen examined–Turkey: Bursa/Osmangazi; Behind Pınarbaşı cemetery, on the bark of *Platanus orientalis,* 06.10.2018, FT25, Yıldırım; Sultan Palace road, on the bark of *Platanus orientalis*, 06.10.2018, FT22.

Physarum notabile commonly found on dead wood and occasionally on the bark of living trees (23), has previously been reported in Turkey checklist (13). It was isolated on the bark of *Platanus* sp. in Kastamonu province (33), just like in our study it was isolated on *Platanus orientalis*. A study by Ocak & Konuk (9), has reported *Physarum notabile* in Konya and Kütahya regions on the bark of *Pinus nigra* and *Prunus domestica*. The large fruiting's and how the sporangia are clustered makes it possible for its identification in the field (23).

Physarum serpula Morgan, Jour. Cinc. Soc. Nat. Hist. 19: 29. 1896 (Figure 12)

Description–Plasmodiocarpous are forming rings, lines, or simple network; sometimes the strands fused laterally so that a broad fruiting surface is formed, 0.2–0.4 mm wide, often interspersed with globose sporangiate fruiting's, dull yellow of ochraceous, rarely bright yellow, fading. Peridium single, thin or closely incrusted lime granules, without calcareous scales. Capillitium dense calcareous, the nodes numerous, large angular, branching, pale yellow or whitish, connected by short hyaline threads or broad limy strands. Spores globose, dull black in mass, dark brown in transmitted light, minutely warted with a paler and smoother area on one side, 10–13 µm in diameter. Plasmodium at maturity is greenish yellow.

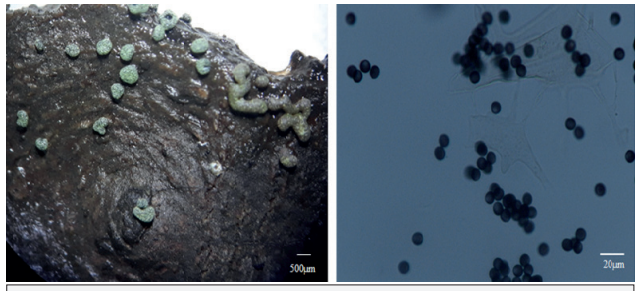

Figure 12. Plasmodiocarp, spore and a capillitium of *Physarum serpula*

Specimen examined – Turkey: Bursa/Yıldırım; Sultan Palace road, on the bark of *Platanus orientalis*, 06.10.2018, FT66.

Physarum serpula is a rare species in Turkey. It was first reported in Bursa province on the bark of *Quercus robur* (18). Recently it has been reported in the Kütahya region on the bark of living *Pyrus communis* and *Malus* sp. (9). In our study, it was isolated from the bark of the *Platanus orientalis* tree. Usually found on dead leaves, bark and lichens, it is closely related to *P. decipiens* and *P. auriscalpium*, and they are normally confused for each other. Although they may be closely related, *P. decipiens* have a more sporangiate and more badhamoid than theother two; also *P. auriscalpium* has prominent limescale (23). This species of Physarum is very uncommon due to it being inconspicuous (23).

Stemonitales

Enerthenema papillatum (Per.) Rost., Mon. App. 28.1876 (Figure 13)

Description–Sporangia disperse or crowded, spherical 0.4– 0.7 mm in diameter, dark brown to almost black. Hypothallus is discoid or continuous under a group. Stalk consisting of a net of opaque fibers, shorter or nearly as long as the sporangium, black. The columella is black extending to the apex of the sporangium. Capillitium consisting of long dark flexuous threads which are connected to the top of the columella dichotomously branched 1–3 times in the outer part. Spore dark brown to almost black, grey-brown in transmitted light and 10–12 µm in diameter with fine warts.

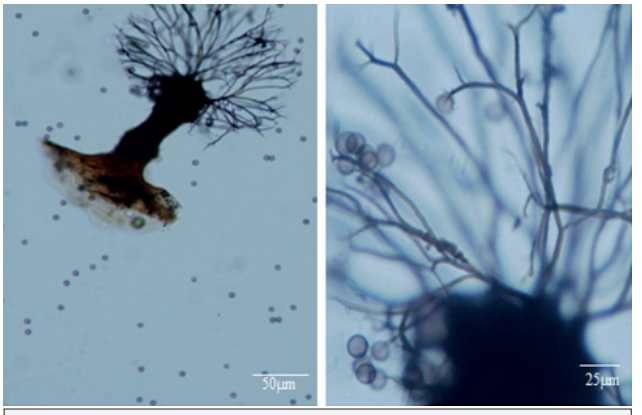

Figure 13. Spore, capillitium. Sporangia characteristic of *Enerthenema papillatum*

Specimen examined–Turkey: Bursa/Osmangazi; In the garden in front of Muradiye Madrassa, on the bark of *Platanus occidentalis,* 05.08.2018, FT58, Çekirge road in front of Çelik Palas, on the bark of *Platanus orientalis,* 05.08.2018, FT89.

Enerthenema papillatum is typically found on the dead wood and bark of living trees. Mostly found in America, Canada, Chile, etc. (29). In our study, it has been isolated from the bark of *Platanus orientalis* and *Platanus occidentalis*. This species of *Enerthenema* has been reported in Turkey since 1983 by Härkönen & Uotila (11) after which it was reported by other researchers in Turkey (13). A study done by Ergül & Akgül (15), reported *Enerthenema papillatum* from the Uludağ region of Bursa. A similar study conducted in İzmir has also reported *Enerthenema papillatum* on the bark of *Pinus brutia* (31).

Macbrideola cornea (G. Lister & Cran.) Alexop., Mycologia 59:112.1967 (Figure 14)

Description–Sporangia scattered or in groups, globose, dark brown, 0.3–2.0 mm high, and 0.1–0.3 mm in diameter. The Hypothallus is small red-brown or yellow disc. Its stalk is slender, straight, sometimes yellow, red-brownish, honey color at the base, dark above, distinctly hollow, becomes darker upward and

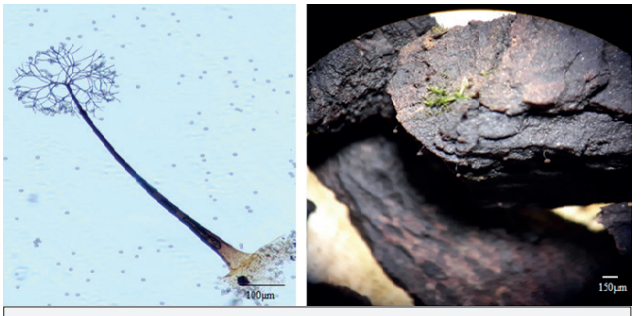

Figure 14. Sporangia, spore, capillitium of *Macbrideola cornea*

its 1/2 to 2/3 of the total height. The peridium is fugacious with the exception of a collar around the sporangium. The columella is brown reaching about the center of the sporangium dividing into the main capillitium branches. Capillitium is dichotomously branching, dark brown, usually 1–3 times, tapering outwards but not very thin at the periphery, branches usually covered with a colorless layer. It has spores that are mass dark brown, lilac-brown or grey-brown in transmitted light, minutely warted and 8–9 µm in diameter. Plasmodium is normally colorless.

Specimen examined–Turkey: Bursa/Osmangazi; Pınarbaşı park in front of picnic tables, on bark of *Platanus orientalis,* 06.10.2018 FT29, In the garden in front of Muradiye Madrassa, on bark of *Platanus occidentalis,* 05.08.2018, FT58, I. Murat road, opposite Mutlu hotel, on bark of *Platanus orientalis,* 05.08.2018, FT03, Çeltik Village Yeniyol street, on bark of *Platanus orientalis,* 06.10.2018, FT18, Yıldırım; Mollaarap *plane tree* road, on bark of *Platanus orientalis*, 06.10.2018, FT09, Sultan Palace road on bark of *Platanus orientalis*, 06.10.2018, FT20.

Macbrideola cornea found on the bark of trees, it has been reported in some European countries like France, Scotland, and Germany (2). Reported in several parts of Turkey as explained in the checklist of myxomycetes in (13). This species has been reported before from bark of trees in the city of Bursa by Ergül & Dülger (35). In İzmir it was isolated on *Pinus brutia* (31) and in Konya from bark of *Pinus nigra* (9). In our study, we isolated *Macbrideola cornea* form several *Platanus orientalis* and *P. occidentalis*. It can be differentiated from the *Paradiacheopsis* by its hollow stalk (23).

Macbrideola decapillata H. C Gilbert, Univ. Iowa Stud. Nat. Hist 16: 158. 1934.Iowa, (Figure 15)

Description–Sporangia is stalked, globose, scattered, dark brown, 175–350 µm in height, 50–100 µm in diameter. The stalk is slender, translucent, yellow at the base, brown above 125–250 µm long. Peridium is leaving a small collar. Columella reaching to three-quarter of the sporangial diameter rounded at the tip or bearing a few short branches. Capillitium is often absent but never more than a few filaments arising from the columella towards the outside. Spores in mass dark brown, violet brown in transmitted light, 7–9 µm diameters, unevenly warted. Plasmodium colorless.

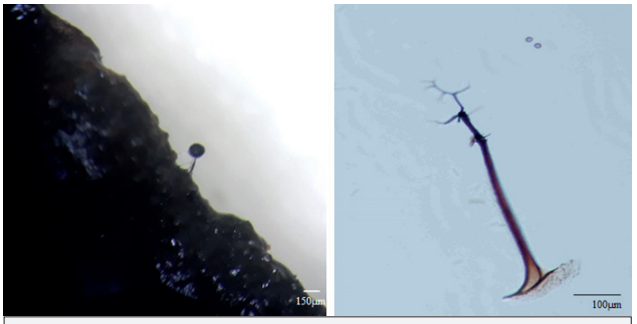

Figure 15. Sporangia, spore and capillitium of *Macbrideola decapillata*

Specimen examined–Turkey: Bursa/Osmangazi; Demirkapı community assisted police volunteer training center, on the bark of *Platanus orientalis,* 06.10.2018, FT54.

Macbrideola decapillata was isolated from a tree of *Platanus orientalis* in this study. This species has been reported a few times in other studies conducted in other parts of Turkey (13) and also recorded in North and Central America and Spain. Mostly found bark of living trees in the moist chamber (23), *Macbrideola decapillata* can be easily distinguished from *Macbrideola cornea* and *Macbrideola scintillans* by the absent of capillitium, long thin stalk, dull spore mass which dispersed quickly leaving only the stalk and columella (2, 23).

Macbrideola martinii (Alexop. & Beneke) Alexop., Mycologia 59: 114. 1967. (Figure 16)

Description–The sporangia are scattered, brown, globose, minute, 0.08–0.15 mm in diameter, a total height of 0.5–0.6 mm. Its peridium is completely evanescent. The stalk is brownish-yellow or honey at the base, brown upwards and continues up to the center of the sporangium as columella, and it is slender. The columella is an extension from the stalk reaching about the center of the sporangium and dividing into main capillitium branches. The capillitium arises from the tip of the columella, it is lax, consisting of three or four slender, brown branches which are fork dichotomously three or four times with no anastomoses. The spores are brown in mass and violet-gray by transmitted light, minutely verrucose, 7–8 µm in diameter. Its plasmodium is unknown.

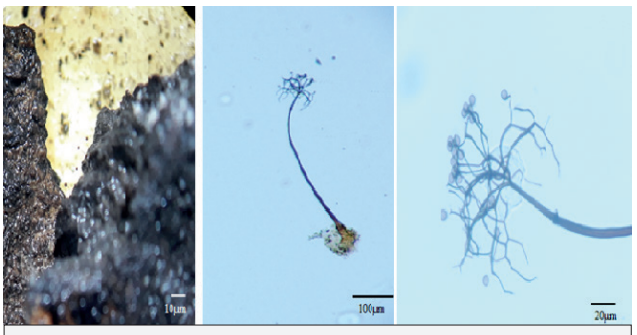

Figure 16. Sporangia, spore and capillitium of *Macbrideola martinii*

Specimen examined–Turkey: Bursa/Osmangazi; In front of Orhangazi tomb, on the bark of *Platanus orientalis*, 05.08.2018, FT07, Çekirge road in front of Çelik Palas, on the bark of *Platanus orientalis*, 05.08.2018, FT89.

Found on dead bark of trees in a moist chamber, distributed in Jamaica and Dominica (2), *Macbrideola martini* has been reported only twice in Turkey according to checklist (13). Isolated from the bark of the Quercus tree in İstanbul, using moist chamber (8). In this study, it has been isolated from barks on the bark of *Platanus orientalis*. Its slender tips of the capillitium make it different from *Macbrideola cornea* (2).

Macbrideola oblonga Pando, F., Lado, C. 1988: (Figure 17)

Description–Sporangia are scattered 0.4–0.9 mm tall. Hypothallus is wide and reddish. The stalk tapered, 16–25% of the total height, reddish, translucent at the base. Sporotheca mostly ellipsoidal, rarely sub-globose or cylindrical, burnt umber, 0.20–0.45 mm diameter. Peridium is remaining as a transparent, red-brown collar. Columella is reaching almost to the apex of the sporotheca. Capillitium arising from along the length of the columella, dichotomously branched, ending free or united near the surface, small sporocarps with few or no anastomoses, somewhat thick, brown, hardly attenuated outwards, free ends blunt, slightly swollen or club-shaped. Spore–mass brown. Spores globose, pale brown, 10.5–11.5 (12.5) µm diameter, densely, regularly verrucose.

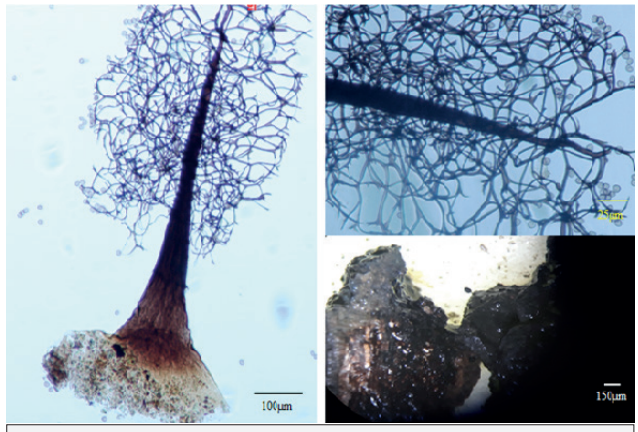

Figure 17. Sporangia, spore and capillitium of *Macbrideola oblonga*

Specimen examined–Turkey: Bursa/Osmangazi; Çeltik Village Yeniyol street, on bark of *Quereus robur*, 06.10.2018, FT45.

Macbrideola oblonga species is rare and commonly found in the desert of Western Kazakhstan (9). It has never been reported in Turkey until recently by Ocak & Konuk (9) from the bark of *Pinus nigra* tree in the Konya region of Turkey. In the city of Bursa and the Marmara region of Turkey, this is the first time it has ever been isolated and reported from the bark of *Quercus robur* tree.

Trichiales

Arcyria cinerea (Bull.) Pers., Syn. Fung.:184. 1801. (Figure 18)

Description–Sporangia scattered or in groups, usually cylindrical or subconical, 0.5–0.8 mm in diameter, almost white, pale gray, beige or rarely ochraceous. Hypothallus is extending under the whole group but discoid under isolated sporangia. The stalk is 0.2–1.5 mm long usually darker than the sporangium, filled with round cells 14–22 µm in diameter. The peridium is fugacious, small, yellowish, and translucent and the surface is smooth or almost smooth. The capillitium net is usually small meshed, rarely rather wide-meshed, mainly expanding upwards to 1.5 times the original height, firmly connected to the cup, strongly warted or spinulose at the base and covered with spines at the periphery. The spore is 6 – 8 µm in diameter, almost colorless in transmitted light, covered with fine warts. Plasmodium is usually white and sometimes grey or greenish.

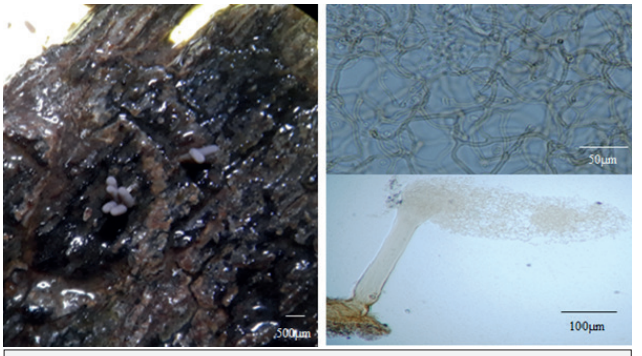

Figure 18. Capillitium, Sporangium, Stalk and spore characteristics *Arcyria cinerea*

Specimen examined–Turkey: Bursa/Osmangazi; Burton visualization center near Çelik Palas, on the bark of *Platanus orientalis*, 05.08.2018, FT84, Sultan Palace road, on the bark of *Platanus orientalis*, 06.10.2018, FT48

Arcyria cinerea has been isolated from *Platanus orientalis* trees in our study. In Bursa-Uludağ, it was isolated from fallen and rotten trees (15). In a similar study in Konya, *Arcyria cinerea* was isolated from the bark of living *Pinus nigra* (9), *Platanus* sp., *Malus* sp. and *Pinus nigra* (32). This species has also been reported by some researchers in Turkey as described in (13). Distributed in France and the British Isles, it is common on logs, dead wood, and bark of living trees (2, 23). In bark culture, this species can be mistaken for *Arcyria pomiformis*. However, it can be differentiated by its development of pinkish plasmodium unlike that of *A. pomiformis* which is yellow.

Arcyria insignis Kalchbr. & Cooke, in Kalchbr., Grevillea 10:143. 1882 (Figure 19)

Description–Sporangia scattered, gregarious, shortly ovate with an acute apex, pale rose, flesh color or pale salmon 0.5–1.5 mm tall. The stalk is short 0.7 mm high brownish-orange, and the base filled with spore-like or sub-globose cells. Peridium is remaining as a shallow saucer shape cup. Capillitium is pale yellow to colorless threads, marked with bands and spines arranged in a loose spiral, in part minutely spinulose to nearly

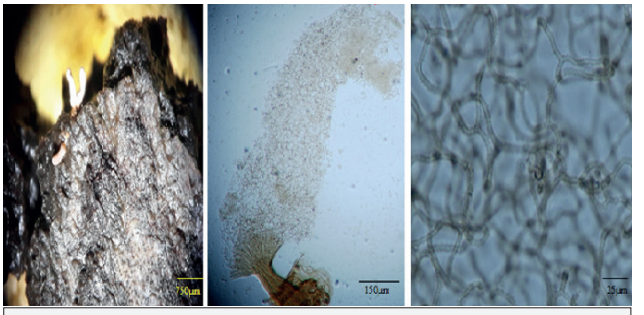

Figure 19. The capillitium, spore, and sporangium of *Arcyria insignis*

smooth attached to the calyculus. Spores are yellowish pinkish in mass and colorless in transmitted light with scattered groups of warts, 8–10 µm in diameter.

Specimen examined–Turkey: Bursa/Osmangazi; In front of Çekirge police center, *Platanus orientalis*, 05.08.2018, FT28.

Arcyria insignis was previously reported in Bursa by Ergül & Akgül (15) from decay logs of *Fagus orientalis* and on bark and branches of *Pinus brutia* (31). In our study, it was isolated from the bark of *Platanus orientalis*. Distributed in New Zealand, the Mediterranean region and Central Europe, it is commonly found on fall branches (23).

Perichaena corticalis (Batsch) Rost., Mon. 293. 1875 (Figure 20)

Description–The sporangia are mainly sub-globose or globose, sessile, gregarious, hemispheric, rarely short ring-shaped plasmodiocarps, 0.2–1.0 mm in diameter, bright reddish brown to nearly black, undulating line around the middle indicates the position of the lids. The Hypothallus is extending under the whole group, thin brown. The Peridium is thick, double and

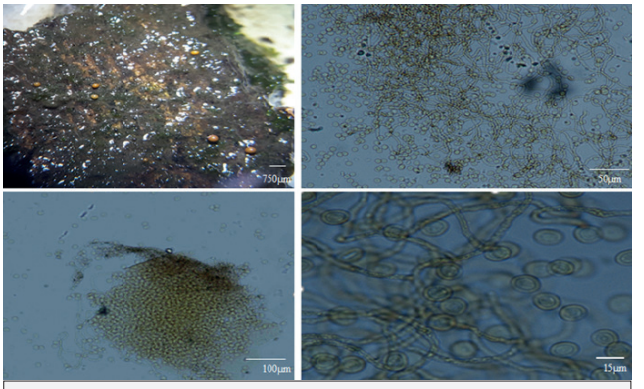

Figure 20. Spore mass, peridium, capillitium and sporangia of *Perichaena corticalis*

the outer layer often impregnated with granular materials and sometimes calcareous, connected to the membranous inner layer, opening with a convex lid leaving a persistent basal cup. Capillitium is abundant, scanty,1.5–4.0 µm in diameter, slender or simple branched yellow threads, irregular compressed, angled or constricted minutely warted or spiny, rarely nearly smooth. Its spores are in mass bright yellow or golden yellow, yellow in transmitted light 10–14 µm in diameter, with small pale warts. Plasmodium is usually watery gray or brown. Common in bark or dead trees and moist chamber culture of barks.

Specimen examined–Turkey: Bursa/Osmangazi; Behind Pınarbaşı cemetery, on the bark of *Platanus orientalis,* 06.10.2018, FT36. Pınarbaşı Park in front of picnic tables, on the bark of *Platanus orientalis,* 06.10.2018 FT29. Pınarbaşı Park, on the bark of *Platanus orientalis*, 06.10.2018 FT44. Pınarbaşı Park opposite war cemetery, on the bark of *Platanus orientalis*, 06.10.2018, FT42. In front of Orhangazi tomb, on the bark of *Platanus orientalis*, 05.08.2018, FT07. Hamza Bey road, on the bark of *Platanus orientalis*, 05.08.2018, FT75. In front of Çukurca mosque, on bark of *Platanus orientalis*, 06.10.2018, FT91. Çeltik Village Yeniyol street, on the bark of *Platanus orientalis,* 06.10.2018, FT18. Sultan Palace road, on bark of *Platanus orientalis,* 06.10.2018, FT10. Sultan Palace road, *Platanus orientalis*, 06.10.2018, FT20.

Mostly found on dead wood and bark of living trees. *Perichaena corticalis* has been mainly distributed in the Americans and Europe. This species of Perichaena has been reported in Turkey in the 1980s by Härkönen & Uotila (11) and also, reported in the bark of trees collected from the Görükle Campus in Bursa (35). In İzmir, it was reported to have been isolated form branch of *Pinus brutia* (31), and in Kütahya province, it was isolated from the bark of living *Prunus domestica* (9). In our study, we isolated *Perichaena corticalis* on the bark of *Platanus orientalis*.

DISCUSSION

The species isolated so far has been reported previously in Turkey. Six of the species (*Physarum gyrosum*, *Physarum notabile*, *Didymium bahiense*, *Didymium difforme, Macbrideola martinii*, and *Macbrideola oblonga*), are new records for the city of Bursa. This study also reports the first *Macbrideola oblonga* in the Marmara region of Turkey. *Cribraria violacea* is the most frequently isolated species isolated from 12 (40%) of the barks of monumental trees. There was no co-existence between the species but the *Enerthenema papillatum* were found together with the *Macbrideola* spp. The species isolated were on the barks of *Platanus orientalis* 27, *Platanus occidentalis* 1 and *Quercus robur* 2. The texture of the tree bark might primarily be a factor on the occurrence of different myxomycetes on a bark of tree, *Platanus* sp.*,* *Quercus* sp., etc. tends to store water and nutrients required for the growth of myxomycetes (36), in our study, even though samples were collected from other plants species, production was only observed on *Platanus* sp. and *Quercus* sp.

The yield of corticolous myxomycetes is low compared to studies done in the Uludağ region, where 35 species were isolated from 61 localities (15). It could be that the Uludağ region is a more natural ecological environment than the city center area. Some studies indicate that there is an ecological simplification in the urban environment leading to the decrease in some of these species (37). A similar study in Spain reported a significant difference between the diversity of myxomycetes in the city are and outside the city area. More myxomycetes were isolated from outside city area due to the decrease in environmental pollutants (14). Not all studies agree to the low yield of myxomycetes in the urban areas. A study by (1) founds no significant difference between the city and outside the city area.

Even though the sample side from the outside city is not much, the species isolated from both areas are the same except for *Macbrideola oblonga* which was isolated from the outside city only. This species was isolated from a village in Konya province (9), and in Spain, a study that compares the effect of environmental factors on the presence of myxomycetes found *Macbrideola oblonga* in a less polluted environment (14).

Some of the species are more tolerant of pollution than others. A cosmopolitan *Licea* sp. which has been isolated frequently in other studies was not isolated in our study. This species was said to be not pollutant tolerant. *Echinostelium minutum* which was isolated from a more acidic environment, in a city area (14) was only found in the city area of our study. The second most diverse species *Perichaena corticalis* was mainly found on barks from the park and the graveyards in the city, unlike *Perichaena corticalis, Cribraria violacea* was found on barks from both the park, graveyard, and the streets.

Even though we do not evaluate the environmental factors that may hinder the habitat of myxomycetes in the city, we can suggest that diversity is low compared to other studies previously done in Bursa city. The collection of other types of specimens from the plants such as the fallen leaves and logs were not possible in this study due to the plants being located in the city area where these debris are swept off daily. Increasing the sample size by increasing the number of localities, may help observe many other species that were not isolated in this study. Specimens collected at different periods throughout the year may also help understand the diversity of the species at different period of the year.

The pollutants in the city area might hinder the diversity of myxomycetes, but in our study, we were able to isolate species that were never isolated from the more natural and floristic areas like the Uludağ region of Bursa, could it be that these species can easily be found in city areas? But since they were recorded in other cities outside of the city area, it could be that environmental pollutants do not affect the myxobiota.

CONCLUSION

This study provides a piece of first information on the diversity of myxomycetes in the city area and on Monumental trees of Bursa. It also added data and 6 new records for the Myxobiota of Bursa.

The effect of urbanization on the distribution of myxomycetes can be studied at a larger scale by increasing the number of samples, collecting environmental pollutant parameters, comparing the distribution of myxomycetes in the city to those in semi-urban and the more floristic areas. This will help have a better understanding of myxomycetes distribution in the city of Bursa. Our study can serve as a basis for future studies on understanding the Myxobiota of Bursa.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of study: C.C.E., F.T.; Data Acquisition: C.C.E., F.T.; Data Analysis/Interpretation: C.C.E., F.T.; Final Approval and Accountability: C.C.E., F.T.; Drafting Manuscript: C.C.E., F.T.; Critical Revision of Manuscript: C.C.E., F.T.; Technical or Material Support: C.C.E.; Supervision: C.C.E.

Conflict of Interest: The authors declare that they have no conflicts of interest.

Financial Disclosure: There are no funders to report for this submission

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ISTANBUL UNIVERSITY PRESS

RESEARCH ARTICLE

Eur J Biol 2019; 78(1): 51-57

Lectin Treatment Affects Malignant Characteristics of TPC-1 Papillary Thyroid Cancer Cells

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Please cite this article as: Sancakli A, Kaptan E. Lectin Treatment Affects Malignant Characteristics of TPC-1 Papillary Thyroid Cancer Cells. Eur J Biol 2019; 78(1): 51-57.

ABSTRACT

Objective: Abnormal glycosylation is a universal aspect of cancer cells. The altered glycosylation pattern has been originated from changings in expression of glycosylation enzymes which are up-regulating in reply to some oncoproteins in the biosynthetic pathway of glycans. In this study, it was aimed to show the presence of terminal α-2,3, α-2,6 sialic acid and α-1,6/α-1,2 fucose motifs in TPC-1 papillary thyroid cancer cells. Also it was aimed to examine the changes in viability and mobility of the cells after exogenously specific lectin treatment.

Materials and Methods: In this study, the presence of terminal sugar residues in glycan chains on the cell surface was demonstrated using lectin histochemistry and lectin blotting techniques in TPC-1 cells. The changes in the cell viability and proliferation after lectin treatment were assessed using the WST-1 test. The Changes in the cell mobility after lectin treatment, however, were assessed using the wound healing test.

Results: α-2,3, α-2,6 sialic acid and α-1,6/α-1,2 fucose motifs were widespread in the surface of TPC-1 cells. MAL-II (*Maackia amurensis* Lectin II) treatment increased the cell proliferation and mobility of TPC-1 cells. Although SNA (*Sambucus nigra* Aglutinin) and AAL (*Aleuria aurantia* Lectin) treatment did not significantly affect the cell proliferation, SNA and AAL treatment supported the mobility of TPC-1 cells.

Conclusion: Lectin treatment affect cancerous properties differently depending on the cell type. Also lectin treatment can support the malignant behaviour of cancer. For this reason, it is necessary to understand the mechanisms of the lectin effect on the cancer cells.

Keywords: Papillary thyroid cancer, lectin treatment, glycosylation

INTRODUCTION

Thyroid carcinoma is one of the highly malignant disease that originates from endocrine glands. Thyroid gland consists of follicular and parafollicular (C cells) cells. There are 4 histological types of thyroid carcinoma; Papillary, follicular, medullary and anapylastic. Medullary carcinoma are originated from parafollicular C cells and the others are from follicular cells (1). This classification is characterized by mutations leading to an increase in cellular proliferation and differentiation (2). Papillary thyroid carcinoma (PTC) is the most common human thyroid cancer with incidence of 80% (3). The most common genetic mutations seen in PTC are *BRAF* and *RET* at the percentage of 40-60% and *RET* at 20% respectively. The gene product of *BRAF* is known to involve in the MAPK/ERK signalling a pathway which plays an important role in various cellular processes such as cell division, differentiation and secretion. *BRAF* is a serine/threonine (S/T) kinase and activated by somatic point mutation occurs in S/T domain (V600E) and the mutated form receives a mitogenic signal from RAS and transmits it to the MAP kinase pathway (4). The *BRAF* mutation is thought to be the key mutation for the treatment of thyroid carcinoma and for the diagnosis of aggressive PTC (4, 5). Another mutation seen in PTC is the telomerase reverse transcriptase (*TERT*) gene. It plays an important role in activity of

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Submitted: 11.04.2019 • **Revision Requested:** 12.04.2019 • **Last Revision Received:** 13.05.2019 • **Accepted:** 17.05.2019

telomerase enzyme during malignant transformation of cells (6, 7). The nucleotide of 1.295.228 C on the 5th chromosome expressing this enzyme is replaced by the T nucleotide (C228T) and the 1.295.250 C nucleotide is replaced by the T nucleotide (C250T). Aberrant expression of *TERT* causes cell proliferation without changing the length of telomerase in human (8, 9).

Glycosylation is a posttranslational or cotranslational modification required in many proteins to perform many cellular functions. Glycan chains linked to proteins plays role in physiological processes such as intracellular protein trafficcing, signal transduction, cell-cell interaction, cell-extracellular matrix (ECM) interaction, differentiation (10). Also it has an important role in the pathophysiological processes (11). Glycoconjugates are glycosylated biomolecule groups and they have been shown to take part in the development and progression of cancer (12). Additionally, cancer-associated glycoconjugates in blood serum and tissues are used as a marker for determining the progression of the disease (13). Altered or aberrant glycosylation is a common pathological processes in the various type of cancers. This processes is performed by many glycosylation enzymes, which participate in the biosynthetic pathways of glycans, up-regulated in reply to some oncogenic proteins (14). Changes in the expression of these kind of enzymes induce differences in the synthesis of the glycan chains found in membrane glycolipids and glycoproteins (15). This phenomena are also observed in the processes such as embryogenesis, regeneration and inflammation (16).

In the present study, we aimed to examine the existence of α-2,3, α-2,6 sialic acid and α-1,6/ α-1,2 fucose motifs, which are common terminal sugars of several cancers. In addition, we aimed to show the possible effects of the lectins having binding ability to terminally located α-2,3 sialic acid, α-2,6 sialic acid α-1,6/α-1,2 fucose motifs on cell viability/proliferation and mobility.

MATERIALS AND METHODS

Cell Culture

TPC-1 cells were cultured at 37°C with 5% CO_2 at 1 atmospheric pressure in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin ve 100 µg/mL streptomycin). Firstly, cells with sufficient density were washed with sterile PBS (Phosphate Buffered Saline) without calcium-magnesium. The cells were removed from petri dish with 2.5% sterile Trypsin-EDTA (Ethylene Diamine Tetraacetic Acid). For freezing of the cells, the freezing medium prepared with 90% FBS and 10% was used and transferred to -152°C for periodic storage.

Cell Surface Lectin-Fluorescent Staining

The aim of this method is to determine α-2,3 and α-2,6 sialic acid and α-1,6/α-1,2 fucose motifs on TPC-1 cell surface. For this purpose, 25.000 cells were transferred to each well of 24-well plate with 12 mm diameter sterile coverslip. The cells with sufficient density were rinsed with HEPES (pH 7.5) and then the cells fixed in 4% paraformaldehyde (pH 7.2) for 10 min in a 37°C incubator. After washing, the cells incubated in HEPES containing 50 mM ammonium chloride (NH_4Cl) for 5 min and blocked with BSA for 10 min. The sugar residues on the cell surfaces were labelled with 2.5 µg/ml biotinylated MAL-II, SNA and AAL (Vector Lab. UK) lectins for 1 hour at room temperature and then cells were incubated with Cy3-conjugated Streptavidin (ThermoFisher-Scientific, USA) and ProLong Gold antifade reagent (ThermoFisher-Scientific, USA) with DAPI which was used to distinguish the nuclei of the cells. To check out lectin binding specificity, two negative control were designed namely, untreated and inhibitory sugars (MAL-II/3'sialillactose, SNA/6'sialillactose, AAL/L-fucose) groups. Lectin binding activity in the surface of the cells were observed with an epi-illuminated fluorescence microscope (Nikon, Eclipse-Ti-U) and photographed with a camera (Nikon, DSRi1).

Lectin Blotting

Membrane Protein Extraction Kit (ThermoFisher-Scientific, USA) was used for the isolation of membrane proteins. The cells were harvested from the flask with the cell scraper and centrifuged at 3000g. Membrane proteins were obtained following the instructions of the product manual. The amount of the proteins was determined using the Bradford protein determination method and 30 µg/ml membrane protein samples were loaded in each well of 4-12% Bis-tris gradient acrylamide gel. The electrophoretically separated proteins in the gel were transferred onto nitrocellulose membrane using Novex iblot gel transfer system (ThermoFisher-Scientific, USA). After transferring step, the membrane was incubated in 1µg/ml biotinylated MAL-II, SNA and AAL lectins at room temperature for 1 hour. The biotinylated lectins were labelled by incubating cells with 2 µg/ml HRP-conjugated Streptavidin in PBST (Phosphate buffered Saline-Tween 20) at room temperature for 1 hour. After that, cells were washed with PBST and the lectin specific sugar motifs on the cell surface were visualized by the gel imaging system (KODAK Gel Logic System 1500) using the Novex ECL HRP Chemiluminescent Substrate Reagent Kit.

Cell Viability and Proliferation Assay

In order to investigate the effect of MAL-II, SNA and AAL unconjugated lectins on the viability of TPC-1 cell lines, WST-1 test was performed with 50.000 cells per well of 96 well plates. The cells treated with the lectins in doses ranging from 0,001 to 1µM for 24 and 48 hours and, 10µl WST-1 reagent was treated to these cells and incubated in a 37°C incubator for 30 minutes. The plate was stirred for 60 min in a shaker and the absorbance at 440nm were taken using µQuant plate reader (BioTek, Vinooski, VT). Cell viability and proliferation data obtained from three independent experiments and performed in triplicate wells.

Wound Healing Assay

According to the data obtained from the cell viability test, two different doses which do not cause any increase in the number of cells were selected for each lectin and used in the wound healing test in order to investigate the effects of the cells on the cell mobility. TPC-1 cells were seeded on 6-well plates in certain numbers. When the cells reached a density of 70-80%,

a wound model was created by carefully drawing horizontal and vertical lines with 200 µl pipette tip to the bottom of the wells. Unconjugated lectins were treated to the wound model for 24 and 48hours and wound width was measured with NIS-Elements D 3.1 software after at 0, 24 and 48 hours. Using the data obtained, the percentage of wound width was calculated in terms of the formula; Wound width % = (Wound width at 24 or 48h)/(Wound width at 0h) ×100.

Statistical Analysis

Statistical analysis were made using SPSS software. The paired comparisons of the parameters with the control groups were performed using the t-test. ANOVA analysis and Tukey postoc test were used for multiple comparisons. Microsoft Excell program was used to draw graphics.

RESULTS

Terminal Sugar Residues of TPC-1 Cell Surface

In order to show α-2,3, α-2,6, terminal sialic acid and α-1,6/α-1,2 terminal fucose motifs on cell surfaces, MAL-II, SNA and AAL lectins specific for these motifs were used respectively. Fluorescent intensities were obtained from lectin staining study with biotinylated lectins and Cy3-streptavidin. The presence of α-2,3 and α-2,6 sialic acid and α-1,6/α-1,2 fucose motifs were evident on the cell surface of TPC-1 cells. However, α-2,3 sialic acid and α-1,6 fucose motifs were significantly higher than α-2,6 sialic acid motifs ($p \leq 0.001$) (Figures 1A and 1B). The data obtained from lectin blotting studies showed that there were terminal α-2,3 and α-2,6 sialic acid and α-1,6/α-1,2 fucose sugars in the membrane proteins of TPC-1 cell. The membrane glycoproteins glycosylated with terminally α-2,3 sialic acid in TPC-1 cells were ranging from 40 to 80 kDa. Its terminally α-2,6 sialillated membrane glycoproteins were ranging from 20 to 110 kDa. Its terminally α-1,6/α-1,2 fucosylated membrane glycoproteins, however, were ranging from 30-160 kDa (Figure 1C). Although α-2,6 sialilation was weaker than the others when considering SNA fluorescent density in TPC-1, α-2,6 sialilated membrane glycoproteins more diverse than the other sugar glycosylation when considering lectin blotted membranes (Figures 1A-C).

Effects of Lectins on TPC-1 Cell Viability

When TPC-1 cells treated with MAL-II lectin for 24 hours, the cell viability of TPC-1 cells increased in a dose dependent manner at doses among 0.001 and 0.05µM. However, the cell viability decreased in dose dependent manner at doses among 0.05-1µM. On the other hand, no significant changes were observed in the cell viability of SNA and AAL lectins 24h treated groups in TPC-1 cells at the dose range of 0.001-1µM (Figure 2A). The results of 48h SNA and AAL lectin treatment in TPC-1 cells at the dose range of 0.001-1µM were not similar to the findings those of 24h SNA and AAL lectin treatment (Figure 2B). Some significant fluctuations in the viability of TPC-1 cells were observed in 48h SNA lectin treatment. A statistically insignificant 38% increase was observed in the 0.1 µM dose compared to the control. However, the viability of SNA treated TPC-1 cells decreased in dose dependent manner. AAL lectin treatment to TPC-1 cells at a dose range of 0.001-1µM for 24 hours increased the cell viability in dose dependent manner up to 25%. It was found that the increase in the viability of these cells was statistically significant at 0.5, 0.1, 0.25 ($p \leq 0.05$) and 0.5µM ($p \leq 0.01$) (Figure 2B).

Effects of Lectins TPC-1 Cell Mobility

Increased cell mobility was observed in both 0.001 µM and 0.005 µM doses in 24h and 48h MAL-II lectin treated TPC-1 cells. A significant enhancement in cell mobility was monitored, especially at the concentration of 0.001µM. The wound closure was quicker than untreated control cells ($p \leq 0.001$). There was also a statistically significant increase at MAL-II concentration of 0.001µM in 48h treatment ($p \leq 0.05$). Insignificant increase in cell mobility were observed in 48h treatment of MAL-II at 0.005µM concentration when compared to control (Figure 3). 24h and 48h SNA lectin treatment for the doses 0.005 µM and 0.01µM augmented the cell mobility of TPC-1 cells in a dose-dependent manner. However, this increase is only statistically significant at 48 hours at a concentration of 0.005µM ($p \leq 0.05$) (Figure 4). A statistically significant increased cell mobility was observed in both concentrations of 0.001 µM and 0.005µM AAL for 24h treatment (Figure 5). The closure was quicker in both doses of AAL than the untreated control group ($p \leq 0.05$). Overall results of MAL-II treatment suppressed wound closure of TPC-1 cells at all doses tested when compared to untreated control. The wound width of 24h and 48h lectin treated cells were narrower than that of untreated control with various statistical significances.

DISCUSSION

Recent studies have shown that most of the lectins have anticarcinogenic feature. This fact generally occurs when lectins link to glycan chains in the tumor cell membrane to produce various effects. Mechanical details are not yet known, but there are so many pieces of evidence that lectins may inhibit or activate protein function by binding to glycan chains of glycoproteins in different cellular compartments (17). Thus, the lectin interaction in different cellular compartments can give rise to some toxic effects such as decrease in protein synthesis or cell death stimulation.

Our results pointed out that MAL-II treatment to TPC-1 cells, which are papillary thyroid cancer cell line, increased the cell proliferation at the dose range of 0.001-1µM. Similar to our findings PNA, which is peanut lectin and recognizes Galβ-1,3GalNAc (TF-antigen) motifs, had also been shown to have a proliferative effect on HT29 colon cancer cells (18, 19). MAL-II treatment at the concentration range of 0.8-8 µM demonstrated to induce apoptosis in lung cancer cell lines (20). Chen et al., (2009) showed that sialic acid specific PCL lectin purified from *Phaseolus coccineus* plant was highly toxic on L929 fibrosarcoma cells. As a result of this toxic effect, apoptotic death in the cells was more dominant but they also observed that it stimulated necrotic death (21).

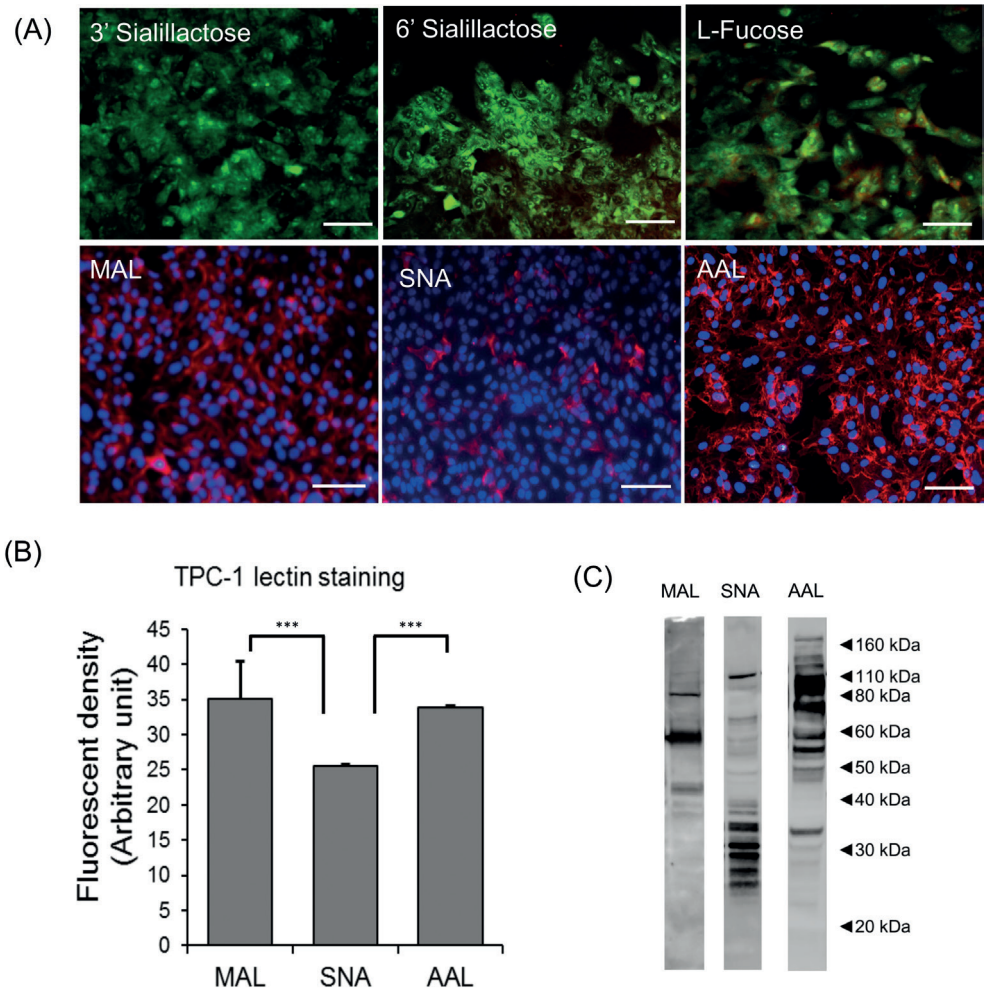

Figure 1. Alpha-2,3/2,6 sialic acid and α-1,6/α-1,2 fucose motifs in the cell surface of human papillay thyroid carcinoma cell lines TPC-1. The fluorescent micrographs of cell surface lectin binding assays and their inhibitory sugar controls (A) and the surface fluorescence intensity of the cells after biotinylated MAL-II, SNA and AAL lectin-staining (B) and the membrane glycoprotein profile having MAL-II, SNA and AAL affinities in the membrane protein fraction of TPC-1 cells blotted on nitrocelluse membrane. The detected bands display the specific glycosylation status (α-2,3, α-2,6 sialic acid and α-1,6/α-1,2 fucose residues) of membrane glycoproteins of TPC-1 cells (C). Fluorescence intensity data are represented as the mean±SD. Asterisc (***) shows significance ($p \leq 0.001$) compared to one another. The scale bar is 50µm.

Similarly, mannose binding lectin acquired from *Sophora flavescens* had a toxic effect on HeLa cells and it caused apoptotic and necrotic death (22). ACA lectin acquire from *Amaranthus caudatus* plant can bind to TF-antigen motifs same as PNA (23, 24) and it was found to significantly increase the proliferation of HT29 colon cancer cells (25). Based on our results, consumption of plant nutrients or extracts containing α-2,3 sialic acid binding lectins seems unfavorable for the papillary thyroid cancer patients. Because lectins are protein or glycoprotein nature and they are protected from gastrointestinal proteolysis and they can enter the circulatory system. The researchers reported that bioactive PNA lectin reached an average serum concentration of 50 nM after 200 gr of peanut consumption (26).

The researchers stated that decrease in invasive character of melanoma cells after MAL-II treatment was originated from increased α-2,3 sialic acid motifs of podoplanin which is extracellular domain of the mucin receptor. Podoplanin is also responsible for invasiveness of melanoma cells (27). In another study, it was found that only β1 unit of α5β1 integrin in melanoma cells was decorated with α-2,3 sialic acid residues and MAL-II lectin treatment gave rise to a significant reduce in the mobility of cells. MAL-II lectin treatment revealed better results than SNA lectin treatment, which have affinity to α-2,6 sialic acid, in the matter of suppressing melanoma cell mobility (28). In addition, MAL-II and SNA lectin treatment to 8505C anaplastic and K1 papillary thyroid cancer cells has been shown to be effective in suppressing

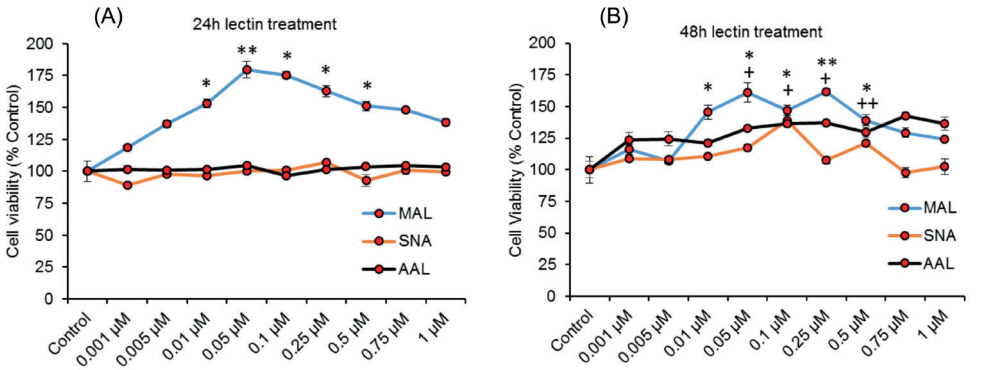

Figure 2. MAL-II, SNA and AAL treatments affect on the survival of human papillary thyroid carcinoma cell lines TPC-1. Changes in the cell viability in 24h (A) and 48h lectin treatment (B) at doses among 0.0001-1µM. The data are represented as the mean±SD. Asteriscs (*/**) show significance (p≤0.05/p≤0.01) between treated and untreated groups.

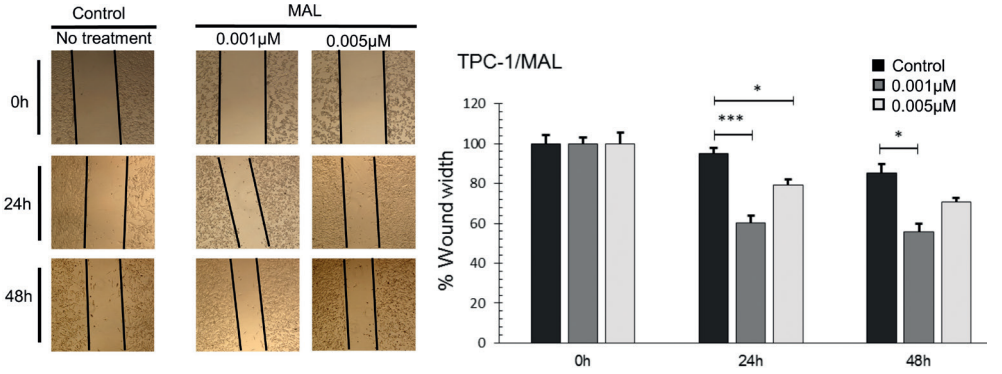

Figure 3. MAL-II treatment affects on the mobility of human papillary thyroid carcinoma cell line TPC-1 and, 0.001 and 0.005µM MAL-II treatments augmented the cell mobility of TPC-1 cell. Wound width percentages are represented as the mean±SD. Asteriscs (*/***) show significance (p≤0.05/p≤0.001) between treated and untreated groups.

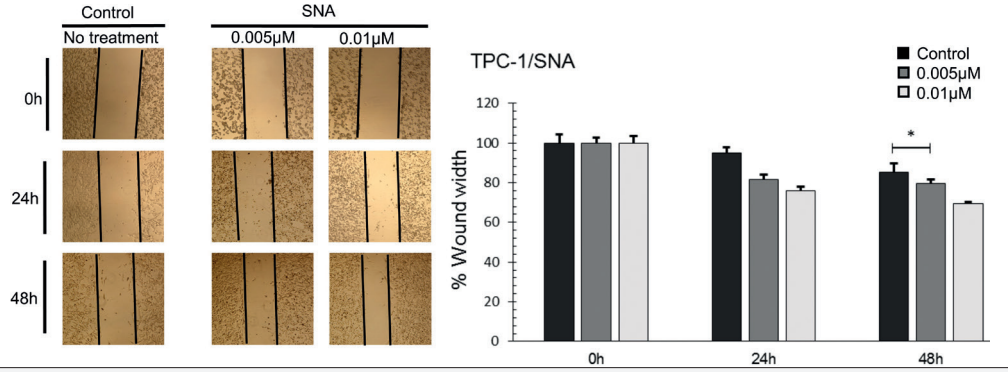

Figure 4. SNA treatment affects on the mobility of human papillary thyroid carcinoma cell line TPC-1 and, 0.005 and 0.01µM SNA treatments enhanced the cell mobility of TPC-1 cell. Wound width percentages are represented as the mean±SD. Asterisc (*) shows significance (p≤0.05) between treated and untreated groups.

cell migration. However, it has been demonstrated that AAL treatment increases the mobility of 8505C cells. It has been also shown that MAL-II and SNA treatment to FTC-133 follicular thyroid cancer cells increases cell mobility (29). Our findings have been pointed out that MAL-II, SNA and AAL lectin treatment significantly increase cell mobility on TPC-1 papillary cancer cells. Although limited number of study have been focused on the effects of lectins related to cell mobility, overall findings have indicated that lectin treatment can affect cell mobility differently depending on the cancer types and even depending on the cancer cell types. It can be suggested that lectin effect comes out in cancer cells

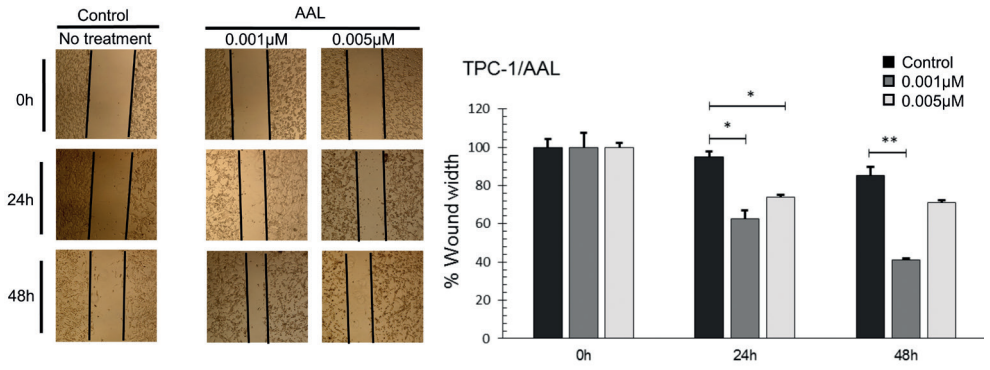

Figure 5. AAL treatment affects on the mobility of human papillary thyroid carcinoma cell line TPC-1 and, 0.001 and 0.005μM AAL treatments increased the cell mobility of TPC-1 cell. Wound width percentages are represented as the mean±SD. Asteriscs (*/**) show significance (p≤0.05/p≤0.01) between treated and untreated groups.

with respect to which cell surface proteins will decorate themselves with the altered sugar chains. Therefore, further studies with a mechanistic perspective should consider glycosidic modifications of membrane proteins.

CONCLUSION

In this study, we examined the effects of lectins targeting to cell surface glycosidic phenotype of papillary cancer cells. Particularly, it was found that the MAL-II treatment supported the aggressive features of the cancer cells. Despite the studies demonstrating the anticancer properties of some lectins, our study have pointed out that lectins can support the poor prognosis of cancer. Also, the present study underscores the need for investigation about the effects of lectins on cancer cells in further details. Because, the nutritional plants contain lectins and they pass through the digestive system without being broken down by proteolytic enzymes and pass into the bloodstream. Therefore, this situation can be considerable for cancer patients. That is why studies on the identification of lectins found in nutritional plants and how they affect cancer cells are of great importance.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of study: E.K.; Data Acquisition: A.S., E.K.; Data Analysis/Interpretation: E.K., A.S.; Final Approval and Accountability: E.K., A.S.; Drafting Manuscript: E.K., A.S.; Critical Revision of Manuscript: E.K., A.S.; Technical or Material Support: E.K., A.S.; Supervision: E.K.

Conflict of Interest: The authors declare that they have no conflicts of interest.

Financial Disclosure: There are no funders to report for this submission

Acknowledgements: This study was supported by the grant from Istanbul University, Unit of Scientific Research Projects (IU. BAP, project no: 46177)

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SHORT COMMUNICATION

Eur J Biol 2019; 78(1): 58-62

Infestation of the Torrent Catfish (Siluriformes, Trichomycteridae) by an Exotic Crustacean (Copepoda, Lernaeidae) in Andean Mountains of Argentina

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Please cite this article as: Fernandez L, Sanabria E, Quiroga L. Infestation of the Torrent Catfish (Siluriformes, Trichomycteridae) by an Exotic Crustacean (Copepoda, Lernaeidae) in Andean Mountains of Argentina. Eur J Biol 2019; 78(1): 58-62.

ABSTRACT

Objective: This study reports the presence of a copepod, affecting adults of the torrent catfish in a tributary of the San Juan River in the southern part of South America.

Materials and Methods: The fish (48.1-54.1 mm standard length) were collected in Los Bretes, San Juan province, Argentina. They were anesthetized, fixed in formalin, preserved in ethanol and deposited in the Ichthyological Collection of the FACEN.

Results: Three torrent catfish (*Trichomycterus corduvensis*) examined were infested with nine copepod parasites (*Lernaea cyprinacea*), which were attached to the gill, urogenital opening, and base pectoral fin.

Conclusion: According to our results, this is the first record of parasitism of Lernaea on the torrent catfish (*T. corduvensis*) and a new locality from the Andean Mountains of Argentina.

Keywords: Anchor worm invasion, *Lernaea cyprinacea*, torrent catfish, *Trichomycterus corduvensis*, andes

INTRODUCTION

Lernaea cyprinacea Linnaeus 1758 or anchor worm is a common parasite and appears almost all over the world in freshwater fish (1). *Lernaea* is an ectoparasitic copepod causing lernaeosis in several species of fishes. In South America, the reports of lernaeosis are scarce, and it should be remembered that there are thousands of native freshwater fish on this continent that have never been examined for parasites (2-5). Argentina is one of the most diverse countries in the world and little is known about the diversity of invertebrate species of ichthyoparasites of inland aquatic ecosystems (6). Lernaeids comprise of a family of Copepoda (100+ species) in which the small free-living adult females become parasitic after copulation, metamorphose, and grow to a relatively large size without molting (7). These ectoparasitic copepods are distributed naturally in Africa, Central Asia, Southern of Siberia, and are reported in Europe, Japan, and Israel (7). In Argentina, the first record of lernaeid parasites infecting freshwater fish is from 1993, parasitizing *Odontesthes bonariensis* in Córdoba (4, 8). The torrent catfish *Trichomycterus corduvensis* is widely distributed in tropical South America and occurring in a remarkable variety of environments such as temporary streams, subterranean drainages in caves, high elevations, fossorial sandy setting, and warm thermal waters (9). *Trichomycterus corduvensis* feed on benthic aquatic macroinvertebrates, such as dipteran larvae, coleopterans, ephemeropterans, trichopterans, plecopterans, and crustaceans and it is distributed in the western part of Argentina, including thermal waters and high elevations (9, 10). Species of Trichomycteridae (Figure 1) have never been reported with infestation by lernaeids, being this study and the first report in San Juan province (Figures 2A-B).

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Submitted: 31.07.2018 • **Revision Requested:** 15.10.2018 • **Last Revision Received:** 21.12.2018 • **Accepted:** 24.12.2018

Figure 1. *Trichomycterus corduvensis* widely distributed in Argentina.

Table 1. Specimens of *Trichomycterus corduvensis* (FACEN 61) infested by *Lernaea cyprinacea*.

n=3 (in mm SL)	**Parasites number and area**
48.1	3 p (right) (Fig. 3B), 1 p (left), 2 g (right)
54.1	1 u (Fig. 3C)
48.1	2 g (left) (Fig. 3A)

Abbreviations: standard length (SL), gill (g), urogenital opening (u), pectoral-fin base (p).

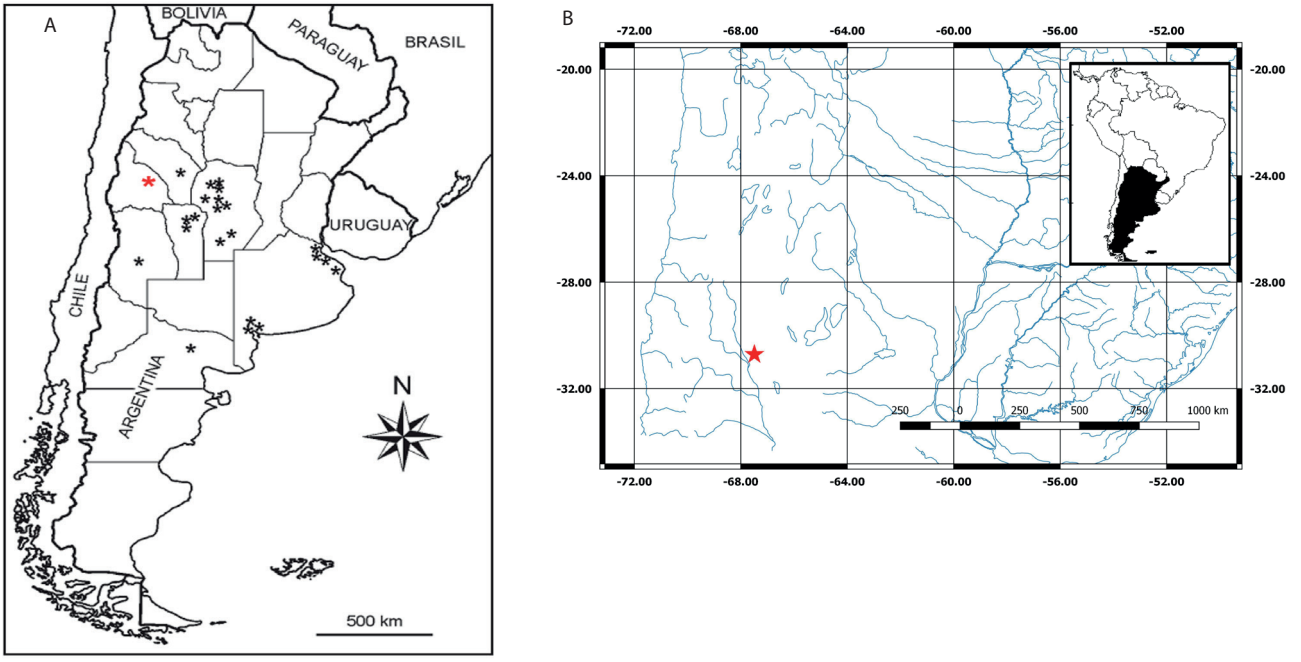

Figure 2. A. Map of *Lernaea cyprinacea* modified from Waicheim et al 2017. B. The new record (red asterisk) is at 1,011 m above sea level in Los Bretes, San Juan.

MATERIAL AND METHODS

The fish were collected in San Juan province, Argentina (67°29´35"W, 30°42´54"S, at 1,011 m elevations) in December 2005 (Figure 2B). Specimens were kept alive and later anesthetized and killed with ice *in situ*. They were fixed in 10% formalin, preserved in 70% ethanol and deposited in the Ichthyological Collection of the Facultad Ciencias Exactas y Naturales, Universidad Nacional de Catamarca, Argentina (FACEN): FACEN 61, 3 specimens, 48.1-54.1 mm Standard Length (SL), Los Bretes, Departamento Valle Fértil, Provincia San Juan, Argentina. The taxonomic identification was based on Gervasonii et al. (8), Moreno et al. (11), and Fuentes Parra (12).

RESULTS AND DISCUSSION

Three torrent catfish showed severe physical damage by the adult parasites of *Lernaea cyprinacea*. They were found macroscopically on various parts of the host's body of *Trichomycterus corduvensis* (Figures 3A-C, Table 1). Nine parasites were attached in: the gill (4 parasites), urogenital aberture (1 parasite), and the base of the pectoral fin (4 parasites). Although *Lernaea cyprinacea* seem to

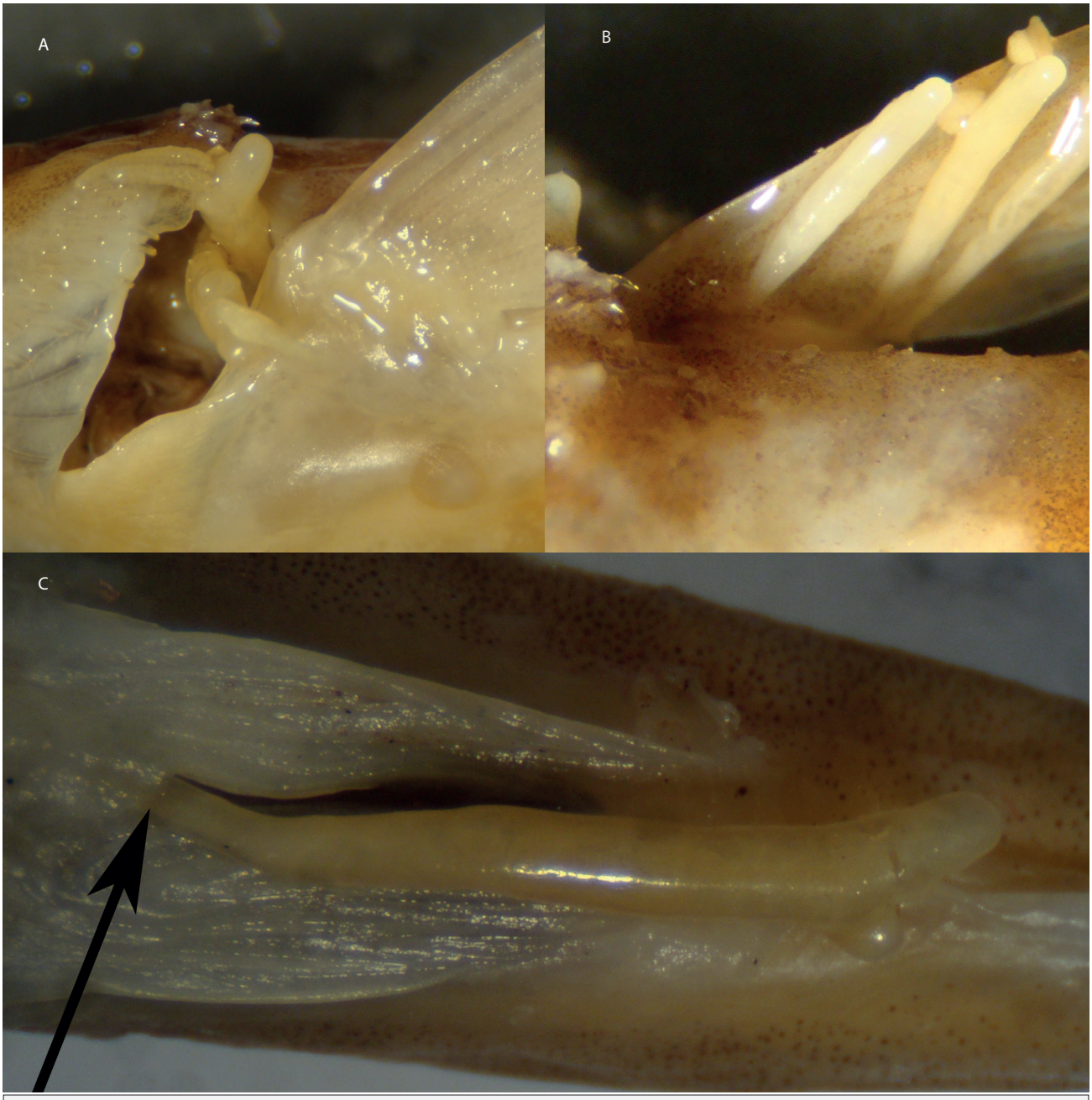

Figure 3. *Trichomycterus corduvensis* infected with *Lernaea cyprinacea*. FACEN 61: Argentina, Provincia de San Juan, Departamento Valle Fértil, Los Bretes, 48.1 mm SL: A. gill (2) and B. pectoral fin base (3); 54.1 mm SL: C. urogenital opening (1).

attach themselves to random parts of the host's body, Bulow et al. (13) and Stavrescu-Bedivan et al. (14) found that *L. cyprinacea* preferred sheltered areas on the fins and skin for attachment. One of the hypotheses proposed to explain this copepod attachment preference is that fins offer greater protection against currents and tissues at the base of the fins may be more easily penetrated (14-16). We agree with that (4 specimens) and we add the gill (4 specimens) and urogenital opening (Table 1 and Figures 3A-C) as sites of preference.

Plaul et al. (5) and Gervasoni et al. (8) summarized in Tables 1 and 2 the localities of *L. cyprinacea* in Argentina (Buenos Aires, Corrientes, Córdoba, Mendoza, Río Negro, San Luis, La Rioja, and Santa Fé) and mentioned as hosts of fish species: Siluriformes: *Corydoras paleatus*, *Hypostomus* sp, *Rhamdia sapo;* Characiformes: *Astyanax bimaculatus, A. eigenmanniorum, A. hermosus, Astyanax* sp, *Bryconamericus iheringii*, *Cheirodon interruptus*, *Oligosarcus jenynsii, Prochilodus lineatus, Cyphocharax voga;* Cypriniformes: *Carassius auratus, Cyprinus carpio, Botia macracantha*; Atheriniformes: *Odontesthes bonariensis;* Cyprinodontiformes: *Jenynsia* sp, *Poecilia* sp, *Xiphophorus helleri*; Salmoniformes: *Oncorhynchus mykiss;* Perciformes: *Percichthys trucha*.

The life cycle of this parasite does not include an intermediate host and it has nine stages in its life cycle, including three free-living naupliar stages, five copepodites stages and one adult stage (12, 17, 18). After male and female adults mate on the fish host (23-24 °C temperature) after which males die (24 hs after), females metamorphose, insert their cephalic region with 4 lateral processes into the host tissue and then produce two conspicuous egg sacs (18-20). In the Los Brete of San Juan, *Cyprinus carpio* is absent and thus other fish species are spreading this copepod upstream.

According to Plaul et al. (5), Piasecki et al. (21), the existence of the parasite and its pathogenic activity in very austral locality demonstrates an increase in temperature generated by contamination. Plaul et al. (5) mentioned that the extreme conditions facilitate the proliferation of the parasites, such as concentration of salts by natural eutrophication or irrigation agriculture. Climatic changes could also increase significantly the geographical dissemination of *L. cyprinacea* in high altitude. *L. cyprinacea* has spread to different parts of the Argentina via movement of aquaculture, such as *Odontesthes bonariensis*. For example Soares et al. (18), Mancini et al. (22), and Bethular et al. (23) mentioned that the silverside (*O. bonariensis*) has been introduced into numerous freshwater environments in Argentina such as other countries (e.g, Japan, Italia, Perú, Bolivia, Uruguay and Chile). Whether accidental or deliberate, the introduction of exotic species is one of the major reasons for the loss of biological diversity, habitat alteration, and overexploitation of natural resources (8, 24-26). This opportunistic ectoparasite is extremely harmful and can be transmitted by water source causing infestation in the Andean basin where others endemic catfishes live (27-29). Parasitological studies are needed to determine the current status of *L. cyprinacea*, especially in the northwestern of Argentina. This new report demonstrates the absence of host specificity and the high adaptability to numerous habitats of freshwater, including polluted streams or endorheic systems in high elevation. For this reason, the provincial and municipal commissions on the environment must promote strong policies directed to prevention of disease in protected areas and the conservation of Andean catfish endangered by exotic fish and its parasitic infestations.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of study: L.F., E.S., L.Q.; Data Acquisition: L.F., E.S., L.Q.; Data Analysis/Interpretation: L.F., E.S., L.Q.; Final Approval and Accountability: L.F., E.S., L.Q.; Drafting Manuscript: L.F., E.S., L.Q.; Critical Revision of Manuscript: L.F., E.S., L.Q.; Technical or Material Support: L.F., E.S., L.Q.; Supervision: L.F., E.S., L.Q..

Acknowledgement: We thanks to Facultad Ciencias Exactas Naturales, Universidad Nacional Catamarca (FACEN-UNCA) and Instituto Biodiversidad Neotropical (IBN-UNT) by facilities and other assistances. For the loan of specimens we thank to J. Andreoli Bize (FACEN).

Conflict of Interest: The author has no conflict of interest to declare.

Financial Disclosure: PIP (Proyecto Investigación Plurianual CONICET) Project nº 11420090100321

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