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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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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.

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Multi-Mode Assessment Approach on Anti-Cancer Potency of Vanadium on Breast Cancer Cells

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ABSTRACT

Objective: The anti-cancer activities of vanadium and its compounds have been widely investigated in cancer research recently. This is mainly attributed to vanadium and its compounds' near ideal properties for being an anti-cancer agent. Most of the current classical chemotherapeutics used in cancer therapy are known to bring numerous strong side effects. Thus, there is a need to discover new drugs which have mild or no side effects and which are effective in low doses. For the treatment of breast cancer (a disease with a difficult and costly treatment process, high mortality, and which is especially prevalent in woman), novel drugs and approaches are required. With this in mind, this study investigates the potential therapeutic efficacies of vanadyl sulphate, a member of the vanadium compounds with ideal anti-cancer properties such as cytotoxicity, antiproliferative and proapoptotic activities on human breast adenocarcinoma cells (MCF-7) including morphological and ultrastructural changes.

Materials and Methods: A MTT colorimetric assay was used for cell viability assessment. Morphological and ultrastructural changes were evaluated using confocal and transmission electron microscopy methods, respectively. The apoptosis stimulating property of vanadyl sulphate was tested under a flow cytometry. And also, cell cycle and proliferation inhibitory effects were examined using the immunohistochemistry technique.

Results: Consequently, vanadyl sulphate was detected to be cytotoxic on MCF-7 cells and also damaged the morphology and ultrastructure of cells, stimulated the expression of cyclins and E-cadherin, which in turn triggered apoptotic cell death.

Conclusion: According to our findings, vanadyl sulphate was determined to be a strong, potent candidate for anti-cancer drug development and is advisable for further investigations in this area.

Keywords: Breast cancer, vanadium, cytotoxic activity

INTRODUCTION

Cancer occurs with an uncontrolled proliferation and differentiation of the normal functions of cells. The disease causes irreversible functional abnormalities in tissues and organs of the body. Statistical reports have shown that cancer remains one of the most serious public health problems with its high global mortality rates. In addition, it is estimated that cancer will be ranked highest among those diseases that result in death in the next five years. Breast cancer has been reported as ranked second highest among those cancer cases with high mortality and morbidity (1). Breast cancer occurs in the mammary tissues of woman and men and this problem has become more intense in the last few years (2). Moreover, it is well document-

ed that breast cancer is the primary cause of death in women-sufferers of cancer with a high mortality rate worldwide (3). The main issue underlying breast cancer-caused death is the metastasis of the cancer cells to the other organs or the whole body because of delayed diagnosis or inappropriate treatment approaches (4). Therapy approaches for breast cancer mainly include surgery, radiation and chemotherapy (3). Metal-based compounds such as cisplatin, ruthenium and gold are widely used in cancer treatments due to their growth inhibition efficacies. However, they have several limitations such as severe side effects, narrow bioavailability and difficult administration. Furthermore, development of resistance to current chemotherapeutic agents is frequently reported (5). Based on the above mentioned limitations, investigations



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into finding novel agents for cancer therapy have increased over the last decades. Today, metal-based agents are one of the most investigated compounds because of their anti-cancer potencies. Vanadium and its various compounds are the focus of cancer research into using metal-based agents. Ovary cancer, testicular cancer, basophilic leukemia, lymphomas, nasopharyngeal tumors, bone tumors, and neuroblastomas, were investigated as cancer types against which the anti-cancer efficacies of vanadium and its compounds were demonstrated (5,6). Vanadium as a micronutrient as well as vanadium compounds were determined to be effective in killing cells of a variety of human cancers (7). Vanadium enters the human body mostly via the daily consumption of food such as black pepper, parsley, mushroom etc. and is found in two oxidation states (+4 and +5). The oxidation state of +4 is called vanadyl cation and diffuses into the cell through the cell membrane or uses anion channels. Vanadium also exists in extracellular fluids in the form of metavanadate (+5 oxidation state). Also, human blood contains vanadium in the range of 0.42 and 0.08 µg/L (8). Furthermore, vanadium and its compounds were found to accumulate in cancerous cells/tissues more than normal cells (9). Accordingly, it was shown that the accumulation of vanadium and vanadium compounds in the heterochromatin sides of the nuclei temporarily suppresses mitosis and leads to a reversible inhibitor of cell cycle at late S and G2 phases (10).

The first anti-cancer research using vanadium salts dates from 1965. Thereafter, a variety of vanadium salts were investigated in several malign cell lines such as B and T cell lymphoma, hepatoma, osteosarcoma and testis, uterus, lung, kidney, nasopharynx and esophagus carcinoma cells (10,11). In addition, the salt vanadyl sulphate (+4), was found to have high anti-cancer activity under lymphoma, neuroblastoma, T cell, basophilic and eritroleukemia, liver, ovary, testis, esophagus and bone tumor cell lines (6,12,13). Also, the cytotoxic effect of vanadyl sulphate was reported to be lower in normal cells than in the cancer cells (14). Moreover, a greater accumulation of vanadium in cancerous breast tissues than in normal tissues has been well documented (9). The most important property of vanadium compounds to be investigated in terms of cancer research is that they have the potential to be an ideal/near ideal agent for cancer treatment in terms of inhibiting cell growth, causing cytotoxicity, stimulating cell death (apoptosis/necrosis), decreasing/inhibiting metastasis as well as reducing resistance development in cancer cells (15). In addition, their antiproliferative and proapoptotic activities vary among cell types exposed to them (12). Despite all of the above given properties, the anti-cancer activities of vanadyl sulphate on human breast cancer cells MCF-7 are still poorly investigated. Based on this knowledge, herein it is aimed to explore the cytotoxic, antiproliferative and proapoptotic potencies of vanadyl sulphate on human breast cancer cells MCF-7 along with vanadyl sulphate-derived morphological and ultrastructural changes in the cells.

MATERIALS AND METHODS

Materials

MCF-7 (ATCC® HTB-22™) cells were purchased from the American Type Culture Collection (Manassas, USA). Vanadium (IV)-oxid sulphate pentahydrate pure (VOSO_4) obtained from (Riedel-de Haen cat: 14229 Lot: s29267-275, CA) was used as a test agent. Dimethyl sulfoxide (DMSO), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-2H-tetrazolium bromide) (M2003), Dulbecco's Phosphate Buffered Saline (PBS) were purchased from Sigma-Aldrich (St. Louis, USA), Roswell Park Memorial Institute medium (RPMI-1640) was obtained from GIBCO (Grand Island, USA), fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Merck Schuchardt (Darmstadt, Germany). Osmium tetroxide, glutaraldehyde, araldite, propylene oxide, uranyl acetate, lead citrate were from Electron Microscopy Science (USA). Cell Cycle DNA test plus reagent kit and Annexin V apoptosis detection kit were from BD, Pharmingen (USA) and phalloidine, Anti-E cadherin were obtained from Thermo Scientific (USA). Fluo-3, ATP, Anti-cyclin B1 and Anti-cyclin D1 were purchased from Santa Cruz (CA, USA).

Methods

Cell Culture

Breast cancer cells MCF-7 (ATCC® HTB-22™) were cultured in RPMI 1640 medium (Gibco, USA) supplemented with penicillin-streptomycin (1%), fetal bovine serum (10%) at 37°C in a humidified atmosphere with CO_2 (5%). Passage 8 cells with the confluency of 85% were used as test cells in all experiments.

MTT Colorimetric Assay

For the cell viability assessment, a MTT assay was used. In short, a stock solution of vanadyl sulphate was prepared in distilled water. MCF-7 cells were plated at a concentration of 1×10^3 cells per well into 96-well plates. Concentrations ranging from 20-170 µM were exposed to the cells and incubated for 24 hours under incubator conditions of 37 °C in a humidified atmosphere of 5% CO_2 in air. After incubation, the media were removed and MTT (20 µL in 200 µL fresh medium/per well) was added to the cells and re-incubated for 2 hours under the same incubation conditions. Following, incubation media from each well were replaced with 200 µL DMSO and plates were read on an ELISA reader at a wavelength of 570 nm ($n=3$). Viability percentages and the IC_{50} value were determined from the absorbances from the ELISA reader by using the TRAP Version 1-22 programme of the United States Environmental Protection Agency (EPA).

Analyses of the Morphological Changes

For detecting any morphological changes in the MCF-7 cells caused by vanadyl sulphate, the confocal microscopy method was used. In this method, MCF-7 cells were plated on coverslips in 6-well plates at (3×10^5 /well) and exposed to IC_{50} dose of vanadyl sulphate for 24 hours at 37 °C for 24 h. A plate of cells was kept untreated as control cells. Following the incubation period, cells were double stained with phalloidine and acridine orange at room temperature. Stained cells were imaged under a

confocal microscope Leica TCS-SP5 II supplemented with Leica Confocal Software Version 2.00 (16).

Semiquantitative Measurement of Intracellular Calcium Level

The confocal microscopy technique was used for detecting any changes in intracellular calcium level. In this respect, MCF-7 cells were seeded onto circular coverslips (3×10^5) and incubated for 24 hours at 37°C, 5% CO₂ incubator conditions with the IC₅₀ value of vanadyl sulphate. Untreated MCF-7 cells were grown under the same conditions as control cells. After incubation, all the cell groups were washed in PBS and incubated with fluo-3 dye solution containing pluronic acid for one hour under the same incubation conditions. Following this, coverslips were re-washed, placed on a sample holder and analysed after adding ATP solution under a confocal microscope Leica TCS-SP5 II supplemented with Leica Confocal Software Version 2.00 (17).

Analysing the Ultrastructural Changes

For ultrastructural analyses vanadyl sulphate (IC₅₀ value) treated MCF-7 cells were fixed in glutaraldehyde and post fixed in osmium tetroxide (2%). Following fixation, the cells were dehydrated in graded ethanol then embedded in Epon 812 epoxy. After polymerisation for 48 hours at 60°C, samples of thin sections were prepared at a maximum thickness of 100 nm. The sections were placed under copper grids and were stained with lead citrate and uranyl acetate, respectively. Stained samples were imaged under a TEM (FEI Tecnai BioT-WIN, The Netherlands) (18).

Analysing the Cell Death Mode

The flow cytometry technique was used for detecting the cell death mode caused by vanadyl sulphate on MCF-7 cells. For this process, the MCF-7 cells were plated in 6-well plates at a density of 5×10^5 cells/well. After this, cells were exposed to IC₅₀ value vanadyl sulphate for 24 hours at 37°C, in a 5% CO₂ incubator. At the end of incubation period, the cells were harvested by trypsinization, washed (2xPBS) and the cell count in 1 mL of

medium was adjusted to 1×10^6 cells. For the staining process, 5 µL of annexin and 5 µL of PI were added to a facs tube, then 100 µL of cell suspension was added to the tubes containing the fluorescent dyes. Samples were incubated in the dark at room temperature for 15 minutes. After the incubation period the samples were read on a flow cytometer (BD FACSCalibur™, USA) according to the user manual processes laid out in the Annexin V-FITC apoptosis detection kit (BD, Pharmingen, USA). All steps were done in triplicate.

Labelling the Cyclins and E-cadherin Proteins by Immunohistochemical Analyses

The vanadyl sulphate treated and untreated MCF-7 cells were incubated in the above mentioned incubator conditions. After the incubation period, the cells were fixed in glutaraldehyde (4%), placed on slides using a CytoSpin 3 (Thermo Scientific Shandon) device and were allowed to air dry. The dried samples were washed in distilled water and kept in PBS containing tween 20 for 3 minutes at room temperature. Then, all the samples were exposed to hydrogen peroxide (3%) for 10 minutes in a humidified staining chamber at room temperature. After this step, the samples were washed again for 3 minutes at room temperature in PBS (with tween 20) and kept in the staining chamber with ultra V block solution for 5 minutes under the same conditions. After that, the samples were incubated with the primary antibodies (Anti-cyclin B₁, Anti-cyclin D₁ and Anti-E-cadherin) for 1 hour in dilutions as indicated in the user manuals of Santa Cruz and Thermo Scientific, respectively. Then, an amplifier was added to the samples and treated with secondary antibodies for 30 minutes at room temperature. At the end of incubation time, AEC chromogene and haematoxylin retrieval were performed and samples were washed with distilled water and mounted with coverslips for imaging under a light microscope (Leica DM6000 B).

Statistical Analysis

For detecting the IC₅₀ value and confidence intervals of 95%, TRAP Version 1-22 software of the United States Environmental Protection Agency (EPA) was used. The results were evaluated by using one way anova for multiple comparisons and Tukey post-test of Graphpad Prism 6.0 for Windows. The data showed as Mean±SDs and $p < 0.05$ was taken as significant.

RESULTS

MTT Cytotoxicity Assay Findings

The viability percentages of the MCF-7 cells exposed to different concentrations of vanadyl sulphate for 24 hours decreased according to dose (Figure 1). The half maximal inhibition concentration (IC₅₀) of vanadyl sulphate for 24 hours on the MCF-7 cells was detected to be 85µM (82,29-91,01 µM with confidence interval of 95%). A statistically significant decrease ($p < 0.05$) was detected at the IC₅₀ concentration applied to the cells.

Confocal Microscopic Findings

Intensive alterations were detected on the confocal microscopic evaluation of the MCF-7 cells treated with IC₅₀ concentration of vanadyl sulphate for 24 hours when compared to the

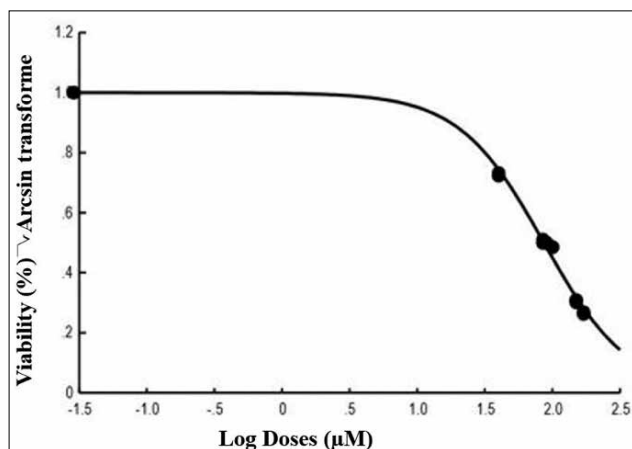


Figure 1. Curve of viability decrease in vanadyl sulphate applied MCF-7 cells. $p < 0.05$ was detected for IC₅₀ inhibition concentration.

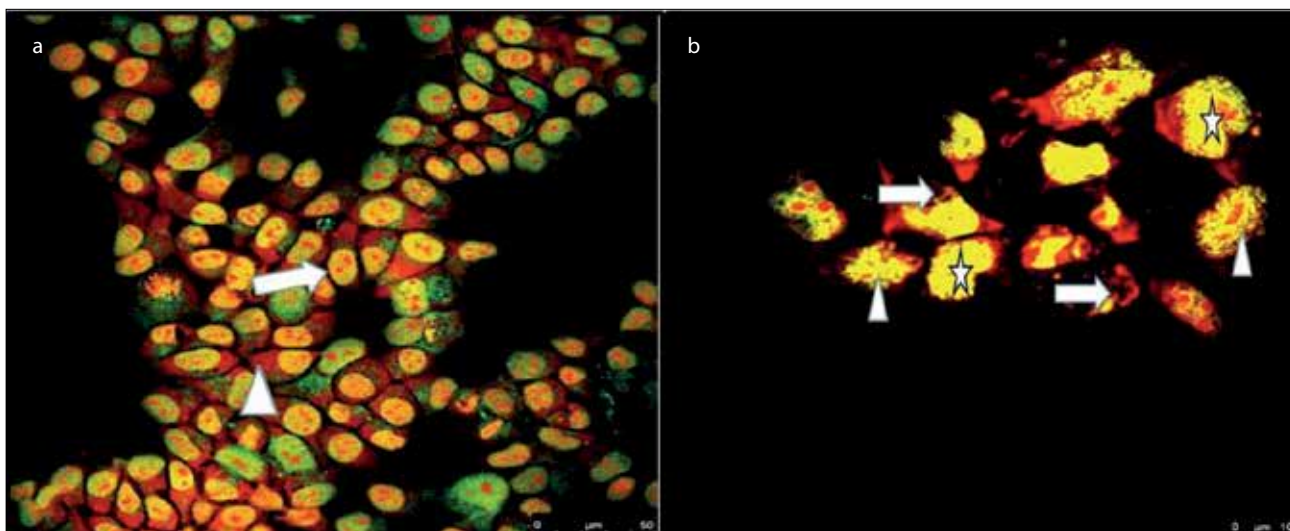


Figure 2. a,b. (a) Morphology of the untreated MCF-7 cells (40x); Arrowhead-cytoskeleton arrow-nucleus. (b) Morphology of vanadyl sulphate treated MCF-7 cells (40x); Arrow-fragmentation of the cytoskeleton, arrowhead-chromatin condensation and asterisk-pyknotic (horse-shoe) nuclei.

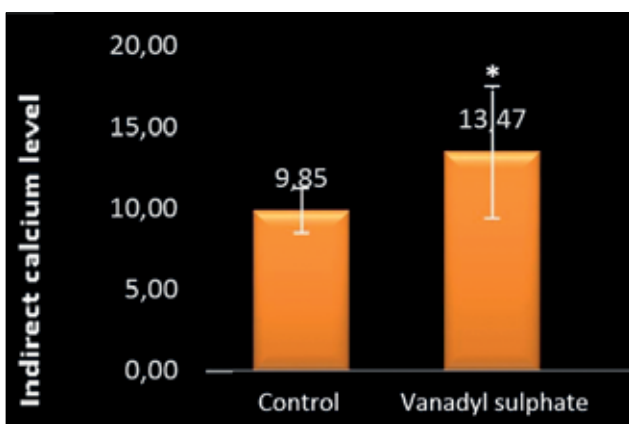


Figure 3. Basal intracellular calcium measurement results of untreated and vanadyl sulphate treated MCF-7 cells. * $p < 0.05$ versus control group.

untreated MCF-7 cells (Figures 2a and 2b). The fusiform shape of the untreated cells (Figure 2a) changed to a shrunken, circular shape in the treated cells (Figure 2b). The compact nuclei and cytoskeleton of the untreated cells were perforated, disintegrated as well as fragmented with the application of the vanadyl sulphate. In addition to the chromatin condensation that was shown in almost all cells exposed to the agent, a pyknotic nucleus formation was detected and the micronuclei of the treated cells were raised in number (Figure 2b).

Semiquantitative Measurement Evaluation Results of Basal Calcium Levels

The basal calcium level of untreated MCF-7 cells was detected to be 9.85 whereas this level in vanadyl sulphate treated cells increased to 13.47 ($p < 0.05$) (Figure 3). This was a semiquantitative evaluation of basal calcium levels of cells. The emission of fluo-3 dye was taken for evaluation thus the result is given with-

out a unit. Each examination was done in triplicate. The standard deviations for the replicates of each group of cells were slightly increased (Figure 3). This was attributed to the property of MCF-7 cells that were grown on cell culture flasks as multilayers of different thicknesses. Thus the emission of fluo-3 dye might be detected at different levels with confocal microscopy.

Transmission Electron Microscopy Findings

Ultrastructural changes derived from vanadyl sulphate being applied to MCF-7 cells were evaluated under a TEM. All the alterations detected in the TEM images of vanadyl sulphate treated cells were compared with the TEM images of the untreated MCF-7 cells. The cell membrane, cytoskeleton, membranous organelles, nuclear membrane, nucleolus and the chromatin of the untreated MCF-7 cells were compact, without disintegrations or fragmentations (Figure 4a). In contrast with the control cells, the cellular membrane of vanadyl sulphate treated cells was found to undulate and make blebs (Figure 4b). The cytoskeleton of treated cells lost their integrity and the formation of autophagosomes, secondary lysosomes and lipid droplets as well as vacuolization were detected (Figures 4c, d, and e). The membranous organelles of vanadyl sulphate treated MCF-7 cells were lacerated (Figure 4e) of which the most affected organelle was found to be the mitochondrion. Mitochondria were found disintegrated with swollen compartments and clear loss of cristae (Figures 4f and g) that might lead to their dysfunction.

Flow Cytometry Findings

In the annexin-V/PI analyses findings the apoptosis triggering activity of vanadyl sulphate was underlined. 100% of untreated MCF-7 cells stained with annexin-V/PI were found to be alive (Figure 5a). The population of MCF-7 cells exposed to the half maximal inhibition concentration of vanadyl sulphate for 24 hours resulted in 51.7% total apoptotic cells of which 9.9% were early apoptotic and 41.8% late apoptotic cells. The percentage

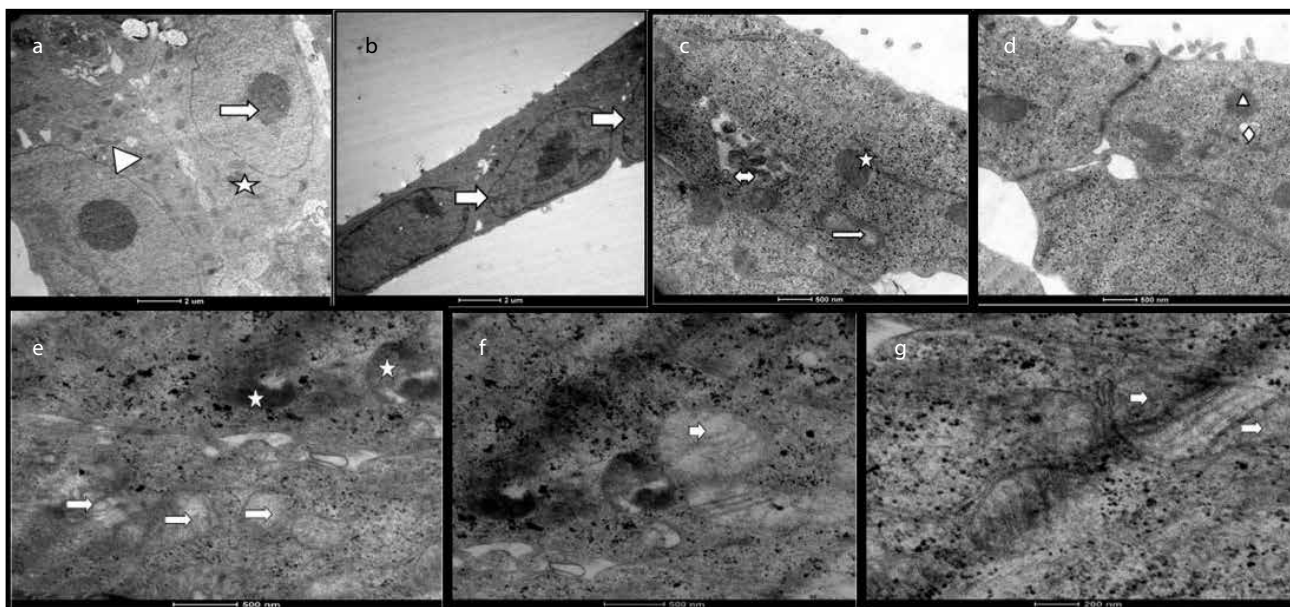


Figure 4. a-g. TEM micrographs of untreated (a) and vanadyl sulphate treated (b, c, d, e, f and g) MCF-7 cells. A; Arrowhead-nuclear membrane, arrow-nucleolus, asterisk-cytoskeleton and organelles. (b) Arrow-undulation of cell membrane. (c) Arrow-loss of cristae, double headed arrow-autophagosome, asterisk-disintegrated mitochondrion. (d) Arrowhead-lipid droplets, rhomboid-vacuolization. (e) Arrow-dysintegrated membranous organelles, asterisk-secondary lysosome. (f) Arrow-enlarged mitochondrial compartments and (g) Arrow-Loss of cristae.

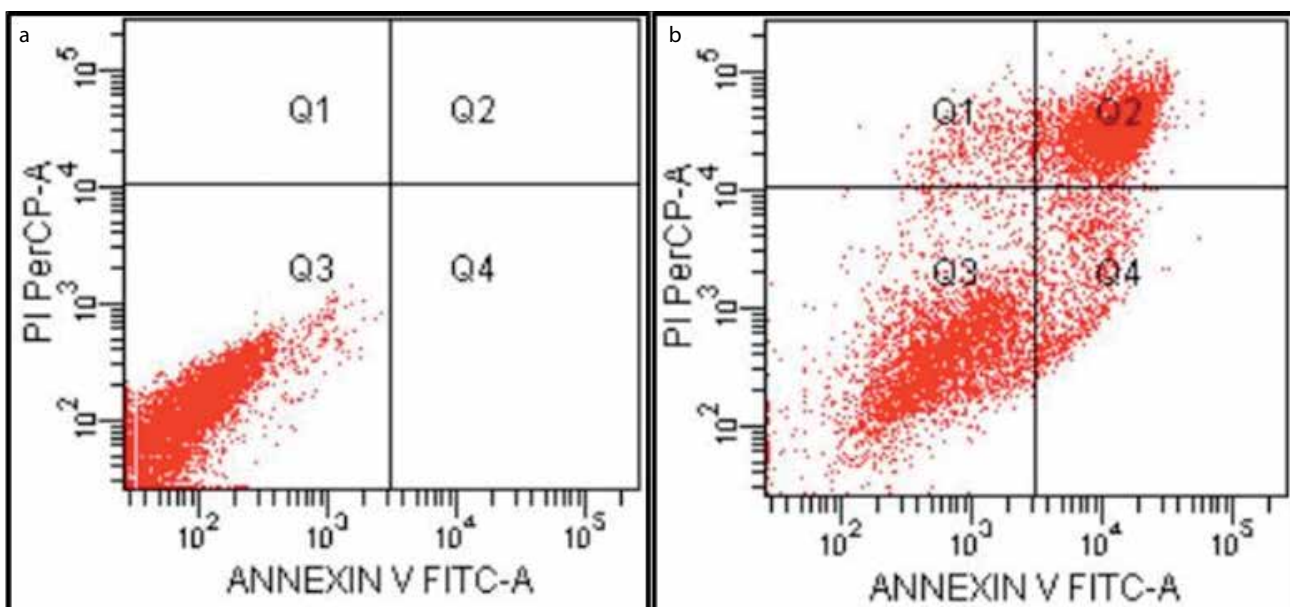


Figure 5. a,b. Cell death mode of untreated (a) and vanadyl sulphate treated (b) MCF-7 cells. (a) Q1-Necrotic/dead cells (0.00%), Q2-Late apoptotic cells (0.00%), Q3-Live cells (100%) and Q4-Early apoptotic cells (0.00%). (b) Q1-Necrotic/dead cells (4.3%), Q2-Late apoptotic cells (41.8%), Q3-Live cells (44.0%) and Q4-Early apoptotic cells (9.9%).

of necrotic cells in this group was 4.3% and 44% of the treated cells were live cells (Figure 5b).

Immunohistochemical Results

The semiquantitative evaluation results of cyclin B₁ and D₁ and E-cadherin are shown in Table 1. The average staining score of the untreated MCF-7 cells was 0.66. The scoring method for vanadyl

sulphate treated MCF-7 cells was made in comparison to untreated cells. The cyclin B₁ staining average score decreased to 0.33 in the treated cells. In contrast, the cyclin D₁ average increased threefold to 1.66 in the same group of cells. The E-cadherin staining score of vanadyl sulphate treated MCF-7 cells was raised to 2, i.e. almost three times bigger than that of control cells (Table 1).

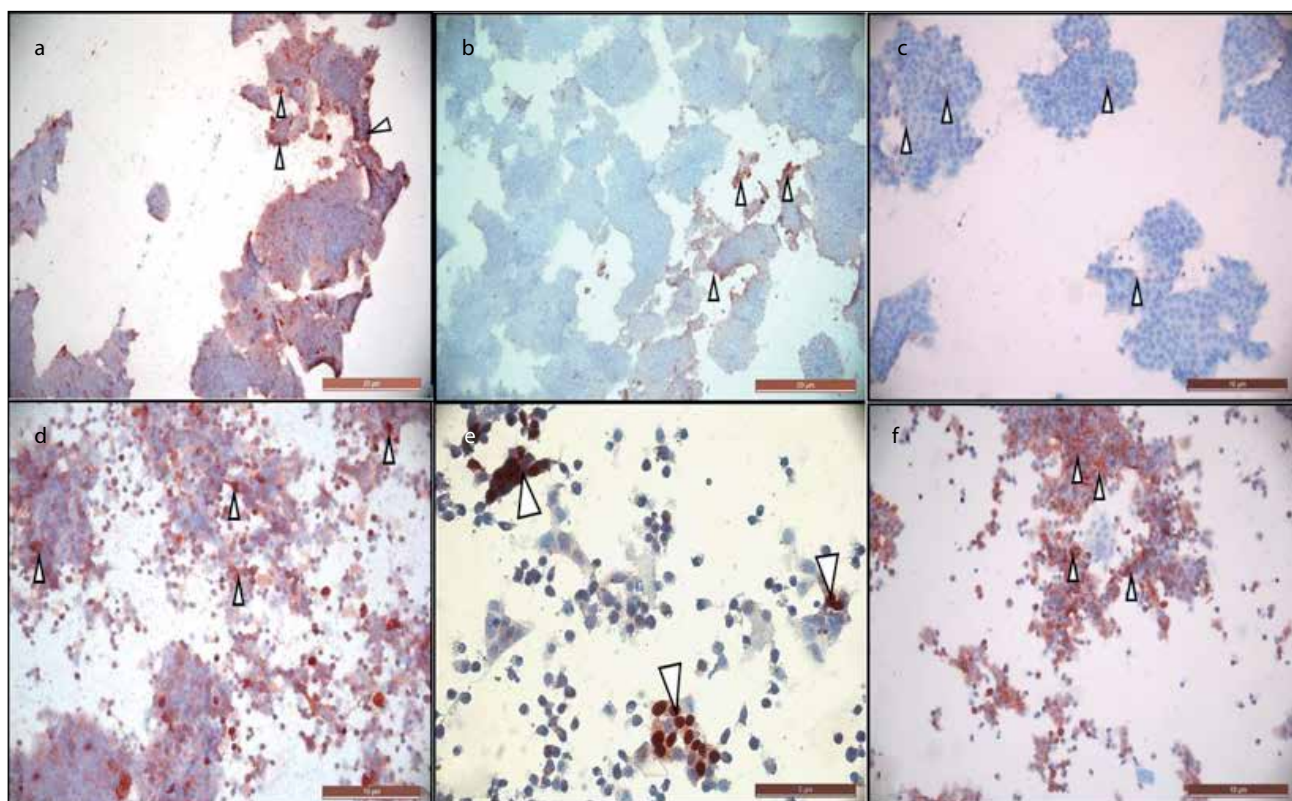


Figure 6. a-f. Light microscopy images of cyclin B1 (a), cyclin D1 (b) and E-cadherin (c) proteins labelled in untreated MCF-7 cells (40x). In images (d), (e) and (f) are shown cyclin B1, cyclin D1 and E-cadherin stainings of vanadyl sulphate treated MCF-7 cells (40x), respectively. Intensively stained cells are indicated by arrowheads.

Table 1. Semiquantitative evaluation of labelings of cyclins (B1, D1) and E-cadherin in untreated and vanadyl sulphate treated MCF-7 cells. In this table, unstained cells were indicated by a 0, slightly stained by 1, moderately stained by 2 and intensively stained cells were indicated by the number 3

Cyclin B1	1. Replication	2. Replication	3. Replication	Average
Untreated cells	1	1	0	0.66
Vanadyl sulphate treated cells	0	0	1	0.33
Cyclin D1	1. Replication	2. Replication	3. Replication	Average
Untreated cells	1	0	1	0.66
Vanadyl sulphate treated cells	1	2	2	1.66
E-cadherin	1. Replication	2. Replication	3. Replication	Average
Untreated cells	1	0	1	0.66
Vanadyl sulphate treated cells	2	2	2	2

The stainings of cyclins (B₁, D₁) and E-cadherin of MCF-7 control cells are shown in Figures 6a, b and c, respectively. In Figures 6d, 6e and 6f the stainings of cyclin B₁, cyclin D₁ and E-cadherin for vanadyl sulphate treated MCF-7 cells are given. These images were used in the scoring method whose results are given in Table 1. Images were given to show the staining type of the cells.

DISCUSSION

Cancer treatment with metal based chemotherapeutics has become an intensively applied method in current therapy. Current therapeutics such as cisplatin, gold and ruthenium are quite effective in killing cancer cells due to their biochemical properties on inhibiting the growth of cancer cells (5). How-

ever, their strong side effects and development of resistance make their application and effectiveness on cancer therapy limited (19). Thus, novel agents for cancer treatment are really needed. Vanadium and its compounds have recently been reported as good candidates for anti-cancer potency (5). Based on this, in this study, we evaluated the anti-cancer potency of a vanadium salt, vanadyl sulphate, with a multi-mode assessment approach on its antiproliferative, cytotoxic and proapoptotic properties on human breast cancer MCF-7 cells. According to the MTT findings, the viability of MCF-7 cells decreased dependent on dose when applied for 24 hours with different vanadyl sulphate concentrations. As shown in Figure 1, the half maximal inhibition concentration (IC_{50}) of vanadyl sulphate on MCF-7 cells was determined to be 85 μ M for this length of exposure. Similarly with our results, in L929 mouse fibrosarcoma cells and human hepatocarcinoma HepG2 cells vanadyl sulphate was shown to act as an antiproliferative agent according to dose and time (14). In addition, in another study, A549 human lung adenocarcinoma cells were inhibited with an IC_{50} value of 15 μ M. In the same study, human prostate cancer cells DU145 were totally inhibited by the application of IC_{50} concentration of 15 μ M (20,21). This can be attributed to the dependence of the antiproliferative activity of vanadyl sulphate on the cells type and application dose and time. This property of vanadium and vanadium compounds has been well described in a previous study (14).

Apoptosis triggering activity on malign cells is the most common property of vanadium compounds (14). For vanadium compounds, it is described that they are proapoptotic via DNA fragmentation (9,14). Based on the investigations into finding an apoptosis stimulating agent for cancer therapy (19), we evaluated the mode of cell death triggered by vanadyl sulphate application to MCF-7 cells. On the morphological analyses on confocal microscopy, on MCF-7 cells exposed to IC_{50} value of vanadyl sulphate for 24 hours clear apoptotic sparks such as chromatin condensation, fragmentation of cell and the nuclei, hole formation in the cytoskeleton and horse-shoe nucleus formation were detected (Figure 2). In line with our results, on A549 and DU145 cells exposed to vanadyl sulphate researchers have shown apoptotic cells with fragmented nuclei (21).

In one piece of research it was reported that increased intracellular basal calcium level lead to apoptosis (22). The death of T-cell hybridoma cells is found to be related to the increased intracellular calcium level (23). In accordance with these findings in our study, the basal intracellular calcium level of MCF-7 cells exposed to vanadyl sulphate for 24 hours was found to be augmented (Figure 3). The apoptosis triggering activity of vanadyl sulphate on MCF-7 is underlined in our study based on the morphological and ultrastructural changes (Figure 4) of the applied cells as well as annexin V-FITC and PI evaluations of cell death mode (Figure 5) in the same group of cells. In annexin-V analyses results, apoptosis was shown in 51.7 % of vanadyl sulphate applied cells. In addition the detected increase in basal calcium levels of the treated cells suggest apoptosis in accordance with the findings of other researchers on vanadate ap-

plied cells that reporting inhibition of Ca-ATPase activity, and in turn causing intracellular calcium accumulation and apoptosis (24). Results of a study on H35-19 rat hepatoma cells exposed to $VOSO_4$, Na_3VO_4 ve $NaVO_3$ salts, showed undulations in the nuclear membrane, trabecular nucleus, fragmentations on the cisternae of Golgi apparatus as ultrastructural changes (25). Similarly, a loss of cristae and swelling of mitochondrion, shrunken cell structure, secondary lysosome formation, chromatin condensation and pyknotic nuclei were detected as ultrastructural changes after exposure to vanadyl sulphate for 24 hours.

Sodium orthovanadate was reported to stop cell cycles by causing increased cyclin B_1 expression on HepG2, Sk-Hep-1 and Hep3B liver carcinoma cells relative to dose (14). Moreover, oxovanadium compounds were declared to inhibit cell cycles at a concentration of 100 μ M over the course of a 24 hours application (26). In our study, in vanadyl sulphate treated MCF-7 cells, cyclin B_1 expression was relatively decreased (Table 1) compared to the control cells. On the contrary, cyclin D_1 expression on these cells was significantly increased (Table 1).

The expression of E-cadherin in a variety of aggressive human cancers has been reported to be reduced (27). In breast cancer cells the expression of this protein was declared to be partly or totally absent. The absence of E-cadherin expression makes breast cancer cells more invasive and metastatic (28). Stimulating expression of E-cadherin in breast cancer cells was determined to significantly inhibit the proliferation of cells *in vitro* and *in vivo* (20). Herein, it was detected increased E-cadherin expression in MCF-7 cells caused by vanadyl sulphate application. Our result is supported by a study that declared vanadium (IV) compounds to inhibit the invasion and adhesion of osteosarcoma cells relative to dose (29).

In conclusion, vanadyl sulphate was found to be cytotoxic and antiproliferative on MCF-7 cells when applied for a short time. It triggered apoptosis by changing the morphology and ultrastructure of the exposed cells. Thus, vanadyl sulphate might be suggested for further investigations for its usage in cancer therapy due to its anti-cancer properties considered above.

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Serum Concentration of Ghrelin, Oxidative Stress and Lipid Parameters in Obese Subjects

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ABSTRACT

Objective: Ghrelin is a hormone with peptide structure. It has fatty tissue and increases appetite. Obesity is a multifactorial chronic disease characterized by an increase in fat tissue. Fat tissue, like the endocrine organ, triggers oxidative stress and can lead to the development of obesity-related pathologies. The purpose of this study is to examine the relationship between the ghrelin in blood, and oxidative stress and lipid parameters.

Materials and Methods: The study was conducted with 61 obese and 24 healthy individuals. Ghreline levels were measured using the ELISA method, while total antioxidant status (TAS) and oxidant status (TOS), triglyceride (TG), total cholesterol (TC), HDL-cholesterol (HDL-C) and LDL cholesterol (LDL-C) levels were measured using the photometric method.

Results: A negative correlation was found between body mass index (BMI) and ghrelin levels in the obese group ($p < 0.05$). But there was no significant difference of ghrelin levels in obese and control groups ($p > 0.05$). TAS was observed to be lower in obese compared to control group, while The Oxidative Stress Index (OSI) was found to be significantly higher than the obese group ($p < 0.05$). TG levels were found to be increased in obese; whereas ghrelin, TC, LDL-C and HDL-C levels did not show any difference ($p > 0.05$).

Conclusion: Increasing obesity level (BMI) and decreasing ghrelin level were found to be correlated. New studies are needed in order to discover the changes in ghrelin level connected to oxidative stress.

Keywords: Ghrelin, obesity, oxidative stress

INTRODUCTION

Obesity is an increase in the amount of fat in the body, which occurs when energy intake is more than energy spent.

Today fatty tissue is no more regarded as a mere fat storage since it carries an important duty. That is, it affects other organs and carries communication between them, therefore fatty tissue is regarded as an endocrine organ which synthesizes and releases many chemical messengers, the cytokine of fatty tissue (adipokine) (1-3).

In obesity, an increased fatty tissue brings many physical and biochemical pathologies (1,2).

Ghrelin is an acylated peptide which contains 28 amino acids and it is primarily produced in the stomach and the proximal small intestine (4). Ghrelin activates the hypothalamus and other related systems in the brain, therefore increasing gastrointestinal motility and decreasing insulin secretion (5). The growth hormone secretagogue receptor (GHS-R) mediates the different actions of the synthetic growth hormone secretagogues (GHS) and the endogenous ligand of this

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receptor, ghrelin (6). This endogenous ligand for this GHS receptor (GHS-R) was generally identified by Kojima et al. in 1999 and named 'ghrelin' (7). Currently it's the only known orexigenic hormone (8). Cancer patients with loss of appetite were reported to gain back appetite when administered ghrelin (9).

In healthy cells, oxidation of molecular oxygen is a well-controlled process. However, in cases of cell damage and disease, superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) amounts increase. In the case of increased reactive oxygen radicals (ROS), insufficient antioxidants lead to oxidative stress. In the case of oxidative stress, proteins, lipids and DNA are damaged. Several studies report that increased oxidative stress in obesity contributes to development of atherosclerosis (10-14).

The aim of this study was to investigate the relationship between blood levels of ghrelin hormone, body mass index (BMI), oxidative stress and lipid parameters, which are important in carbohydrate and fat metabolism.

MATERIALS AND METHODS

Study Design and Data Collection

This study includes 24 controls (13 male and 11 female) and 61 obese (37 male and 24 female) who have consulted Mega Medipol Hospital Laboratory of Medipol University between September and October 2015. After taking the approval of ethical committee of Medipol University, all the patients were informed and confirmed consent documents were taken from all them. Groups were classified according to their BMI into two groups: $BMI > 18.9$ and $BMI < 24.9$ kg/m² are considered as normal weight and $BMI > 30$ kg/m² are considered as obese. BMI values were obtained by dividing the weight (in kg) by the square of height (m²).

The mean BMI (kg/m²) in the control group was 23.52 ± 0.89 , while in the obese group it was 33.76 ± 6.15 .

Exclusion Criteria in the Study

Exclusion criteria in the study was as follows: younger than 18, over 75 years old, smoking habits, hypertension, heart diseases, osteoarthritis, cancer, polycystic disease, inflammation and infectious diseases not included in the study. The study started after the approval of Medipol University Ethics Board. All the subjects were informed about the study and their approved consent forms were received.

Blood Collection and Storage

Venous blood was collected in the early morning before breakfast and after overnight sleep. Blood samples were collected in yellow covered flat tubes and purple covered (EDTA containing) tubes. Yellow covered tubes were centrifuged at 2400 rpm for 10 minutes in the clinical biochemistry laboratory of Medipol University, and blood cells were separated from serum. Separated serums were taken into Eppendorf tubes and kept at -80°C until the analysis.

Methods Used

Following analyses were carried out: Ghrelin, triglyceride (TG), total cholesterol (TC), low density lipoprotein cholesterol

(LDL-C), high density lipoprotein cholesterol (HDL-C), total antioxidant status (TAS), total oxidant status (TOS) and oxidative stress index (OSI) levels.

Serum ghrelin levels were determined by using Ray Bio EIA-GHR-1 Elisa kit; TAS and TOS were determined by colorimetric method. OSI was calculated using the formula given below: $OSI = [(TOS, \mu\text{mol } H_2O_2 \text{ equivalent/l}) / (TAS, \mu\text{mol Trolox equivalent/l})] \times 100$ (15). Serum TC, HDL-C, LDL-C and TG levels were measured with Roche/Hitachi C501 instrument photometrically with the kits recommended by the instrument company.

Statistical Analysis

The Statistical Package for the Social Sciences (SPSS) Windows version 22.0 (IBM Corp.; Armonk, NY, USA) program was used to evaluate the statistical analysis of the study. Variables were defined in \pm SD limits. T-test was used for the comparison of measured variable average values that obey normal distribution, in two groups. In order to compare dependent variables paired t-test was applied. Also, Mann-Whitney U-test was preferred to compare the average values obtained that do not obey normal distribution. For all the test $p < 0.05$ was considered significant.

RESULTS

As it was shown in Table 1, there was no significant change in the plasma levels of ghrelin ($p > 0.05$) in obese and control groups.

Serum TG levels were increased in obese group ($p < 0.05$) whereas other lipid parameters such as TC, LDL-C and HDL-C were normal levels ($p > 0.05$).

There was a significant decrease in serum TAS in obese group ($p < 0.001$), serum TOS were not significantly changed ($p > 0.05$) and OSI were significantly high in obese compare to normal subjects.

When correlation analyses were examined, a positive relation was found between TOS and OSI ($r: 0.77, p < 0.05$) TOS and TG ($r: 0.52, p < 0.05$), a negative correlation was observed between TOS values and HDL ($r: -0.34, p < 0.05$). In addition, positive relation was found between OSI and TG ($r: 0.33, p < 0.05$).

The plasma levels of ghrelin were significantly negatively correlated with BMI ($r: -2.65, p < 0.05$).

DISCUSSION

Known as the "orexigenic hormone", ghrelin maintains the energy balance of the organism together with neuroendocrine regulation, intestinal and pancreatic peptides (4,5,16). Despite these systems for protecting the organism, the prevalence of obesity is increasing in the world. Obesity results in insulin resistance, inflammation, oxidative stress in parallel with increasing fat tissue (1).

In this study, we investigated the relationship between ghrelin serum concentration and oxidative stress and lipid parameters

Table 1. Laboratory findings of control and obese group.

	Control group (Mean±SD)	Obese group (Mean±SD)	p*
Ghrelin (pg/mL)	110.78±25.46	110.43±25.43	0.955
TAS (μmol Trolox Equiv./L)	0.92±0.11	0.81±0.13	<0.05
TOS (μmol H ₂ O ₂ Equiv./L)	26.96±5.49	28.26±6.31	0.386
OSI (AU)	2.92±0.62	3.56±0.86	<0.05
HDL (mg/dL)	53.22±17.82	47.65±13.52	0.149
LDL (mg/dL)	119.35±34.45	121.98±37.88	0.768
TG (mg/dL)	126.14±82.92	158.13±85.36	0.033
TC (mg/dL)	195.46±41.36	198.35±40.99	0.771
TAS: total antioxidant response; TOS: total oxidant status; OSI: oxidative stress index value; AU: Arbitrary unit; TC: total cholesterol *p<0.05 for t test.			

in the obese and healthy control group, which increased appetite and food intake.

Mucioli et al. (17) reported that ghrelin, one of the peptides in the appetite center, increases appetite and causes obesity. As reported by Kara et al. (18), Ghrelin injections to mice caused an increase in fat tissue by reducing fat use. Ghrelin's fat tissue and appetite-enhancing effects are independent of GH effects and are thought to be regulated by specific neurons in the CNS where leptin is also a mediator.

Wren et al. reported that when ghrelin is administered intravenously to normal weight healthy people, the desire to eat is increased. Blood levels of ghrelin decrease after fasting and after a sugary and fatty meal (5).

Tschöp et al. (19) reported that ghrelin levels were lower in obese subjects than in weaker subjects. In the Suematsu et al. study, ghrelin was measured in 17 obese and 17 healthy subjects and the ghrelin level was found to be lower in the obese group than in the control group (12). Participants had an increase in serum ghrelin levels as a result of their weight loss after the diet (14,17-19).

In the study of Cinaz et al. (20), hunger and satiety ghrelin levels were measured in 38 obese and 19 healthy children. In both obese and control groups fasting ghrelin levels were higher than satiety ghrelin levels ($p<0.05$). The researchers also showed that the hunger and satiety ghrelin levels of obese children were lower than the control group ($p<0.05$). The study also found a negative correlation between BMI and hunger strike levels in the obese group.

In our study, the ghrelin concentration was not statistically different in the control and obese group ($p>0.05$). However, increased obesity level (BMI) was found to be correlated with decreased ghrelin level ($r: -2.65$ $p<0.05$). This situation is caused by positive energy balance which suppresses ghrelin secretion in obese people. This finding is consistent with stud-

ies suggesting that ghrelin levels are reduced in obese individuals.

In our study, TAS was observed to be lower in obese compared to control group, while OSI was found to be significantly higher than the obese group ($p<0.05$). Obese group with oxidative stress do not differ in terms of ghrelin levels ($p>0.05$). The only study in this area in the literature was reported by Suematsu et al. In their study, free 8-epi-prostaglandin $F_{2\alpha}$ was measured as a systemic marker of oxidative stress and, independently from obesity, it was discovered that increases in oxidative stress decreases ghrelin (12).

In the literature, in general, ghrelin levels were found to be decreased in obese subjects, but the mechanism of this decrease has not been explained (19).

The LDL-C, HDL-C and TC levels did not significantly change when compared to control group and remained within the normal reference limits when the lipid profile in both groups were examined and TG levels were found to be statistically higher in the obese group ($p<0.05$). Positive correlation between TG and TOS and OSI shows the role of TG increase in the formation of oxidative stress.

In conclusion, ghrelin levels did not significantly change in obese group when compared to control group. This situation is caused by positive energy balance suppresses ghrelin secretion in obese people. Understanding obesity and its associated diseases with the appetite hormone ghrelin will help to develop new strategies for the prevention of obesity. New studies are needed for guidance in this area.

Ethics Committee Approval: Ethics Committee Approval was received for this study from the ethics committee of Medipol University.

Informed Consent: Written informed consent was obtained from patients who participated in this study.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – S.Z., T.Y., N.E.; Design – S.Z., T.Y., N.E.; Supervision – T.Y., N.E.; Resource – N.E.; Materials – N.E., G.Ü.; Data Collection and/or Processing – S.Z., G.Ü.; Analysis and/or Interpretation – S.Z., T.Y., N.E.; Literature Search – S.Z., N.E.; Writing – S.Z., N.E.; Critical Reviews – T.Y., N.E.

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Serum Protein Profile of Juvenile Pacu (*Piaractus mesopotamicus*) and Dourado (*Salminus brasiliensis*) Fed Bovine Colostrum as Partial Source of Protein in the Diet

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ABSTRACT

Objective: The electrophoretic profile of serum proteins was investigated in juvenile *Piaractus mesopotamicus* and *Salminus brasiliensis* fed with diets containing bovine colostrum, a nutraceutical food, in lyophilized form (LBC) for either 30 or 60 days.

Materials and Methods: Blood samples were collected from juveniles of *P. mesopotamicus* and *S. brasiliensis* fed for either 30 or 60 days with diets containing 0%, 10% or 20% of LBC. Serum protein fractions were then determined by electrophoresis in agarose gel.

Results: The *P. mesopotamicus*, an omnivorous fish, showed six serum protein fractions, while the *S. brasiliensis*, a carnivorous fish, showed four serum protein fractions. In both species the albumin fraction showed higher protein content at 30 days than at 60 days ($p < 0.05$), indicating an inverse relationship with growth. The 5th and 4th fraction in mobility from the *P. mesopotamicus* and *S. brasiliensis* serum, respectively, were positioned in a gamma-globulin zone. In *P. mesopotamicus*, the concentration of protein in the gamma-globulin zone was higher at 60 days than at 30 days ($p < 0.05$). In *S. brasiliensis*, in turn, the concentration of protein in the gamma-globulin zone was higher at 30 days than at 60 days ($p < 0.05$). In juvenile *P. mesopotamicus*, a higher concentration of protein was also observed in the gamma-globulin zone in the 0% LBC compared to 10% and 20% LBC ($p < 0.05$). Thus, the feeding period influenced only the fractions that were positioned in the albumin and gamma-globulin migration zone in both species.

Conclusion: The consumption of bovine colostrum decreased the concentration of protein in gamma-globulin fraction of the *P. mesopotamicus*, indicating that these juveniles had either less stimulus for their own synthesis of defense elements or an immunosuppressive effect of bovine colostrum ingestion.

Keywords: Colostrum, teleost, omnivorous, carnivorous, electrophoresis

INTRODUCTION

Fish farming has increased mainly due to a greater demand for healthier meats for human consumption (1). However, the intensive production of these species presents a great challenge for the producer as a concentration of large populations increases the predisposition to bacterial and parasitic diseases (2). This problem is especially critical in the early stages of life of teleosts (3). Therefore, the formulation of diets that contribute to the health and well-being of juveniles are of great relevance.

Bovine colostrum is the first mammal lacteal secretion that is crucial for nutritional, metabolic and endocrine status of neonates (4,5). The effects of colostrum consumption include the protection of the enteric and respiratory tract and reduction in mortality and morbidity of the newborn (6-8). The high nutritional value and concentration of biological molecules, such as antioxidant and antimicrobial factors, designate colostrum as a nutraceutical food (9). This lacteal secretion from bovine has been widely used as an immunological and nutritional source for sev-

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eral species, including swine and rodents (10,11). In fish, the supply of lyophilized bovine colostrum is an innovative conception and the effects of this food on fish health has been explored by different approaches (12-14).

In Brazil, *Piaractus mesopotamicus* and *Salminus brasiliensis*, popularly known as pacu and dourado, are neotropical species with favorable features for commercial production. Besides be-

ing a species of interest for the sport of fishing, *Piaractus mesopotamicus* and *Salminus brasiliensis* present good performance and acceptance by the consumers (2,15,16). However, information about their physiology and the effects of nutraceutical foods on their health is still being gathered. The electrophoresis of proteins from blood plasma or serum can be used in humans and animals to provide information on acute and chronic inflammatory processes and consequently health (17,18). In recent decades, the study of blood protein fractions from different species has increased, including works involving fish, which focus on the relation between water pollution and fish health (19-22). The effects of diet on protein profile, especially gamma globulin fraction where antibodies are positioned, are of great relevance to evaluate the health and immunity of fish.

Since electrophoresis has been proposed to aid in the diagnosis of disease, normal reference ranges for serum protein levels still need to be established in different species of fish. Considering colostrum as a nutraceutical food, there is the possibility of this food modulating immunity, and consequently, fish health. Thus, this study investigates the electrophoretic profile of serum proteins of juvenile *Piaractus mesopotamicus* and *Salminus brasiliensis* fed diets with bovine colostrum, a nutraceutical food, in the lyophilized form (LBC).

MATERIAL AND METHODS

Diets

The first lacteal secretion from multiparous Holstein cows was frozen at -20°C and, thereafter, lyophilized. This powder was homogenized to obtain a homogeneous sample, which was analyzed for the bromatological composition. Considering the nutritional composition of bovine colostrum, isonitrogenous and isoenergetic pelleted diets were formulated to attend to the requirements of juveniles *Piaractus mesopotamicus* and *Salminus brasiliensis* (Tables 1 and 2). After the homogenization of the ingredients, the diets were pelletized, dried (at a temperature of approximately 46 °C) and stored in a dry place until the time of supply. Chemical compositions of experimental pelleted diets were subsequently submitted to chemical analysis (23).

Experiment

Juvenile *Piaractus mesopotamicus* and *Salminus brasiliensis* (8.5±0.7 and 13.3±0.9 g; 7.8±0.3 and 10.8±0.3 cm, respectively) were randomly distributed in 36 tanks (324 pacu in 18 tanks and 270 dourado in 18 tanks) with controlled water quality parameters (26.8±1.5°C, 5.8±1.0 mg L⁻¹ of dissolved oxygen and <0.05 mg L⁻¹ dissolved ammonia). After adaptation for 10 and seven days, respectively, diets containing 0%, 10% or 20% bovine colostrum in LBC were hand-fed to apparent satiety twice a day (08h30 and 16h30) for either 30 or 60 days (authorized by the ESALQ/USP ethics committee).

Seven fish were sampled on the 30th and 60th experimental day after 24 h fasting and anesthesia with benzocaine (0.1 g L⁻¹). Blood samples obtained from the caudal vein were centrifuged and the serum was stored at -20°C.

Table 1. Chemical composition of experimental pelleted diets fed to juvenile *Piaractus mesopotamicus*

Ingredient (g kg ⁻¹)	Diets ¹		
	0%	10%	20%
Bovine colostrum (679 g kg ⁻¹ CP)	-	100	200
Soybean meal (45 g kg ⁻¹ CP)	265	76.7	-
Wheat meal	238	311.8	311.8
Poultry by-product meal	200	200	131
Broken rice	188	198	200
Fish meal (55% CP)	50	50	50
Fish oil	46.2	40	45.7
DL-methionine	2.4	3.4	4.7
L-lysine HCl	-	6	11.5
BHT	2	2	2
Cellulose	-	3.4	-
Calcareous	-	-	14.7
Corn grain	-	-	9.3
Premix ²	10	10	10.5
Chemical composition (g kg ⁻¹) ³			
Dry matter	940.6	933.4	936.4
Crude protein	324.6	314.9	322
Crude fiber	30	27.7	30.7
Fat	90.5	98.2	109.3
Ash	106.4	101.1	92.8
Gross energy (MJ kg ⁻¹)	18	18.2	18.3

¹0% - diets containing 0% of lyophilized bovine colostrum; 10% - diets containing 10% of lyophilized bovine colostrum; 20% - diets containing 20% of lyophilized bovine colostrum.

²Guabi Nutrição Animal, Campinas, São Paulo (ingredient per kg). Vitamins: A, 2,500 UI; D3, 600.000 UI; E, 37.500 UI; K3, 3,750 mg; C, 50,000 mg; B1, 4,000 mg; B2, 4,000 mg; B6, 4,000 mg; B12, 4,000 mg; calcium pantothenate, 12,000 mg; biotin, 15 mg; acid folic, 1,250 mg; niacin, 22,500 mg. Mineral: Cu, 2,500 mg; Zn, 12,500 mg; I, 375 mg; Se, 87.5 mg; Co, 125 mg; Mn, 12,500 mg; Fe, 15,000 mg; BHT, 15,000 mg.

³Original matter basis.

Analyses

The total serum protein was determined (24) and an electrophoretic analysis was performed in an agarose gel (CELMGEL) for 30 min at 90 volts. After staining with 0.2% starch black for 5 min., a reading of the protein fractions in a densitometer (CELM DS35) with a wavelength of 520 nm was performed. The software CELM SE-250 was used to calculate the relative percentage of each protein fraction from the area under the curve created by the protein fraction. No attempt has been made to identify protein components of the sera studied as albumin or globulin. The electrophoretic fractions were numbered according to the increasing mobility.

Statistical Analysis

Serum variables were analyzed based on a 2x3 completely randomized factorial design using SAS software (SAS Institute Inc., 2004), taking into consideration the three diets and two feeding periods (30 and 60 experimental days). An analysis of variance was per-

formed using the general linear model and comparisons between pairs of means were made using the Tukey test considering $p < 0.05$.

RESULTS

Electrophoretic profile of serum proteins of juvenile pacu (*Piaractus mesopotamicus*)

Using the software SDS-60 (Celm), six fractions in the serum of juvenile *Piaractus mesopotamicus* were identified (Figures 1 and 2). These fractions were quantified by the software considering the value of serum total protein (Table 3). The effect of the period was observed for the 2nd fraction ($p < 0.05$), with a higher value at 30 days compared to 60 days. The 6th fraction was affected by the diet and period ($p < 0.05$), with higher values at 60 days and in the group 0% LBC.

Electrophoretic profile of serum proteins of juvenile dourado (*Salminus brasiliensis*)

Using the software SDS-60 (Celm), four fractions in the serum of juvenile *Salminus brasiliensis* were identified (Figures 3 and 4). These fractions were quantified by the software considering the

Table 2. Chemical composition of experimental pelleted diets fed to juvenile *Salminus brasiliensis*

Ingredient (g kg ⁻¹)	Diets ¹		
	0%	10%	20%
Bovine colostrum (679 g kg ⁻¹ CP)	-	100	200
Soybean meal (45 g kg ⁻¹ CP)	230	230	230
Poultry by-product meal	204.8	119.8	24.5
Fish meal (55 g kg ⁻¹ CP)	320	300	300
Fish oil	95	90	85.2
Premix ²	10	10	10
BHT	0.2	0.2	0.2
Cellulose	20	30	30
Corn (whole grain)	120	120	120
Chemical composition (g kg ⁻¹) ³			
Dry matter	924.4	936.7	925.7
Crude protein	422	425.1	444.7
Crude fiber	18.9	29.2	37.3
Fat	140.7	140.6	133.7
Ash	121.9	114.5	91.1
Gross energy (MJ kg ⁻¹)	20.8	21.1	20.8

¹0% - diets containing 0% of lyophilized bovine colostrum; 10% - diets containing 10% of lyophilized bovine colostrum; 20% - diets containing 20% of lyophilized bovine colostrum.

²Guabi Nutrição Animal, Campinas, São Paulo (ingredient per kg). Vitamins: A, 2,500 UI; D3, 600,000 UI; E, 37,500 UI; K3, 3,750 mg; C, 50,000 mg; B1, 4,000 mg; B2, 4,000 mg; B6, 4,000 mg; B12, 4,000 mg; calcium pantothenate, 12,000 mg; biotin, 15 mg; acid folic, 1,250 mg; niacin, 22,500 mg. Mineral: Cu, 2,500 mg; Zn, 12,500 mg; I, 375 mg; Se, 87.5 mg; Co, 125 mg; Mn, 12,500 mg; Fe, 15,000 mg; BHT, 15,000 mg.

³Original matter basis.

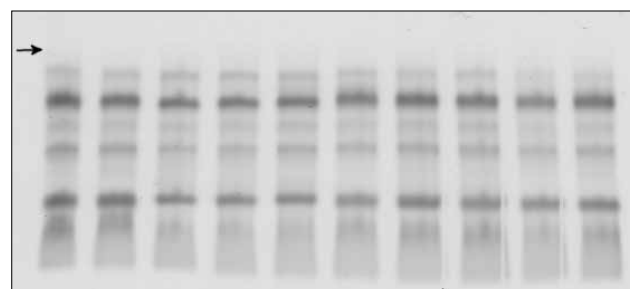


Figure 1. Electrophoretic analyzes of 10 serum samples of juvenile pacu, *Piaractus mesopotamicus*. Arrow: start of the race

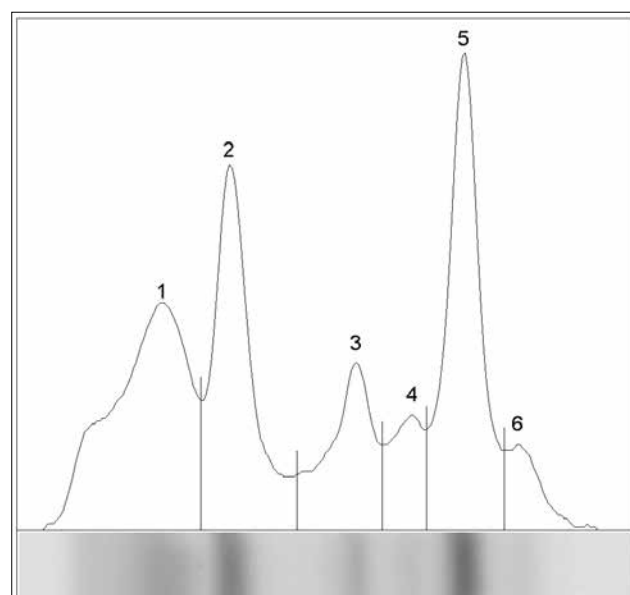


Figure 2. Electrophoretic profile of serum proteins of juvenile pacu, *Piaractus mesopotamicus*, revealing six fractions (in order from fastest to slowest)

Table 3. Serum protein fractions (in order from fastest to slowest) and total protein (g dL⁻¹) in juvenile *Piaractus mesopotamicus* (mean ± standard error) fed lyophilized bovine colostrum

	Diet ¹			Feeding period (days)		Effect ²		
	0% LBC	10% LBC	20% LBC	30	60	D	P	DXP
Total protein	4.00±0.23	4.14±0.16	3.95±0.11	4.04±0.14	4.03±0.12	NS	NS	NS
Band 1	1.07±0.09	1.18±0.06	1.11±0.02	1.09±0.05	1.16±0.03	NS	NS	NS
Band 2	0.80±0.04	0.79±0.05	0.76±0.06	0.86±0.03	0.69±0.03	NS	*	NS
Band 3	0.54±0.03	0.56±0.02	0.54±0.02	0.53±0.02	0.56±0.01	NS	NS	NS
Band 4	0.29±0.02	0.32±0.01	0.29±0.01	0.29±0.01	0.32±0.01	NS	NS	NS
Band 5	0.97±0.05	1.00±0.04	1.00±0.05	1.02±0.04	0.97±0.03	NS	NS	NS
Band 6	0.32±0.03 ^a	0.27±0.01 ^b	0.25±0.02 ^b	0.24±0.01	0.31±0.02	*	*	NS

*p<0.05; NS-p>0.05; means followed by the same letter (a,b) differ (p<0.05); 10% LBC-juveniles fed 0% of lyophilized bovine colostrum; LBC 10%-juveniles fed 10% of lyophilized bovine colostrum; 20% CBL-juveniles fed 20% of lyophilized bovine colostrum; 2D-diet effect, P-period effect; DxP-interaction between diet and period.

Table 4. Serum protein fractions (in order from fastest to slowest) and total protein (g dL⁻¹) in juvenile *Salminus brasiliensis* (mean ± standard error) fed lyophilized bovine colostrum

	Diet ¹			Feeding period (days)		Effect ²		
	0% LBC	10% LBC	20% LBC	30	60	D	P	DXP
Total protein	3.37±0.08	3.42±0.07	3.38±0.12	3.46±0.05	3.33±0.08	NS	NS	NS
Band 1	1.01±0.03	1.13±0.04	1.11±0.07	1.16±0.03	1.01±0.03	NS	*	NS
Band 2	1.45±0.05	1.37±0.06	1.37±0.05	1.35±0.04	1.44±0.04	NS	NS	NS
Band 3	0.12±0.01	0.13±0.01	0.13±0.01	0.13±0.01	0.13±0.01	NS	NS	NS
Band 4	0.79±0.03	0.79±0.01	0.76±0.03	0.81±0.02	0.75±0.01	NS	*	NS

*p<0.05; NS-p>0.05; 10% LBC-juveniles fed 0% of lyophilized bovine colostrum; LBC 10%-juveniles fed 10% of lyophilized bovine colostrum; 20% CBL-juveniles fed 20% of lyophilized bovine colostrum; 2D-diet effect, P-period effect; DxP-interaction between diet and period.

value of serum total protein (Table 4). The effect of the period was observed for the 1st and 4th fractions (p<0.05), with higher values at 30 days compared to 60 days.

DISCUSSION

The serum protein profile of juvenile pacu, *Piaractus mesopotamicus*, showed six fractions. Approximately 28%, 19%, 14%, 8%, 25%, and 7% of proteins were observed in the 1st, 2nd, 3rd, 4th, 5th and 6th fraction, respectively. In the juvenile dourado, *Salminus brasiliensis*, four fractions were identified. The 1st to 4th fraction corresponded approximately to 32%, 41%, 5% and 22% of the serum total protein (25). Work with four species of *Tilapia*, seven serum fractions were observed in the *T. nilotica*, *T. galilaea* and *T. aurea*, while de *T. zillii* showed six serum fractions. The authors also observed differences in the protein concentration in each band and suggest that these results might be related to diet.

The 2nd fraction in the serum of pacu and the 1st fraction in the serum of dourado, *Salminus brasiliensis*, showed a higher protein

concentration at 30 days compared to 60 days, indicating an inverse relationship with growth. As an additional investigation, bovine serum albumin (BSA, Sigma-Aldrich Co) was added to fish serum and samples were submitted again to electrophoresis analyses. The electrophoretic profile was not changed in both species; however, the 2nd fraction from *Piaractus mesopotamicus* serum and the 1st fraction from dourado serum showed a significant increase in its participation of total protein from 19% to 43% and 36% to 53%, respectively. These results indicated that these fractions are in the albumin migration zone and are probably the corresponding protein in fish. Thus, in juvenile pacu, the calculation of globulin by the difference of serum total protein and albumin is not adequate, since near 28% of serum proteins are in the 1st fraction, which corresponds to pre-albumin proteins and are not considered in this calculation.

According to Atamanalp et al. (26) and Maceda Veiga et al. (27), environmental changes can affect the physiological and biochemical characteristics of fish blood, and the hematological and serum protein profiles are important indices in monitoring the effects

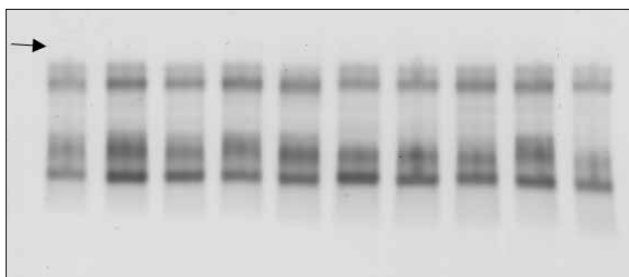


Figure 3. Electrophoretic analyzes of 10 serum samples of juvenile dourado, *Salminus brasiliensis*. Arrow: start of the race

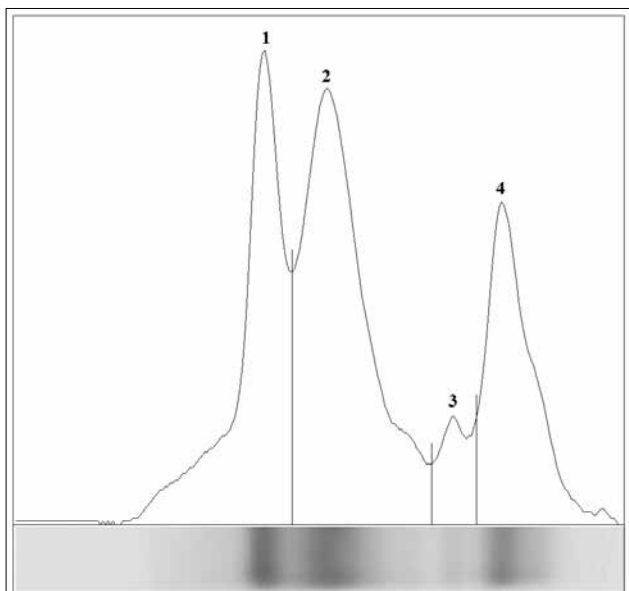


Figure 4. Electrophoretic profile of serum proteins of juvenile dourado, *Salminus brasiliensis*, revealing four fractions (in order from fastest to slowest)

of aquatic habitat changes suggests that the reduction of serum protein concentration is related to an adverse environment. The authors observed lower serum protein concentration in the *Mugil cephalus* exposed to different pHs. In the present work, *Piaractus mesopotamicus* and *Salminus brasiliensis* showed a reduction of protein concentration in the 2nd and 1st fractions, respectively, after 60 experimental days, suggesting a less favorable condition for fish. According to Fazio et al. (22), the reduction of protein concentration is due to protein catabolism, i.e. the process of converting blood and structural proteins to energy to produce higher energy, resulting in fish adaptation to a wide range of environmental conditions.

The 3rd and 4th fractions from *Piaractus mesopotamicus* serum and the 2nd and 3rd fractions from *Salminus brasiliensis* serum were positioned in an alpha-beta-globulin zone. Further studies should be conducted to better characterize each serum protein fraction since this information can be useful to fish health.

The 5th and 6th fraction from pacu serum and the 4th fraction from dourado, *Salminus brasiliensis*, serum were positioned in a gamma-globulin zone, where immunoglobulin are usually expected. In this study, the concentration of this protein fraction in pacu,

Piaractus mesopotamicus, serum was higher at 60 days compared to 30 days while in dourado serum it was lower at 60 days compared to 30 days. According to Scapigliati et al. (28), the immunoglobulin levels increase consistently with age and size; however, other events like water oxygenation and season can also affect concentrations of this protein in serum. The authors observed that the hyperoxygenation of seawater resulted in a two-fold increase of immunoglobulins, from 3.9 mg mL⁻¹ in running seawater, to 7.1 mg mL⁻¹ at 12 ppm O₂ L⁻¹. In juvenile *Piaractus mesopotamicus*, great concentrations of proteins were also observed in the 5th fraction in the group that was not fed lyophilized bovine colostrum, 0% LBC. This result suggests that the first lacteal bovine secretion, rich in immune and antimicrobial elements such as immunoglobulins, lactoferrin, lactoperoxidase and lysozyme (29), may have contributed to the protection of the juvenile. Consequently, the juveniles had less stimulus for their own synthesis of defense elements, probably immunoglobulin M. On the other hand, this result can indicate an immunosuppressive effect of colostrum. According to Mandalapu et al. (30) human colostrum contains a factor (colostrum inhibitory factor XX) that inhibits the induction of interleukin 2 in T lymphocyte cell lines. Aldridge et al. (31), in turn, observed that colostrum feeding reduces the number of immunoglobulin positive cells in the lymphoid tissues of newborn calves.

As found by other authors using SDS-PAGE, the presence, position and amount of protein in serum fractions can be affected by pollutants, water quality and phylogenetic distance (20, 32-36). Kicking et al. (37) states that plasma protein electrophoresis may be useful as a health assessment tool for evaluating injured sea turtles. The authors determined reference intervals for plasma protein fractions of wild Atlantic loggerhead sea turtles, *Caretta caretta*. In the present study, the values of protein concentration could be useful as reference ranges for the health of juvenile *Piaractus mesopotamicus* and *Salminus brasiliensis*.

The two species studied, *Piaractus mesopotamicus* and *Salminus brasiliensis*, showed different electrophoretic profiles of serum proteins. The first species, an omnivorous fish, showed six fractions while the second, a carnivorous fish, showed four fractions. Fractions that were positioned in the albumin and gamma-globulin migration zone were affected by feeding period, 30 or 60 experimental days, in both species, pacu and dourado. Bovine colostrum influenced the serum protein profile of the juvenile *Piaractus mesopotamicus* with some indications of positive protective effects.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of CEUA-ESALQ.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – R.M.N., D.M.; Design – R.M.N., D.M., W.N.; Supervision – R.M.N.; Resource – R.M.N., D.M.; Materials – R.M.N.; Data Collection and/or Processing – D.M., W.N.; Analysis and/or Interpretation - D.M.; Literature Search - D.M.; Writing - D.M.; Critical Reviews – R.M.N.

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

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Plant Preferences of *Halictus* Latreille (Halictidae: Hymenoptera) in the Mediterranean Region of Southern Turkey

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ABSTRACT

Objective: Lack of information regarding the floral preferences of wild bees is one of the major problems in understanding plant-bee interactions. Therefore, we investigated the plant preferences of *Halictus* Latreille (Halictidae: Apoidea: Hymenoptera) species that are distributed in the Mediterranean Region of southern Turkey.

Materials and Methods: Bees were collected through field studies that were performed during the spring and summer seasons between 2008 and 2009. The flowers visited by bees were also recorded. In total, 516 bee specimens belonging to 19 species were collected, and 54 plant taxa were found to be related to these *Halictus* species. In addition to field study data, information collected from the literature was included in the study. The most commonly visited plant families, genera, and species are described using diversity indices scores in terms of the plant taxa preferences of the bees.

Results: A total of 516 bee and 195 plant specimens were sampled from 76 stations located in 14 provinces. In total, 54 plant taxa were found to be related to 19 *Halictus* species. Among such large plant taxa preferences, the most commonly visited ones were *Onopordum*, *Centaurea*, and *Carduus* members.

Conclusion: This study suggests that members of the genus *Halictus* primarily prefer to visit plants belonging to the Asteraceae family. However, they may also visit several other types of flowers such as those belonging to Rosaceae and Brassicaceae families.

Keywords: Apoidea, *Halictus*, plant preference, fauna, flora, Asteraceae, Mediterranean, Turkey

INTRODUCTION

The flowering plants need pollination to set fruits or seeds. Bees (Apiformes: Apoidea: Hymenoptera) are one of the most important pollinators, in this respect (1). They generally feed their offspring with pollen which is a nutrient rich food source. Nearly 20,000 bee species exist, belonging to eight different families (1). Halictidae is one of the most diverse ones of all these families. It contains more than 70 genera and 3000 species found around the world (2). Among them, *Halictus* Latreille is

one of the largest genera. It contains 74 species in the West Palearctic Region. In addition, this genus is mostly distributed throughout the Mediterranean Region (3). Furthermore, there are 35 *Halictus* species in Turkey and 20 of them are found in Mediterranean Turkey (4). However, the studies for establishing the bee fauna of Turkey and data on the floral associations of these wild bees are insufficient.

The information on the plant preferences of bees is very important in two ways. Firstly, it helps researchers cap-

This study was partly presented at the XIII. OPTIMA Meeting with the title "Floral Associations of *Halictus* (Halictidae: Apoidea: Hymenoptera) of Turkey's Mediterranean Region", held on 22-26 March 2010, Antalya, Turkey.

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ture bees more quickly by using the distribution data of plants which directly give the address of bees. Secondly, this kind of observations and records would give a broad perspective in evaluating the co-evolution of plants and bees. Since, some bee species show narrow host-plant preferences, it would be an informative step to expose bee-plant associations in relation with host-plant evolution. Therefore, we cannot ignore the importance of plant preferences data.

Halictidae members have a unique feature which is that they exhibit nearly every degree of sociality, ranging from solitary to eusociality, these kinds of life strategies might have caused diversity in food selections which could possibly be an important reason why Halictidae is one of the dominant pollinator of angiosperms. Moreover, the accumulation of this data also will be helpful in finding out the exact biogeographical explanations of diversification of bees and related plants. Due to the fact that the Mediterranean region is known for containing a large amount of plant diversity (5), the richness of the species in the genus *Halictus* of this region needs to be questioned in this respect. Hence, the main aim of this study is to analyze the plant preferences of the members of the genus *Halictus* species that were found in the Mediterranean region of Southern Turkey.

MATERIALS AND METHODS

The study comprised of all the habitats related with the Mediterranean region of southern Turkey (Figure 1). Fieldwork was carried out through Spring and Summer between 2008 and 2009. Bee material were collected via nets and aspirators. Meanwhile, the flowers that had been visited by bees were also collected for identification. In addition to field study records, foraging flower information (4,6-11) was reviewed and added to the study. Bee and plant specimens were inspected via stereo-microscopes for diagnosis. Identification of the bee specimens were made according to Pesenko, Pesenko et al., Amiet et al. and Ebmer (10,12-14). Plant identifications were made according to Davis and Güner et al. (15-17). Distribution map for the studied area was prepared via CFF 2.0 (18). Bee records were analyzed by ecological diversity indices via PAST (19). Shannon diversity (Shannon H) and Evenness ($\text{Evenness}_e^H/S$) indices were evaluated in order to find out the most preferred plant species.

RESULTS

In total, 516 bee and 195 plant specimens were sampled from 76 stations located in 14 provinces. As a result, 54 plant taxa (Table 1) were found related with 19 *Halictus* species (Table 2). From those plants, seven of them were endemic to Turkey, six of them were Irano-Turanian elements and eight of them were Mediterranean. All the rest were typical for many habitats in Turkey. From the collected bees, there was only one endemic species, *Halictus pentheri* Blüthgen, 1923 and one Mediterranean species, *H. berlandi* Pérez, 1903.

Diversity indices were performed to each taxa and the total numbers of bee individuals captured from plants were analyzed. Also presence/absence data matrix of relevant records

was used to calculate more precise results. All those attempts figured out that *Centaurea iberica* Trev. ex Sprengel is the most preferred plant species (Table 3) and *Onopordum* L. is the most preferred plant genus by means of Shannon index scores (Shannon_H: *Onopordum* spp.: 1.986; *Centaurea* spp.: 1.923; *Echinops* spp.: 1.863; *Rubus* spp.: 1.749; *Picnoman* sp.: 1.667).

On the other hand, evaluation of the flower visit records for *Halictus* from literature (6-11) showed similar results (Table 4). *Centaurea* sp., *Carduus* sp., and *Onopordum* sp. were found as the most frequently visited plants.

DISCUSSION

Halictidae members exhibit nearly every degree of sociality ranging from solitary to eusociality (10). Michener (20) also reported that both solitary and primitively eusocial species are found in the genus *Halictus*. Such life strategy diversity might have caused diversification of food selections among *Halictus* members. Evaluating of the plant preference data of *Halictus* (Table 1) seems to confirm this idea. Such food diversity might also be one of the important reasons for their high abundance in nature.

For example, *H. maculatus* is reported as one of the most widely distributed species within Turkey (4). It is also known as a widespread species through Palaearctic region (10). According to the foraging plant or visited flower information this species was recorded to prefer more than 40 plant taxa belonging to various families (4). Pesenko et al. (10) reported that this species is known as primitively eusocial. Michener (20) reported that in such polylectic bees, even though they are primitively social, there is no any communication or social interaction to share the location of food source information. But such groups that have polylectic behavior (pollen loads contains a variety of flowers) show a tendency to forage on a single flower at each trip (20). That might be the possible explanation for their great amount of variation on visited flower information.

According to our study, *H. resurgens* is reported as the most common member of the group that is recorded from all around the Mediterranean region of southern Turkey (21). When we evaluate the foraging plant or visited flower data of this species, we see the same situation. At different stations we observed that different flower types are visited by members of this species.

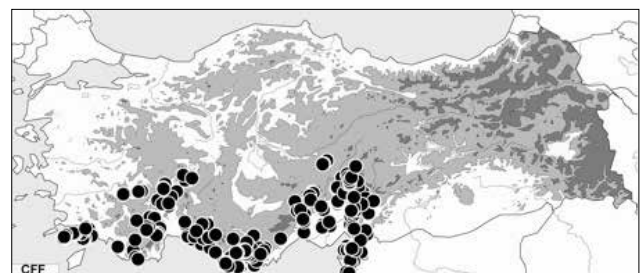


Figure 1. CFF map of studied area (black circles indicates the locations of stations).

Table 1. Determined plant taxa ("*" indicates the endemic species)

<i>Acantholimon</i> sp.	* <i>Marrubium parviflorum</i> Fisch & Mey subsp. <i>oligodon</i> (Boiss.) Seybold
* <i>Anchusa leptophylla</i> Roemer & Schultes subsp. <i>incana</i> (Ledeb.) Chamb	<i>Melilotus officinalis</i> (L.) Desr
<i>Cardaria draba</i> (L.) Desv. subsp. <i>draba</i>	<i>Mentha longifolia</i> (L.) Hudson subsp. <i>typhoides</i> (Briq.) Harley var. <i>typhoides</i>
<i>Carduus nutans</i> L. <i>nutans</i> sensu lato	<i>Mentha spicata</i> L. subsp. <i>spicata</i>
* <i>Carduus olympicus</i> Boiss. subsp. <i>hypoleucus</i> (Bornm.) Davis	<i>Nasturtium officinale</i> R.Br.
<i>Carduus pycnocephalus</i> L. subsp. <i>albidus</i> (Bieb.) Kazmi	<i>Onopordum acanthium</i> L.
<i>Centaurea iberica</i> Trev. ex Sprengel	* <i>Onopordum anatolicum</i> (Boiss.) Eig
<i>Centaurea solstitialis</i> L. subsp. <i>solstitialis</i>	* <i>Onopordum boissieri</i> Willk.
* <i>Centaurea solstitialis</i> subsp. <i>carneola</i> (Boiss.) Wagenitz	* <i>Onopordum bracteatum</i> Boiss. & Heldr. var. <i>arachnoideum</i> Erik & Sümbül
<i>Chondrilla juncea</i> L. var. <i>acantholepis</i> (Boiss.) Boiss.	<i>Onopordum bracteatum</i> Boiss. & Heldr. var. <i>bracteatum</i>
<i>Chondrilla juncea</i> L. var. <i>juncea</i>	<i>Onopordum carduchorum</i> Bornm. & Beauverd
<i>Chrysanthemum segetum</i> L.	<i>Onopordum majori</i> Beauverd
<i>Cichorium intybus</i> L.	<i>Onopordum sibthorpiatum</i> Boiss. & Heldr.
<i>Convolvulus arvensis</i> L.	<i>Peganum harmala</i> L.
<i>Crepis alpina</i> L.	<i>Picnemon acarna</i> (L.) Cass.
<i>Crepis foetida</i> L.	<i>Picris altissima</i> Delile
<i>Crepis foetida</i> L. subsp. <i>commutata</i> (Spreng.) Babcock	<i>Pulicaria arabica</i> (L.) Cass
<i>Crepis foetida</i> L. subsp. <i>rhoadifolia</i> (Bieb.) Celak	<i>Ranunculus marginatus</i> d'Urv var. <i>trachycarpus</i> (Fisch. & Mey) Azn
<i>Echinops orientalis</i> Trautv.	<i>Reseda lutea</i> L. var. <i>lutea</i>
<i>Echinops pungens</i> Trautv. var. <i>pungens</i>	<i>Rubus canescens</i> DC. var. <i>glabratus</i> (Godron) Davis & Meikle
<i>Echinops ritro</i> L.	<i>Rubus sanctus</i> Schreber
<i>Echinops viscosus</i> DC. subsp. <i>bithynicus</i> (Boiss.) Rech.	<i>Scabiosa atropurpurea</i> L. subsp. <i>maritima</i> (L.) Arc
<i>Eryngium campestre</i> L. var. <i>virens</i> Link	<i>Scabiosa celocephala</i> Boiss.
<i>Glaucium leiocarpum</i> Boiss.	<i>Silene vulgaris</i> (Moench) Garcke var. <i>vulgaris</i>
<i>Hirschfeldia incana</i> (L.) Lang.- Foss.	<i>Verbascum</i> sp.
<i>Malva neglecta</i> Wallr.	<i>Vicia villosa</i> Roth subsp. <i>eriocarpa</i> (Hauskn) P.W.Ball

Table 2. Determined *Halictus* species

<i>Halictus adjikenticus</i> Blüthgen, 1923	<i>Halictus pentheri</i> Blüthgen, 1923
<i>Halictus alfenellus</i> Strand, 1909	<i>Halictus quadricinctus</i> (Fabricius, 1776)
<i>Halictus asperulus</i> Pérez, 1895	<i>Halictus resurgens</i> Nurse, 1903
<i>Halictus berlandi</i> Pérez, 1903	<i>Halictus sajoi</i> Blüthgen, 1923
<i>Halictus brunnescens</i> (Eversmann, 1852)	<i>Halictus sexcinctus</i> (Fabricius, 1775)
<i>Halictus cochlearitarsis</i> (Dours, 1872)	<i>Halictus simplex</i> Blüthgen, 1923
<i>Halictus compressus</i> (Walckenaer, 1802)	<i>Halictus squamosus</i> Lebedev, 1911
<i>Halictus luganicus</i> Blüthgen, 1936	<i>Halictus tetrazonianellus</i> Strand, 1909
<i>Halictus maculatus</i> Smith, 1848	<i>Halictus tetrazonius</i> (Klug, 1817)
<i>Halictus patellatus</i> Morawitz, 1874	

The Mediterranean region itself could be another factor for this diversity in food preference. Due to the fact that the Mediterranean region is characterized by a high diversity of plants (5), it is not surprising to see such a diversity of bees, and such different food choices in parallel with this diversity.

In this concept the most common visited flower families and genera were reviewed to analyze the plant preferences of *Halictus* species. The results showed that *C. iberica* is widely preferred by the *Halictus* species (Table 3). However, this selection is not so strict and cannot indicate a monolectic or oligolectic feeding behavior. The results also suggest that there is a choice on

Asteraceae members on family level and especially a choice on the genera *Onopordum*, *Echinops* L., *Carduus* L., *Centaurea* L. and *Cirsium* Adans (Tables 3-4).

Whether such results and the data recorded in the literature (6-11) display a wide oligolectic behavior on family level to Asteraceae, we do not have concrete results to suggest such a strict relation, or any sort of specialization either. According to Larkin et al. (22), even some polylectic bees may display a short-term specialization such as the "flower fidelity" behavior in honeybees.

After we analyzed our results according to Michener (20) and Larkin et al. (22) we can suggest that such short term foragers may have affected our results and displayed somehow specialization on certain plant taxa.

However, as our data did not depend on one single location and just a few samples, depending on our large sample size and sampling locations we can conclude that members of the genus *Halictus* mostly prefer to visit plants belonging to the Asteraceae family but also may visit many types of other flowers such as Rosaceae and Brassicaceae.

These results do not allow us to make concrete remarks on the foraging habit of this genus. However, the information on plant preferences of bees may help us design our further field studies more accurately since by knowing the direct address (the preferred flowers) may help us capture and follow bees more easily. Moreover, such further studies focusing on the plant-bee interactions, orientation of the food source and understanding the social behavior of bees would be more convenient by preferred plant data of the target bee species.

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Table 3. Shannon diversity (*Shannon_H* scores) index scores of related plant species

Plant Species	Shannon_H Scores
<i>Centaurea iberica</i> Trev. ex Sprengel	1.902
<i>Onopordum carduchorum</i> Bornm. & Beauverd	1.786
<i>Onopordum acanthium</i> L.	1.685
<i>Picnomon acarna</i> (L.) Cass.	1.667
<i>Echinops orientalis</i> Trautv	1.565
<i>Onopordum bracteatum</i> Boiss. & Heldr. var. <i>bracteatum</i>	1.465
<i>Rubus canescens</i> DC. var. <i>glabratus</i> (Godron) Davis & Meikle	1.427
<i>Echinops pungens</i> Trautv. var. <i>pungens</i>	1.396
<i>Echinops ritro</i> L.	1.388
<i>Centaurea solstitialis</i> L. subsp. <i>solstitialis</i>	1.24

Table 4. Shannon diversity scores of the data

Genera	Shannon_H ₁	Evenness_e ^{AH} /S	Shannon_H ₂	Genera	Shannon_H ₃
<i>Onopordum</i>	1.986	0.5604	2.565	<i>Centaurea</i>	2.398
<i>Centaurea</i>	1.923	0.5261	2.565	<i>Carduus</i>	2.197
<i>Echinops</i>	1.863	0.6446	2.303	<i>Onopordum</i>	2.197
<i>Rubus</i>	1.749	0.8212	1.946	<i>Taraxacum</i>	1.792
<i>Picnomon</i>	1.667	0.8831	1.792	<i>Cirsium</i>	1.609
<i>Carduus</i>	1.189	0.5471	1.792	<i>Salix</i>	1.609

Shannon_H₁: The scores calculated by individual numbers; Shannon_H₂: The scores calculated by presence/absence data; Shannon_H₃: The scores calculated by presence absence data of the literature; Evenness_e^{AH}/S: The Evenness index

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

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Antioxidant Activity, Total Phenol and Total Flavonoid Contents of *Trachystemon orientalis* (L.) G. Don

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ABSTRACT

Objective: For many years, plants have been considered a source of alternative medicine and are used for the treatment of several diseases. These medicinal plants are excellent sources of phytochemicals and antioxidant activity. *Trachystemon orientalis* (L.) G. Don (Boraginaceae) originates from East Bulgaria but can be found mostly in West Caucasia and the Black Sea region of Turkey. Its flowering branches, rhizomes, and leaves are used as food.

Materials and Methods: The antioxidant activity of a *T. orientalis* aqueous infusion was investigated using various antioxidant tests, such as reducing power and radical scavenging activity. The phenolic and flavonoid contents were also determined. Results were compared with natural and synthetic antioxidants.

Results: The results demonstrated that *T. orientalis* (L.) is a good source of antioxidants.

Conclusion: This study suggested that *T. orientalis* extract can be considered a useful natural antioxidant source because of its flavonoid, phenolic, and anthocyanin contents. The food and cosmetic industries might employ *T. orientalis* extract as an alternative additive to other highly toxic synthetic antioxidants.

Keywords: Antioxidant activity, oxidative stress, phenolic compounds, reducing power, radical scavenging activity, flavonoid, *Trachystemon orientalis* (L.) G. Don.

INTRODUCTION

Oxidative stress is defined as the disturbance or lack of balance in the production of free radicals and the antioxidant system (1,2). Disturbances in this normal redox status can cause toxic impacts by producing peroxides and free radicals that damage cell and tissue components such as lipids, proteins and DNA (3,4). In humans, oxidative stress causes many diseases such as tissue injury, cell death, cancer, cardiovascular diseases, arteriosclerosis, neural disorders, Parkinson's disease, Alzheimer's disease, ageing, skin irritations and inflammations, diabetes mellitus and chronic fatigue syndrome (5-8). Antioxidants are known to delay or inhibit the oxidation of substrates even when present

in low concentrations (9). Synthetic antioxidants such as propyl gallate (PG), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertiary butyl hydroquinone (TBHQ) protect lipids found in foods from the harmful effect of oxidation. However, both BHT and BHA are restricted from use because of their toxic and carcinogenic effects (10). Because of the negative effect of synthetic compounds, natural antioxidants rather than synthetic antioxidants are much more preferred by consumers. Thus, the demand for natural and safer antioxidants is growing.

Studies have shown that higher plants are excellent sources of functional compounds such as anthocyanins, vitamins, flavonoids, dietary glutathione and carotenoids

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- which are responsible for antioxidants' function. Therefore, they are considered as important nutraceuticals with many health benefits (11,12). *T. orientalis* is a plant of East Bulgarian origin. In Turkey, it is popularly known as Galdirek, Kaldirik, Hodan, Kalduruk (Bolu), Tamara (Trabzon), Burgi (Artvin) and Zilbit, Ispit (Zonguldak). The plant is chiefly found in the West Caucasia and Black Sea regions and is consumed as a vegetable in Istanbul and other regions of the Black Sea (13,14). A concoction of their fresh roots is used like a tonic on the skin against rheumatism and/or for the healing of inflamed wounds (15). Additionally, it has diuretic effects and can be used as a blood purifier (16). *T. orientalis* is reported to contain nitrate salts, tannins, mucilage, essential oils, resin and saponins (17).

A quantitative assessment of total phenols, flavonoids and anthocyanine contents of *T. orientalis* was carried out in this study. In addition, the reducing power of the aqueous infusion extract, ferric thiocyanate antioxidant activity and The 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of the plant was conducted.

MATERIALS AND METHODS

Plant Materials

The leaves of *T. orientalis* were acquired from Istanbul, Turkey in the month of March 2017. The fresh sections were cleaned with water and dried at 20°C, then stored at -20°C until use.

Preparation of Infusion Extract

Plant extract was prepared as a 10% (w/v) aqueous infusion. A dried sample (20g) was extracted with boiling water (100 mL) for 15 min while stirring.

Phytochemical Tests

The infusion extract was subjected to preliminary qualitative phytochemical analysis for the detection of major chemical groups (Table 1). The details are below:

1. Phenols were analysed after the aqueous solution of *T. orientalis* infusion was filtered using filter paper. A drop of molybdophosphoric acid reagent was added to the sediment and absorbed into NH_3 vapour.
2. Braemer's test for tannins was adopted for tannins determination. A 10 % alcoholic FeCl_3 solution was added to 2-3 mL of infusion extract.
3. Steroids and terpenoids were determined using the Liebermann-Burchardt test. Into one mL of infusion extract solution, one mL of CHCl_3 , two / three mL acetic anhydride and one to two drops of 98 % H_2SO_4 were added.
4. Dragendorff's reagent was added to 1mL of infusion extract for alkaloid determination.
5. Bornträger's test for anthraquinones was carried out by heating approximately 50 mg of the infusion extract with 10% FeCl_3 solution and one mL of 37 % HCl. Diethyl ether was used to rinse the cooled extract after filtration, before further extraction with concentrated NH_3 .

Determination of Antioxidant Activity of the Extract

Determination of Total Phenolic Compounds

Total phenolic compounds of *T. orientalis* were determined with the Folin-Ciocalteu reagent using Slinkard and Singleton's method (1977), pyrocatechol was used as a standard for phenolic compound (18). Absorbance was measured spectrophotometrically at 760 nm.

Determination of Total Flavonoid Content

Total content of flavonoids in *T. orientalis* was determined spectrophotometrically at 510 nm (19). The results are presented as the mean (\pm SD) mg of (+)-catechin equivalents per gram of extract.

Determination of the Anthocyanin Content

The anthocyanin composition of *T. orientalis* was estimated by the modified method of Padmavati et al. (20). The anthocyanin concentration was determined spectrophotometrically at 530 and 657 nm. The absorbance of anthocyanin was calculated using the extinction coefficient of $31.6 \text{ M}^{-1}\text{cm}^{-1}$.

Ferric Thiocyanate (FTC) Antioxidant Activity

Osawa and Namiki's method was employed for the determination of FTC activity (21). The measurement of absorbance was made spectrophotometrically at 500 nm. α -Tocopherol was used as positive control. All tests were conducted in triplicate and the average was calculated.

FTC antioxidant activity was calculated with the following equation:

$$\text{Inhibition (\%)} = [(A-B)/A] \times 100$$

A represents the control and B is the sample.

Reducing Power

The reducing potential of *T. orientalis* was analysed using Oyaizu's method (22). Absorbance was measured spectrophotometrically at 700 nm, high absorbance values indicate a strong reducing power.

DPPH Radical Scavenging Potential

DPPH radical scavenging activity of antioxidants developed by Brand-Williams et al. (23) was adopted in this study. The equation below was used for the calculation of the DPPH radical activity.

$$\text{DPPH radical scavenging activity (\%)} = (A_0 - A_1/A_0) \times 100$$

A_0 indicates the absorbance of the control and A_1 indicates the absorbance of the sample.

RESULTS

In this study, the presence of tannins, phenols, and anthraquinones was detected in the *T. orientalis* aqueous infusion extract (Table 1).

The level of total phenolic compounds of *T. orientalis* is presented in Table 2. An equivalent of 36 μg of pyrocatechol was obtained in 1 mg/mL of aqueous infusion extract. The concen-

tration of flavonoids in 1 mg of the *T. orientalis* aqueous infusion extract was found to be 29.34 ± 0.62 μg catechin equivalents. This suggests that the antioxidant activities of *T. orientalis* might be due to its high level of flavonoid content. As indicated in Table 2, the anthocyanin level of *T. orientalis* in this study was 0.35 ± 0.06 $\mu\text{mol/g}$ extract.

These primary products (i.e. peroxides) can be measured by the ferric thiocyanate method in the linoleic acid system, with

α -tocopherol employed as standard. As a result, high peroxide formation during the emulsion incubation causes a high absorbance value. The effects of varied concentrations (20 and 60 $\mu\text{g/mL}$) of *T. orientalis* of linoleic acid emulsion on lipid peroxidation are given in Table 3. At forty-eight hours of testing, the percentage peroxidation inhibition of 20 and 60 $\mu\text{g/mL}$ of aqueous infusion extracts on the linoleic acid system was 64.57 ± 3.53 % and 75.48 ± 0.62 % respectively. These values were higher than that of 100 $\mu\text{g/mL}$ α -tocopherol (61.19 ± 0.61 %).

More so, a high absorbance value in reducing power test signifies a strong reducing power. The reducing power of *T. orientalis* increased with increasing concentration of the extract (Figure 1). However, its reducing effect was weak when compared

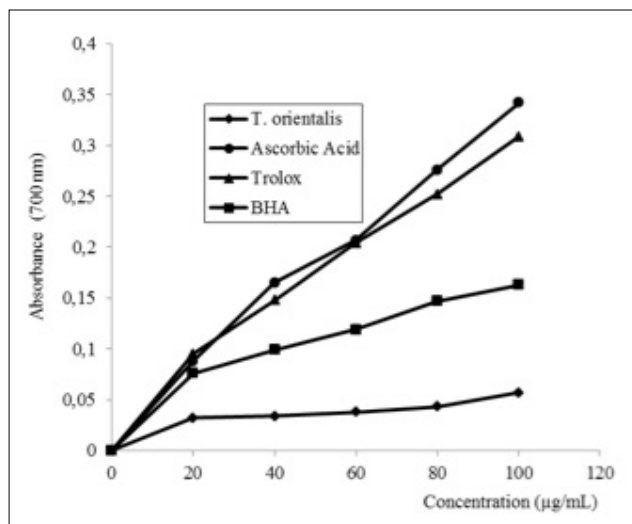


Figure 1. Reducing power of *T. orientalis* (L.) G. Don. aqueous infusion extract.

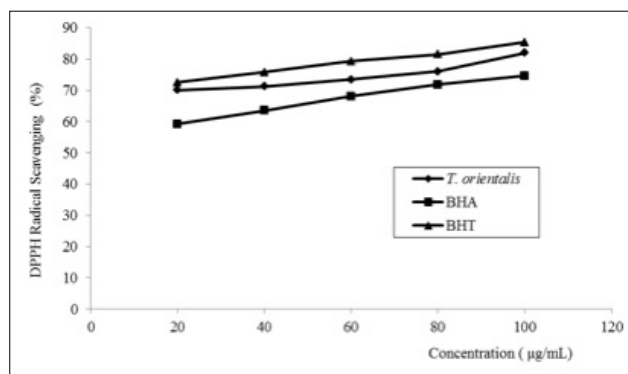


Figure 2. DPPH radical scavenging activity of aqueous extracts of *T. orientalis* (L.) G. Don.

Table 1. Preliminary phytochemical screening of aqueous infusion extract of *T. orientalis* (L.) G. Don

Tested for	Detection*	Test performed
Phenols	++	Phosphomolybdic acid test
Tannins	+++	Braemer's test
Steroids and Terpenoids	---	Liebermann- Burchardt test
Alkaloids	---	Dragendorff's test
Anthraquinones	+++	Bornträger test

* --- = Absent; ++= Moderate; +++ = Abundant

Table 2. Total phenolic compounds (as pyrocatechol equivalent), total flavonoids (as catechin equivalent) and total anthocyanin of *T. orientalis* (L.) G. Don. aqueous infusion extract

Concentration (µg/mL)	Phenolic Compounds (µg pyrocatechol/mg extract)	Flavonoids (µg catechin/mg extract)	Anthocyanins (µmol/g extract)
250	29.36 ± 0.87	7.94 ± 0.31	0.35 ± 0.06
500	32.00 ± 0.32	13.92 ± 0.24	-
750	34.17 ± 1.10	21.86 ± 0.74	-
1000	36.00 ± 0.59	29.34 ± 0.62	-

Values: Mean \pm SD (n=3).

Table 3. Antioxidant activity (FTC) of aqueous infusion extracts of *T. orientalis* (L.) G. Don in comparison to α -tocopherol as reference antioxidant at 48 hours

Compounds	(Inhibition %)
<i>T. orientalis</i> (20 μ g/mL)	64.57 \pm 3.53
<i>T. orientalis</i> (60 μ g/mL)	75.48 \pm 0.62
α -Tocopherol (100 μ g/mL)	61.19 \pm 0.61
Values: Mean \pm SD (n=3). FTC: Ferric Thiocyanate	

to the reducing power of positive controls like BHA, Trolox and ascorbic acid.

The DPPH assay is a highly effective and easy to apply spectrophotometric method for understanding the effect of radical compounds, as well as radical scavenging capacities of antioxidants. Figure 2 represents the DPPH radical scavenging activity of *T. orientalis* extract, and that of BHA and BHT as positive control. Both the extract and the standards exhibited scavenging properties according to BHT, *T. orientalis* and BHA had the following activities respectively 85.45 \pm 9.78% > 81.99 \pm 7.45% > 74.61 \pm 9.04%.

DISCUSSION

Reactive oxygen species and reactive nitrogen species are highly reactive oxidizing molecules which are constantly generated during normal cellular activities. For instance, the activity of the mitochondrial respiratory chain and inflammation could generate these compounds, which could lead to damage of other biological molecules such as proteins and DNA. In the last decade, there has been a growing interest in natural antioxidants. Studies shows that diets rich in fruits, vegetables and derived products have been defined to alleviate chronic diseases. These food containing herbs are rich in phytochemical molecules such as vitamins, phenolic antioxidants etc, thus aids normal health and wellbeing (24-27).

Polyphenols are a diverse group of plant secondary metabolites, encompassing subgroups such as tannins and flavonoids among others. They are found throughout the plant kingdom, with their biological function lying mostly within their defensive capabilities against herbivores, pathogens, and UV-B radiation. Tannins are structurally the most complex group of polyphenols, they are large ringed, present in several plant families and are reported to exhibit anticancer or cancer preventive activity and antioxidant capability (28-29). Therefore, *T. orientalis* aqueous infusion extract which is a good source of tannins may possess significant anticancer activity and antioxidant potentials. In recent years, several clinical trials, as well as other evidences have indicate that diseases precipitated by oxidative stress can be prevented or managed with high flavonoids rich foods (30-32). Previous studies by Özen (33) report a phenolic content of 82.1 \pm 1.5 mg pyrocatechol/g dry weight of *T. orientalis*. Moreover, Ayvaz (34) reported a phenol concentration of

68.9 mg pyrogallat/g and 17.5 mg pyrogallat/g respectively in water and ethanolic extracts. Conversely, the phenolic content of *T. orientalis* in this study (at 500 μ g/mL aqueous infusion extract concentration) was below those of the aqueous infusion extracts in earlier reports (32.00 \pm 0.32 mg pyrocatechol/mg). The difference in concentration may be attributed to the concentration of extract used for the qualitative analysis as well as the extraction methods employed.

In many herbs, antioxidant activities correlate positively with phenolic contents (35). Several works of literatures have reported the key role of phenolic compounds in scavenging free radicals, as well as the high scavenging ability of phenol rich plant samples (36-39).

Flavonoids in plants are involved in providing pigmentation for flowers, fruits and seeds to attract pollinators and seed dispersers, improving fertility, aiding germination of pollen, protecting against stress, and acting as signal molecules in plant microbe interactions. Flavonoids are greater antioxidants than natural phenolic compounds (40). Moreover, the antioxidant activity of plant products is also correlated to their total flavonoid content. Thus, it is suggested that the consumption of flavonoid-containing nourishments is beneficial for protection from free radical damage. The flavonoids content quantified from *T. orientalis* in this study at 1000 μ g/mL aqueous infusion extract (29.34 \pm 0.62 μ g catechin/mg extract) was comparable to that of ethanol extract (29.4 mg catechin /g extract), but lower that of aqueous extract (56.88 mg catechin /g extract respectively) reported by Ayvaz (33).

Anthocyanins which are a type of antioxidant flavonoids have diverse functions in higher plants. They act as insect and/or animal attractors thanks to the coloration they confer to plants. It is also suggested that they have a protective effect against UV light, infection by pathogens as well as scavenging reactive oxygen species. Furthermore, they confer drought, low temperature and high salinity resistance in plants. In general, anthocyanins find their way into human diet through vegetables, fruit, tea, beans, coffee, cereals, herbs and spice extract. They are famous for their pharmacological effects such as antioxidant, antitumor, anti-inflammatory and antimutagenic activities (41). Comparatively, the anthocyanin content of *T. orientalis* in this study was extremely low (0.35 \pm 0.06 μ mol/g extract), in relation to the 15.2 \pm 0.1 mg cyanidin 3-glucoside/g dry weight reported by Özen (34).

Lipid peroxidation mediated chain reactions are processes initiated by free radicals that result in the production of peroxide implicated in several biological complications (42). The mechanism for spectrophotometric estimation of lipid hydroperoxidation involves the oxidation of ferrous ion to ferric ion, followed by coupling to thiocyanate to produce a complex. By this, the amount of peroxide produced during the initial stages of oxidation (the primary product of lipid oxidation) is quantified. The findings of this study suggest that *T. orientalis* aqueous infusion extract has a high peroxidation inhibition capacity at 20 and 60

µg/mL as compared to α-tocopherol and therefore, may serve as an alternative source of antiperoxidants.

Reducing power is related to antioxidant activity due to the fact that antioxidant can give off their electrons for the reduction of reactive radicals. Thus, reducing power can be used to give information about antioxidative potentials of prospective antioxidants (43). It can be measured by the direct reduction of $\text{Fe}[(\text{CN})_6]_3$ to $\text{Fe}[(\text{CN})_6]_2$, forming intense Perl's Prussian blue complex measured at 700 nm. An increase in absorbance indicates an increase in reducing capacity as a result of an increase in the formation of the complex (44). The *T. orientalis* aqueous extract in comparison, had a reducing power value (0.05 at 60 µg/mL) less than the 0.2 previously reported by Özen (34). This difference may be attributed to the variation in antioxidant phytochemical level between the sample used, arising from growth conditions and environmental effects on the plants.

A common method for determining antioxidant capability is the DPPH radical scavenging reaction. A reverse in formation of DPPH radicalisation occurs when antioxidants are added to radicals, thus decolourisation occurs. The potential of plant extracts to act as antioxidants depends on the redox properties and electron delocalization of phenolic hydroxyl groups of their constituent polyphenolic compounds. In this study, *T. orientalis* aqueous infusion extract was found to contain a moderate amount of phenolic compounds, and an above average DPPH radical scavenging activity (81.99 %). A previous study (34) reported an approximate 50% inhibition at 100 µg/mL of extract. Also, Ayvaz (33) reported an IC_{50} DPPH radical scavenging potential of aqueous extract of 0.41 mg/mL and 2.6 mg/mL of the ethanolic extract. The DPPH radical scavenging capability of aqueous *T. orientalis* extract can be attributed to its ease in abstracting a hydrogen atom from the hydroxyl group of its phenolic constituents.

CONCLUSION

This study suggests that *T. orientalis* extract can be considered as a useful natural antioxidant source because of its flavonoids, phenolic and anthocyanin contents. Furthermore, it may serve as a cheap and readily accessible source of natural antioxidant. Therefore, the food and cosmetic industries might employ *T. orientalis* extract as an alternative additive to the more toxic synthetic antioxidants.

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Pathology of *Flavobacterium* sp. KG3 in Experimentally Challenged Ornamental Goldfish *Carassius auratus* (L.)

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ABSTRACT

Objective: This study investigated the pathogenicity and pathology of the gill rot-associated bacterium *Flavobacterium* sp. KG3 in experimentally challenged *Carassius auratus*.

Material and Methods: The pathogenicity of *Flavobacterium* sp. KG3 was assessed by intraperitoneal injection (i/p) and abrasion-bath treatment followed by histopathology.

Results: *Flavobacterium* sp. KG3 challenge caused extensive damages to the gills and internal organs of *C. auratus*, resulting in cellular and tissue-level alterations. The i/p challenge resulted in significant mortalities, with an LD₅₀ value of 2.5×10^5 CFU/fish. The abrasion-bath challenge resulted in 60% mortality at 6.0×10^6 cells/mL in 5 days. The kidney of the challenged *C. auratus* exhibited extensive haemorrhages, polymorphic and constricted nephritic tubules, fibrosis, glomerulopathy, degeneration of nephritic tubular epithelium, disruption of blood vessels, cellular and nuclear hypertrophy, granuloma formation, necrosis of haematopoietic area, vacuolation in haematopoietic tissue, widening of lumen, and thickening of the luminal lining.

Conclusion: Like other recognized bacterial pathogens, *Flavobacterium* sp. KG3 was moderately virulent to *C. auratus* and can produce systemic pathology in the gills, muscle, spleen, and kidney.

Keywords: *Carassius auratus*, *Flavobacterium* sp., Flavobacterial infection, virulence, histopathology

INTRODUCTION

Aquarium keeping is amongst the most popular hobbies with millions of enthusiasts worldwide (1). Among ornamental fish, goldfish *Carassius auratus* is the most common and of international significance. Ornamental fish are cultured on a large scale in various localities of West Bengal, India in earthen ponds and cemented tanks. Diseases of infectious and non-infectious origin are affecting the ornamental fish production and the livelihood of aquarists. A variety of diseases including bacterial and viral diseases have been documented in goldfish (2-4). Among the bacterial diseases, flavobacteriosis caused by *Flavobacterium* spp. is regarded as a predominant disease of ornamental fish (5). They are Gram-negative rods

with 0.3-0.5 µm in diameter and 1.0-40.0 µm in length and known for their opportunistic pathogenic role in fish (6). Diseases caused by *Flavobacterium columnare*, *F. psychrophilum*, *F. branchiophilum*, and other *Flavobacterium* spp. have been documented frequently in fish as primary or opportunistic pathogens (6-11). The number of formally described species of the genus *Flavobacterium* has rapidly expanded from 26 (7) to over 100 (11).

Over the years, there are increasing incidences of flavobacterial infection in cultured Indian fish. Bacterial gill disease by *F. branchiophilum* in Indian major carps (12), *F. columnare* infection in *Catla catla* (13), *Carassius auratus* (14) and *Labeo rohita*, *Ctenopharyngodon idella* and *Anabas testudineus* (15), and infection by *Flavobacterium* spp.



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in *C. auratus* (4) were documented in India. They were also isolated in healthy carps (16). The reports on the pathogenic potential of these bacteria on cultured fish and their systematic pathology are scanty. In an earlier study, we reported the phenotypic and molecular characterization and virulence of gill rot associated *Flavobacterium* sp. KG3 (17). The present study describes the pathogenicity of *Flavobacterium* sp. KG3 in goldfish *C. auratus* as well as the histopathological alterations in different organs.

MATERIAL AND METHODS

Bacterial Strain

The bacterial strain *Flavobacterium* sp. KG3 (NCBI GenBank accession number KP997186) used in this study was isolated from the diseased *Catla catla* gill (17). It was maintained as a glycerol stock in the Department of Aquatic Animal Health, Faculty of Fishery Sciences, Kolkata, India, whose phenotypic and molecular characterizations are described in our earlier report (17).

Pathogenicity of *Flavobacterium* sp. KG3 in *Carassius auratus*

The pure culture of *Flavobacterium* sp. KG3 was revived from the glycerol stock at -20°C and maintained on cytophaga agar (CA) slant. The cell suspension of this strain was prepared and suitably diluted up to 10^{-9} in sterile physiological saline. The number of cells/mL of suspension was determined by spread plating on CA (17).

The experimental goldfish (3.85 ± 0.66 g; 7.99 ± 1.12 cm) were procured from Piyarapur (Lat. $22^{\circ}47'49''\text{N}$; Long $88^{\circ}18'18''\text{E}$), Hooghly district, West Bengal, India. They were packed in oxygen filled polythene bags and brought to the laboratory within 2 hours of collection. At the laboratory, the fish were immersed in 5 ppm KMnO_4 solution for 15 min and transferred to the fiberglass reinforced plastic (FRP) tanks of 500L capacity at the rate of 75 numbers/tank. The weak fish were removed immediately. All fish were maintained in the FRP tanks for 20 days and fed daily with pellet feed twice daily at 2% of the body weight. The challenge experiments were carried out in glass aquaria ($60 \times 30 \times 30$ cm), after thorough washing and drying. The glass aquaria ($n=14$) were filled with 30L each of bore-well water and conditioned for three days. Each aquarium was stocked with 10 healthy goldfish and acclimatized for 3 days with continuous aeration. The fish were fed twice daily with pellet feed at 2% of the body weight and maintained under optimal condition. The wastes and faecal matter were siphoned off and 50% of the water exchanged on alternate days.

The pathogenicity of *Flavobacterium* sp. KG3 was tested by intraperitoneal injection (i/p) and abrasion-bath treatment in duplicate. Aliquots (0.1 mL each) of *Flavobacterium* sp. KG3 cell suspensions from 10^0 to 10^{-3} dilutions were intraperitoneally (i/p) injected, i.e., between the pelvic fins and anal vent to get 10^8 - 10^5 cells/fish, respectively. The control fish were given 0.1 mL each of sterile saline (i/p). The abrasion-bath treatment was done as described previously (17). In brief, the scales of all the fish from each aquarium were scrapped off gently with a scalpel from caudal peduncle to the pectoral fin (abraded). The abraded fish from each aquarium were immersed for 60 min in a suspension (1000 mL) containing 6.0×10^6 cells of *Flavobacterium* sp. KG3/mL. All the fish were then transferred to the respective aquaria containing 30L water. The control group was neither abraded nor challenged. The fish groups were

maintained in the respective aquaria for 28 days. The signs of infection, behavioural abnormalities and mortality were recorded daily. Reisolation of *Flavobacterium* sp. KG3 from the gills and kidney of freshly dead fish was on CA followed by phenotypic confirmation.

Histopathology

Bouin's solution was used to fix the gill, muscle, kidney and spleen samples of *Flavobacterium* sp. KG3 challenged *C. auratus*. The 24 h fixed samples were processed by standard techniques and embedded in paraffin wax. Thin sections of 5 μm thickness were prepared and stained with Haematoxylin and Eosin (18).

RESULTS

Pathogenicity of *Flavobacterium* sp. KG3 in *Carassius auratus*

The gross and clinical signs observed in the experimentally challenged *C. auratus* were lethargy, sluggishness, erratic movement,

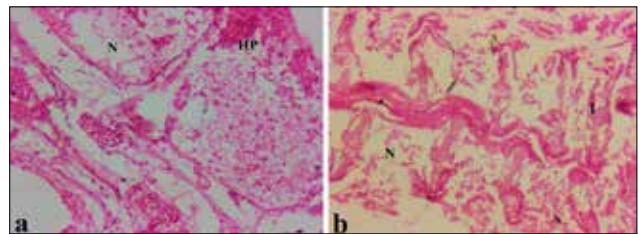


Figure 1. a, b. Photomicrograph of the gill of *Flavobacterium* sp. KG3 challenged *Carassius auratus* showing a) extensive necrosis (N) and hyperplasia (HP) [X200]; b) extensive necrosis (N) and inflammation of cartilaginous tissue (I) [X100]. H&E staining

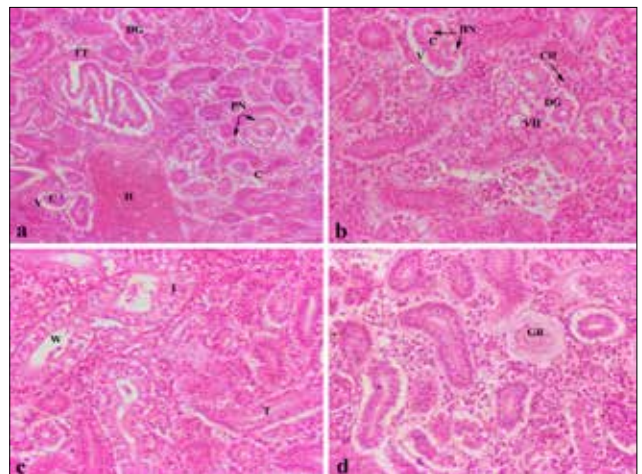


Figure 2. a-d. Photomicrograph of the kidney of *Flavobacterium* sp. KG3 challenged *Carassius auratus* showing a) extensive haemorrhages (H), polymorphic nephritic tubules (PN), fibrosis around the inflamed nephritic tubules (FT), constricted nephritic tubules (C) with vacuolated surrounding (V), degeneration of nephritic tubular epithelium (DG) [X100]; b) vacuolation in haematopoietic tissue (VH), constricted nephritic tubules (C) with vacuolated surrounding (V), cellular (CH) and nuclear hypertrophy (HN), degeneration of tubular epithelium (DG) [X200]; c) nephritic tubule inflammation (I), widen lumen (W) and thickening of luminal lining (T) [X200]; d) granuloma-like formation (GR) [X200]. H&E staining

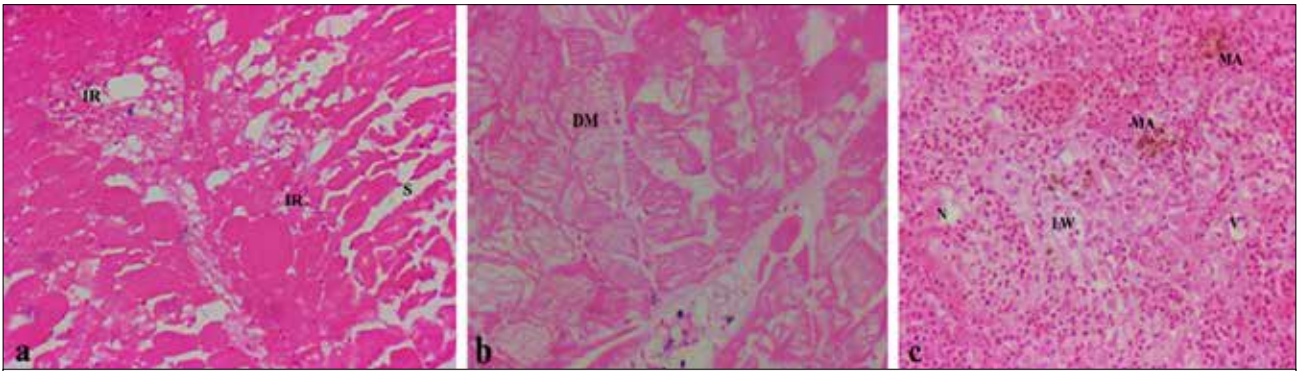


Figure 3. a-c. Photomicrograph of the muscle of *Flavobacterium* sp. KG3 challenged *Carassius auratus* showing a) sarcolysis (S) along with haemocyte infiltration (IR); b) diffused muscle bundle (DM). c) Photomicrograph of the spleen of *Flavobacterium* sp. KG3 challenged *Carassius auratus* showing melanomacrophage aggregate (MA), necrosis (N), loosely packed white pulp (LW) with vacuolated cells (V) [X200]. H&E staining

hanging, anorexia, white patches on the gills, excessive mucus secretion, caudal peduncle lesions, tail rot, cutaneous haemorrhages, scale loss, ulceration in the abraded area, skin discoloration, skin peeling, fluid-filled peritoneal cavity and intestine, pale and discoloured kidney and liver, and haemorrhagic internal organs. About 80-100% mortalities were noted in challenged *C. auratus* at 10^6 - 10^8 CFU/fish levels. The LD_{50} dose of *Flavobacterium* sp. KG3 was determined to be 2.5×10^5 CFU/fish. The abraded fish recorded 60% mortality at a challenge dose of 6.0×10^6 cells/mL.

Histopathology

The gills of experimentally challenged *C. auratus* exhibited extensive necrosis, hyperplasia (Figure 1a) and inflammation of cartilaginous tissue (Figure 1b). In kidney, extensive haemorrhages, polymorphic nephritic tubules, fibrosis around the inflamed nephritic tubules, constricted nephritic tubules with vacuolated surrounding, degeneration of nephritic tubular epithelium (Figure 2a), vacuolation in haematopoietic tissue, cellular and nuclear hypertrophy, degeneration of tubular epithelium (Figure 2b), nephritic tubule inflammation, widen lumen and thickening of luminal lining (Figure 2c) and granuloma formation (Figure 2d) were observed. The challenged goldfish exhibited sarcolysis along with haemocyte infiltration (Figure 3a) and diffused muscle bundle (Figure 3b). Histopathological alterations such as melanomacrophage aggregate, necrosis and loosely packed white pulp with vacuolated cells were noted in the spleen of challenged *C. auratus* (Figure 3c).

DISCUSSION

During the surveillance work on the winter diseases in 2014 and 2015 (November-February), increasing incidences of flavobacterial infection with mild to moderate mortalities were recorded in the cultured food fish and ornamental fish species from diverse localities of West Bengal, India (4,15,17). The surveillance results established that *Flavobacterium* spp. are emerging as major fish pathogens of winter disease in freshwater aquaculture and is a major cause for concern. Therefore, further attempts were made to assess the pathogenic potential of these bacteria on cultured fish as well as the tissue level changes in the infected

fish. In the present study, cent percent mortality was observed in goldfish challenged with *Flavobacterium* sp. KG3 at a level of 10^8 CFU/fish in 24 h. About 80-90% mortalities were recorded at 10^6 - 10^7 CFU/fish within 60 h of the challenge. The LD_{50} value of *Flavobacterium* sp. KG3 was determined to be 2.5×10^5 CFU/fish, which imply that the tested bacterium was moderately virulent to *C. auratus* as per the degree of virulence (19). *Flavobacterium* sp. KG3 also resulted in 60% mortality within 5 days of challenge in abraded and bath treated *C. auratus*. These results suggested that physical or mechanical injuries may facilitate the entry of *Flavobacterium* sp. KG3 to effect significant mortalities in goldfish. In our earlier study, the same bacterium, when challenged by abrasion-bath treatment in *C. catla* fingerlings at 4.7×10^6 cells/mL, resulted in 2.8 times higher mortality (56.7%) within 5 days of challenge than the unchallenged and abraded catla (20%) at 24-28 °C (17). These results corroborate the earlier observations made in zebrafish *Danio rerio* with abrasion-bath experiments using *F. columnare* (5), which recorded LD_{50} values of 1.1×10^6 - 1.1×10^7 CFU/mL. However, their challenge experiments with the same strain by intramuscular and intraperitoneal injection yielded much higher LD_{50} values (3.2×10^{10} CFU/fish and 4.2×10^{10} CFU/fish, respectively) than those observed in the present study by i/p route (2.5×10^5 CFU/fish). The results implied that *Flavobacterium* spp. have varying degrees of pathogenic potential on fish with challenge routes. Irrespective of the challenge route, our strain *Flavobacterium* sp. KG3 demonstrated its virulence and pathogenic potential. Contrarily, in suspension challenge study with *F. branchiophilum* in intact *L. rohita* fingerlings mortalities ranging from nil at 10^6 - 10^8 CFU/mL to 80% at 10^{12} CFU/mL were recorded (12). The results of the present study, thus, demonstrated that with skin injuries or breach of the immune barrier the *Flavobacterium* sp. KG3 can cause mortalities in fish as with other potential bacterial pathogens. The above results, thus, indicated that *Flavobacterium* sp. KG3 may be involved in the pathogenesis of goldfish in union with adverse environmental conditions and/or injuries. The haemorrhagic lesions on the internal organs of challenged goldfish were indicative of septicemia condition.

The histopathological observations in *Flavobacterium* sp. KG3 challenged goldfish also demonstrated extensive damages in the gills and internal organs. The gills of experimentally challenged *C. auratus* had extensive necrosis and hyperplasia, inflammation of cartilaginous tissue similar to those reported earlier (18,20,21). In our earlier study, cartilaginous tissue inflammation, mucus secretion, loss of gill lamellar structure, necrosis of gill filament with the associated reduction in the number of lamellae per filament, obliteration of interlamellar water channels, and fusion of lamellae were noted in naturally infected *C. catla* exhibiting gill rot, from where the *Flavobacterium* sp. KG3 was isolated (17). Likewise, columnaris diseased fish recorded congestion of blood vessels, dissociation of surface epithelium of the lamellae from the capillary bed probably due to the accumulation of oedematous fluid and scattered areas of haemorrhage with globose masses of blood cells (20). On the other hand, proliferative branchitis consisting of epithelial hyperplasia of the gill lamellae and interlamellar space resulting in lamellar fusion was observed during the flavobacterial infection (21). In the present study, the gills of *C. auratus* exhibited extensive necrosis with other cellular and tissue level alterations, which are histologically similar to those of several earlier studies (13,14,20,22).

In the kidney, severe nephritic cellular and tissue level alterations including granuloma formation were observed, thus indicating the systemic pathogenic potential of *Flavobacterium* sp. KG3. Similarly, granulomatous lesions in the kidney of *Mollisia spheonops* affected with *Flavobacterium* sp. was reported (23). Contrarily, in an experimental study with *F. columnare*, kidney lesions were localized in the glomerulus (20). In support of the present study, renal tubular degeneration and proteinaceous casts in the tubular lumen, focal renal tubular degeneration, and necrosis, melanomacrophage, hyperplasia, tubular degenerative changes, necrosis and edema within the renal interstitium of the kidney of an injected (i/p) fish with *Flavobacterium* were noted (21). According to Ferguson (22), inspection of the Hematoxylin and Eosin or Giemsa stained sections from the affected tissue can reveal typical long and slender bacterial cells, where they appear bluish-purple or blue, respectively. But, the present experiment and also in earlier studies (20,24), the stained tissue sections failed to reveal bacteria in the internal organs. Also, the challenged *C. auratus* exhibited diffused muscle bundle, sarcolysis along with haemocyte infiltration; whereas several earlier studies noted severe haemorrhagic muscle with degeneration of the myofibers (21), and degeneration of muscle fibers and necrosis (20).

Histopathological alterations such as necrosis, loosely packed white pulp with vacuolated cells, melanomacrophage aggregate and necrosis were noted in the spleen of challenged *C. auratus*. The presence of melanomacrophage aggregate in the spleen was indicative of immune reactions to ward-off the bacterial challenge. An early study observed no bacilli or microscopic lesions in the liver, spleen and anterior kidney of *Flavobacterium* infected koi carp (25). However, in the present

study, microscopic lesions were noted in all internal organs, which confirm that *Flavobacterium* sp. KG3 has the ability to cause systemic infection. In agreement with this study, a friable and swollen spleen or splenomegaly in columnaris-like diseased fish was reported (21).

CONCLUSION

The histopathological observations of the present study, in general, presented the fact that *Flavobacterium* sp. KG3 can induce pathogenesis both externally and internally in experimentally challenged fish. Since the flavobacterial infection is severe during the winter months appropriate preventive or stress mitigation measures such as avoidance of crowding, use of probiotics, immunomodulators, vaccines or development of disease-resistant stocks, etc are recommended to manage the flavobacterial infection or winter diseases.

Ethics Committee Approval: All the experimental protocols with goldfish as an experimental animal were approved by the Ethical Committee, WBUAFS, Kolkata, India.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - T.J.A.; Design - T.J.A.; Supervision - T.J.A., S.B.; Resource - T.J.A., S.S., H.A.; Materials - S.S., H.A.; Data Collection and/or Processing - S.S., S.B., H.A.; Analysis and/or Interpretation - S.S., S.B., H.A., T.J.A.; Literature Search - S.S., S.B.; Writing - S.S., T.J.A.; Critical Reviews - T.J.A.

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

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The Antiproliferative Activity of *Colchicum umbrosum* Plant Extract and Paclitaxel on C-4 I and Vero Cells

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ABSTRACT

Objective: This study was conducted to determine the antiproliferative effects of paclitaxel (PAC) and *Colchicum umbrosum* plant extract on C-4 I and Vero cells. In addition, the apoptotic effects of plant extract on these cells were compared.

Materials and Methods: C-4 I and Vero cells were treated with PAC and corm extract of *C. umbrosum* for 24 h. The antiproliferative effects of 0.01, 0.1, and 1 mg/mL of plant extract and 7.5, 15, and 30 nM of PAC and the half maximal inhibitory concentration (IC50) values were determined by the MTT method. Morphological changes occurring in the C-4 I and Vero cells were observed under phase-contrast and fluorescence microscopes using DAPI staining.

Results: The IC50 values were found to be 0.01 mg/mL in C-4 I cells and 1 mg/mL in Vero cells for the plant extract and 15 nM in C-4 I and Vero cells for PAC. The apoptotic index (AI) values of the experimental groups of C-4 I and Vero cells were significantly increased compared to those of the control group ($p < 0.01$) after treatment with IC50 concentration of the plant extract. The AI values of the plant extract treatment in C-4 I cells were higher than those in Vero cells at 24 and 48 h.

Conclusion: Treatment with the IC50 concentration of the plant extract induced apoptotic cell death in C-4 I cells. It can worth be considered as a novel promising candidate for the treatment of cancer.

Keywords: C-4 I, Vero, *Colchicum umbrosum*, paclitaxel, cancer

INTRODUCTION

Oncogenes, known as cancer genes, and tumor suppressor genes encode the components of the pathways that regulate the proliferative and social behavior of cells, and a molecular change in these can lead to the development of cancer (1). Natural products are considered a promising approach to cancer therapy with less toxic yet more potent effects. (2-4). Many chemotherapeutic agents used in clinics are produced from plant and analogs of the compound of it contains. Nowadays, the compounds obtained from plants in natural products (secondary metabolites) which have anticancer activity, cause apoptosis and regulate angiogenesis. Additionally, the metastasis potential of tumor cells have been identified (5-8). Some of the compounds and

their derivatives such as paclitaxel, vinblastine, vincristine, etoposide, artemisinin, cannabinoid, resveratrol, camptothecin, derivatives of colchicine (Democolcine, N-deacetylcolchicine, and trimethyl colchicinic acid) are secondary metabolites used as chemotherapeutics in the clinic (3,6,8).

PAC, derived from the bark of *Taxus brevifolia* and having an antimitotic effect, has been used as a drug in some cancer therapy such as cancer of the uterus and breast cancer (9). The drug affects the normal functioning of the interphase as well as the microtubules in the mitosis. This effect of PAC has been shown to result from apoptosis of the resulting cell death (10).



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Colchicine is another secondary metabolite isolated from the species of *Colchicum* genus. Colchicine, an antimetabolic agent that prevents the accumulation of microtubules, has antiproliferative effects due to its ability to inhibit cell division in the metaphase (11-13). Thus, the cells' death was actualized by using the apoptotic pathways (14).

Commonly, cancer cells are more susceptible to apoptosis themselves and the understanding of the molecular pathways that regulate apoptosis is beneficial in exploring the chemotherapeutic targets of cancer, which in turn will allow the discovery and development of new drugs. Recent studies have revealed that there are both natural and synthetic anticancer drugs mediating through apoptosis induction in order to prevent tumor expression, progression, and the emergence of cellular inflammatory responses other than necrosis (15-18). Apoptosis is a genetically controlled death which is found in both physiological and pathological conditions which are not needed or are dangerous to the organism. Given that apoptotic cells can be morphologically identified and counted by using phase contrast and fluorescence microscopy, they have used in the determination of the apoptotic index in tumor growths as a prognostic marker (19).

In this study, the antiproliferative effects of different doses of *Colchicum umbrosum* extract alone and PAC alone treated in C-4 I (cancer-acquired cell) and Vero cells (normal cell) were investigated using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] method for 24 hours. Additionally, its IC₅₀ values were determined. In this context, the aim was to determine the different antiproliferative effects of the chemotherapy drug and the plant extract on both the cancer and normal cells. Morphological changes in cells were shown with phase contrast and fluorescence microscopes with 4'-6-diamidino-2-phenylindole (DAPI). Furthermore, the apoptosis rate was determined using the apoptotic index parameter.

MATERIALS AND METHODS

Colchicum umbrosum Steven (corm, ISTE: 85333) plant was collected from Bolu-Abant in Turkey on 25.06.2008. The plant extract was percolated with pure methanol and lyophilized at Istanbul University Faculty of Pharmacy's Department of Pharmacognosy. Plant extract and PAC (Ebetaksel®, Bristol Myers Squibb Co.) were prepared at different doses and applied on normal and cancer cells used in this study.

Cell Lines

Vero (normal cell line, green monkey kidney) and C-4 I (cancer cell line, human cervix carcinoma) cells were obtained from the ATCC (Vero: ATCC® CCL-81; C-4 I: ATCC® CRL-1594) used in the experiments. 10% Fetal Bovine Serum (FBS, Gibco Lab.) was added to the media used for these cells (Vero cells were MEM medium, C-4 I cells were in Waymouth MB medium) and pH was adjusted to 7.2 with 4.4% NaHCO₃. Cells were incubated in sterile 25 cm² flasks with regular passages twice a week at 37 °C in a 5 % CO₂ and 95 % air mixture (20,21).

Passage Processes of Cells

The Vero and C-4 I cells were passaged when they became monolayers in the culture containers where they grew. Cells were washed with Hank's Buffered Salt Solution and incubated with 2 ml of 0.25% trypsin for 3 minutes to release them from the surface. The suspended cells were taken to the centrifuge tube and 4 ml of the medium was added for the trypsin inactivation. Then, it was centrifuged at 500 xg for 5 minutes. After this process, the supernatant part of the precipitated cells was removed (20,21). The cells were placed in a 96 well plate for the antiproliferative experiment at 30,000 cells/well; and to determine the apoptotic index, the cells were seeded in 12 well plate at 200,000 cells per well. After the cultivation of the cells was carried out, incubation was carried out at pH 7.2 for 24 hours at 37 °C in an incubator containing 5% CO₂ and 95% air (21).

Preparation of Concentration of *Colchicum umbrosum* Extract

The concentration of extracts prepared from the corm of *C. umbrosum* plant used in the experiments was determined with reference to the previous studies (13,22). The dried extract was dissolved in a sterile, serum-free medium to prepare the stock solution. Then, 3 different concentrations of the plant extract (0.01, 0.1 and 1 mg/mL) were prepared by diluting the stock solution with a medium containing 10% FBS.

Preparation of Doses of PAC

The doses of PAC used were prepared taking into account the clinically applied doses. 30 mg/5 ml of sterile stock solution was diluted with PAC (Ebetaksel®, Bristol Myers Squibb Co.) serum-free medium, resulting in an intermediate stock of 7.5 µM. Three different doses (7.5 nM, 15 nM, and 30 nM) to be used in the experiments were prepared sterily diluting them with the prepared intermediate-staged medium containing 10% FBS and then applied to the cells (23).

Treatment of Plant Extract and PAC in Cells

3 different concentrations of the plant extracts alone and 3 different doses of PAC alone were applied to the cells for 24 hours. In the control group, the extract-free medium was used. At the end of these periods, the agents were removed and the analyses were carried out in accordance with the determined parameters.

Determination of Cell Viability

3 different concentrations of the plant extract and 3 different doses of PAC were treated in the cells and the IC₅₀ values were determined for use in the experiments. At the end of the specified experiment period, the cells were removed from the 96-well plate and 40 µL MTT (5mg/mL) was added to the wells and incubated for 4 hours in a shaking incubator. To dissolve the formazan crystals which formed after 4 hours of incubation, 160 µL of dimethyl sulfoxide was added to each well and left in the shaker incubator for 1 hour. Then, the absorbance at 570 nm wavelength reference of 690 nm wavelength was determined using ELISA spectrometry (µQuant, Bio-Tek Instruments INC.) In addition, the samples were photographed using phase-contrast microscopy to determine any morphological changes in the experimental groups (23).

Determination of Apoptosis

At the end of the application times of *C. umbrosum* concentration on C-4 I and Vero cells, 1 mL was taken from the cell suspension in the experimental groups and collected in Eppendorf tubes and centrifuged at 1500 rpm for 5 minutes at 4°C. The supernatant was discharged and 200 µL of the methanol: FTS (1:1) mixture was added. The supernatant was removed by centrifuge at 1500 rpm for 5 minutes. The pellet was resuspended in 200 µL of pure methanol and stored at +4°C. For cleaning the slides, a mixture of ethanol: HCl (1: 1) was used. The slides were left in this mixture for 24 hours, washed in distilled water and stored at +4°C for use. The cells, stored at +4°C in Eppendorf tubes, were centrifuged again for 5 minutes at 1500 rpm, suspended in methanol and then dropped on cleaned slides and dried. The preparations prepared in this way were stained with DAPI for 20 minutes in a non-irradiated container at 37°C in a shaker incubator. After washing in PBS for 20 minutes, the coverslip was closed and examined under a fluorescence microscope. The Apoptotic index (AI) was determined by scoring both the normal nucleus and apoptotic nucleus in the preparations prepared according to the experimental groups. About 100 cells were counted in at least 30 sites for each experimental group, and the percentages of apoptotic cells to total cells for each area was calculated. The AI values were determined by averaging the percentages of the 30 areas for each experimental group (23).

Statistical Analysis

The arithmetic mean of the reproduction speed, the AI data determined by dose applied in all experimental groups, and the standard deviations were calculated. The results were examined to see if they showed any difference at a statistically significant level compared to the control group. For this purpose, the one-way ANOVA test was applied to all the values determined from the experimental groups. The significance of the groups compared to the control group was assessed using the Dunnett's test. A value of $p < 0.05$ was considered statistically significant (GraphPad Prism Version 6.00, GraphPad Software, San Diego California USA).

RESULTS

In this study, the antiproliferative effects of PAC and *C. umbrosum* plant extract in Vero and C-4 I cells were evaluated using the MTT method.

3 different doses of PAC (7.5 nM, 15 nM and 30 nM) and 3 different concentrations of plant extract (1, 0.1 and 0.01 mg/mL) were applied to the cells for 24 hours. In the statistical analysis, it was found that there was a significant difference between the control group and the experimental group where 1 mg/mL concentration of plant extract was applied to the Vero cells ($p < 0.05$) (Figure 1). For C-4 I cells, it was determined that there was a significant difference between the experimental group and control group with 3 different concentrations of plant extract ($p < 0.05$) (Figure 2). In addition, a significant difference was found between the experimental groups and the control group

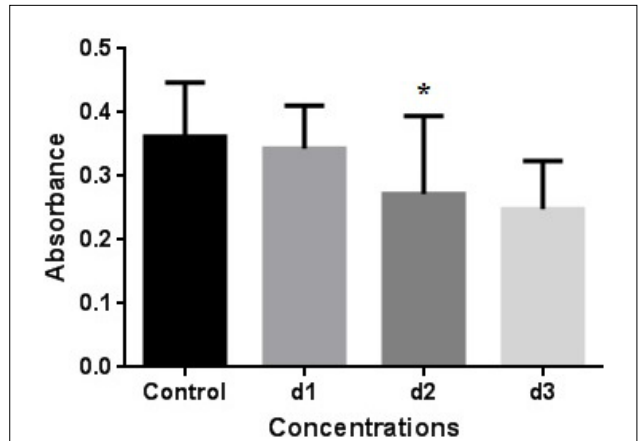


Figure 1. Measured absorbance values of Vero cells treated with different concentrations of *C. umbrosum* plant extract (d1: 0.01 mg/mL, d2: 0.1 mg/mL, d3: 1 mg/mL) for 24 h. *Significantly different from control ($p < 0.05$).

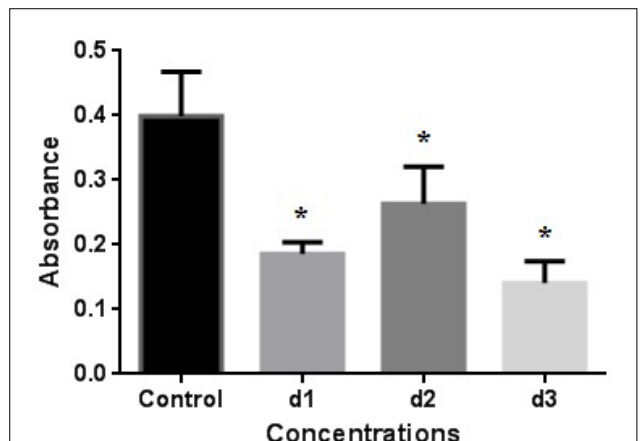


Figure 2. Measured absorbance values of C-4 I cells treated with 3 different concentrations of *C. umbrosum* plant extract (d1: 0.01 mg/mL, d2: 0.1 mg/mL, d3: 1 mg/mL) for 24 h. *Significantly different from control ($p < 0.05$).

where PAC was applied at 15 nM and 30 nM doses to the Vero cells ($p < 0.05$) (Figure 3). It was found that there was a significant difference between all the experimental groups and the control group in which 3 different doses of PAC were applied to the C-4 I cells ($p < 0.05$) (Figure 4).

The viability % values were determined as 94% for 0.01 mg/mL, 75% for 0.1 mg/mL, 68% for 1 mg/mL concentration after the treatment of plant extract in Vero cells for 24 hours (Figure 5). The IC50 concentration was determined as 1 mg/mL in the Vero cells.

The viability % values were determined as 46% for 0.01 mg/mL, 65% for 0.1 mg/mL, 35% for 1 mg/mL concentration after the treatment of plant extract in C-4 I cells for 24 hours (Figure 5). The IC50 concentration was found as 0.01 mg/mL in C-4 I cells.

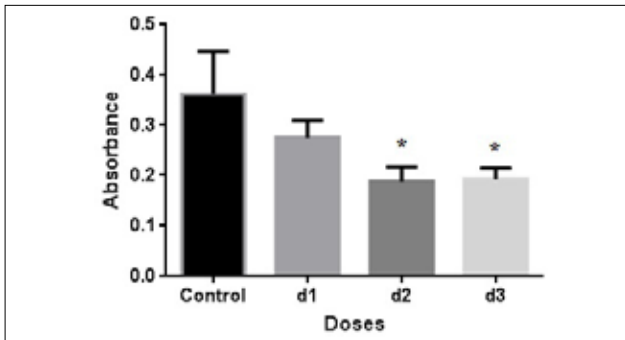


Figure 3. Measured absorbance values of Vero cells treated with 3 different doses of PAC (d1: 7.5 nM, d2: 15 nM, d3: 30 nM) for 24 h. *Significantly different from control ($p < 0.05$).

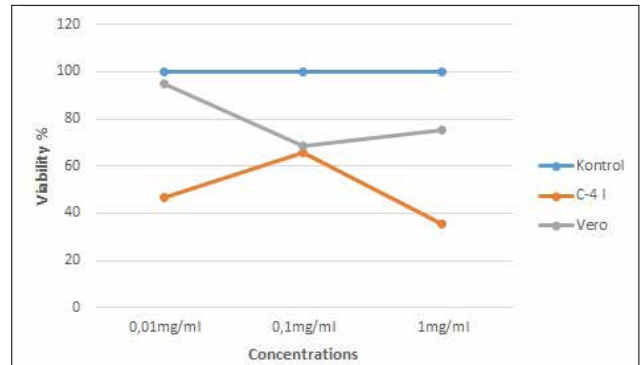


Figure 5. Viability % values of the cells treated with 3 different concentrations of *C. umbrosum* plant extract for 24 h.

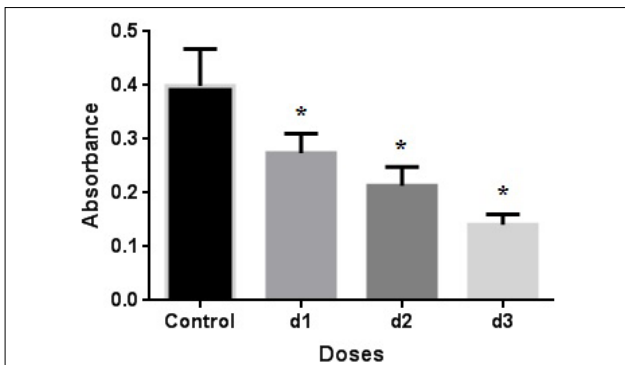


Figure 4. Measured absorbance values of C-4 I cells treated with 3 different doses of PAC (d1: 7.5 nM, d2: 15 nM, d3: 30 nM) for 24 h. *Significantly different from control ($p < 0.05$).

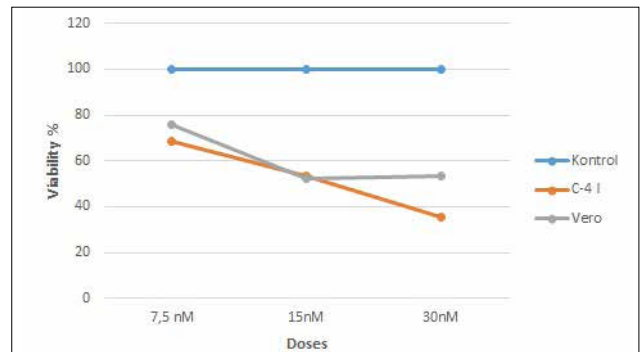


Figure 6. Viability % values of the cells treated with 3 different doses of PAC for 24 h.

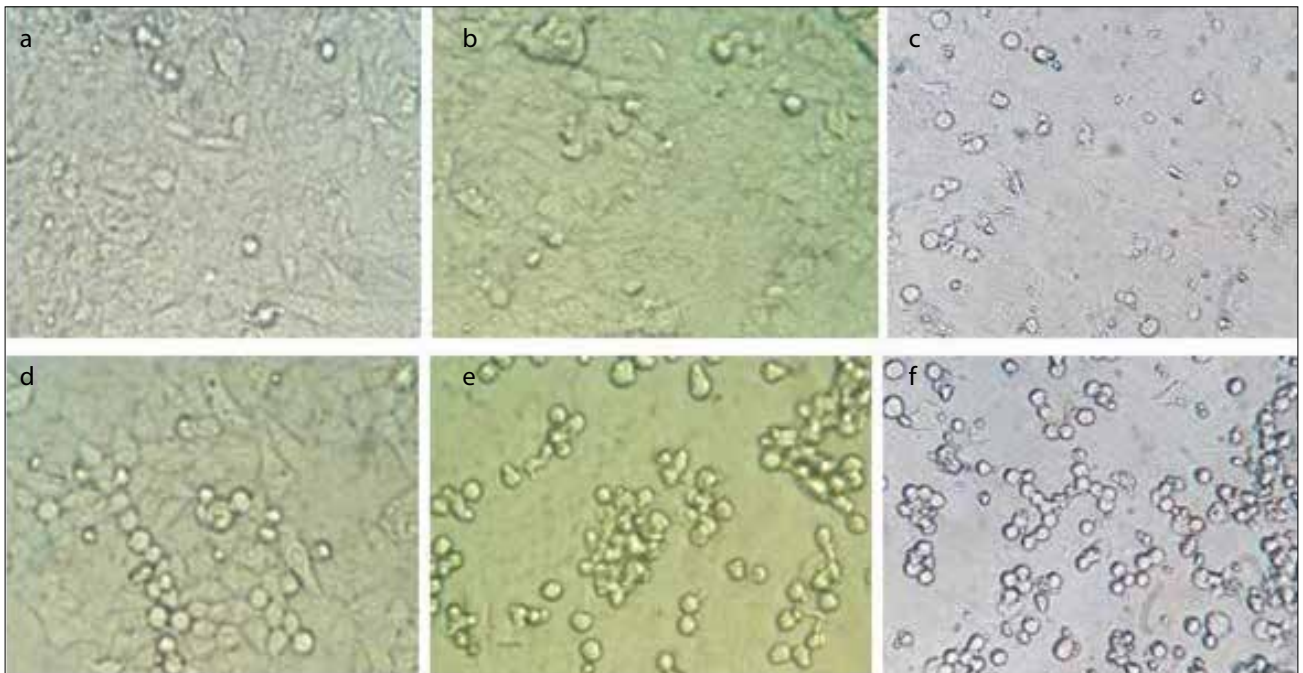


Figure 7. a-f. Phase-contrast microscopy image (x100) treated with IC50 values of plant extracts and PAC for 24 h. a) Vero, Control; b) Vero, *C. umbrosum*; c) Vero, PAC; d) C-4 I, Control; e) C-4 I, *C. umbrosum*; f) C-4 I, PAC.

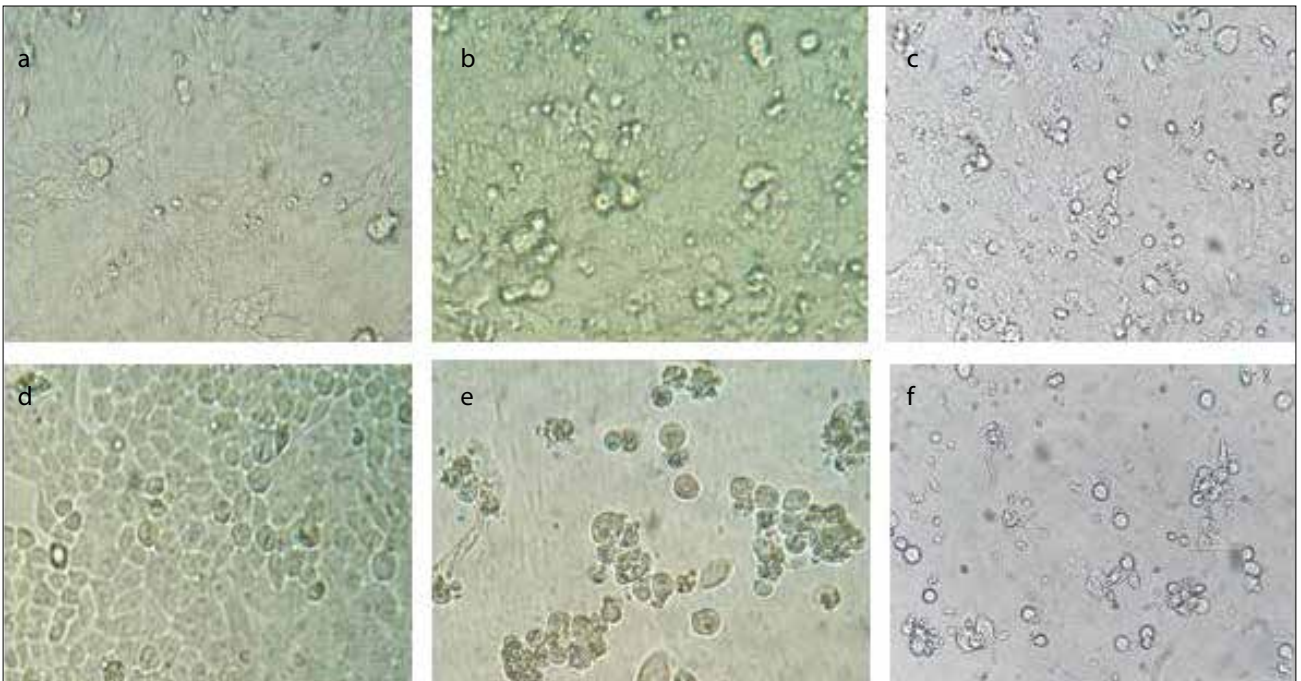


Figure 8. a-f. Phase-contrast microscope image (x100) treated with IC50 values of plant extracts and of PAC for 48 h. a) Vero, Control; b) Vero, *C. umbrosum*; c) Vero, PAC; d) C-4 I, Control; e) C-4 I, *C. umbrosum*, f) C-4 I, PAC.

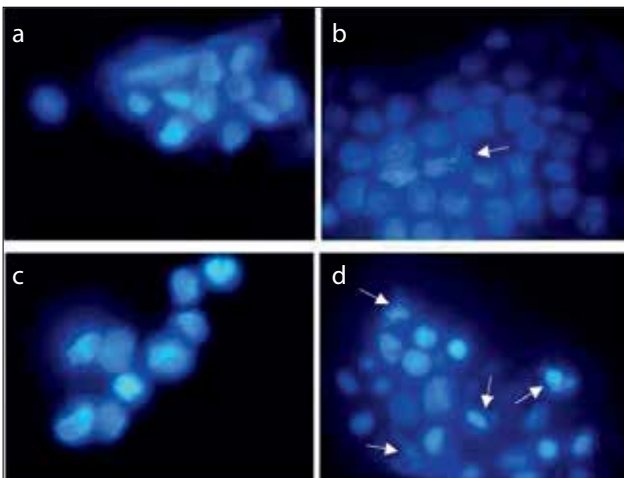


Figure 9. a-d. Fluorescence microscope image (x100) with DAPI of Vero and C-4 I cells for 24 h. a) Vero, Control; b) Vero, experiment group; c) C-4 I, control; d) C-4 I, experiment group. (→: apoptotic cells).

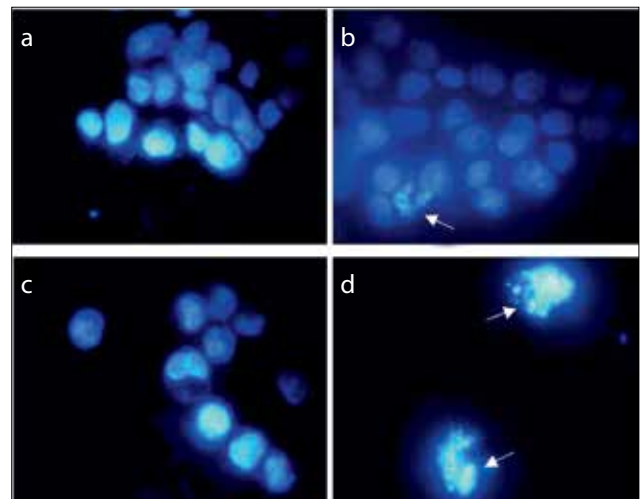


Figure 10. a-d. Fluorescence microscope image (x100) with DAPI of Vero and C-4 I cells for 48 h. a) Vero, Control; b) Vero, experiment group; c) C-4 I, control; d) C-4 I, experiment group. (→: apoptotic cells).

The viability % values were determined as 76% for 7.5 nM, 52% for 15 nM, 53% for 30 nM doses after the treatment of PAC in Vero cells for 24 hours (Figure 6). The IC50 dose was determined as 15nM.

The viability % values were determined as 68% for 7.5 nM, 53% for 15 nM, 35% for 30 nM doses after the treatment of PAC in C-4 I cells for 24 hours (Figure 6). The IC50 dose was determined as 15nM.

Any morphological changes in the cells were shown using phase-contrast microscopy after the treatment of IC50 concentration of plant extracts and IC50 doses of PAC for 24 and 48 hours (Figures 7 and 8).

Nuclear condensation and apoptotic bodies formed during apoptosis induced by the plant extract were shown with a fluorescence microscope at x100 magnification (Figures 9 and 10).

The AI values were determined from the preparations prepared after administration of the IC50 concentration of the plant extract for 24 and 48 hours on the cells. This was done using fluorescence microscopy with DAPI, by counting at least 30 apoptotic and normal cells per 100 cells for each test group.

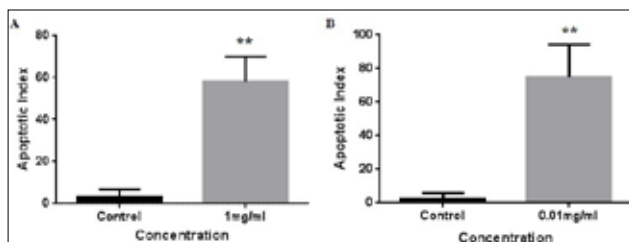


Figure 11. a, b. Apoptotic index values (a) Vero; b) C-4 I) of cells treated with IC50 concentration of the plant extract for 24 h. **Significantly different from control ($p < 0.01$).

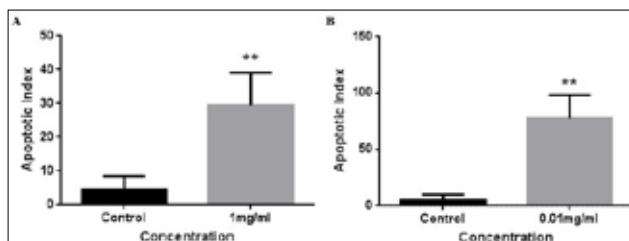


Figure 12. a, b. Apoptotic index values (a) Vero; b) C-4 I) of cells treated with IC50 concentration of the plant extract for 48 h. **Significantly different from control ($p < 0.01$).

At the end of 24 hours, the AI values were found as 3% for the control group and 58% for the experiment group of Vero cell; 2% for the control group and 74% for the experiment group of C-4 I cell (Figure 11). It was determined that the difference between the experimental groups and the control groups was significant ($p < 0.01$).

At the end of 48 hours, AI values were found as 4% for the control group and 29% for the experiment group of Vero cell; 4% for the control group and 77% for the experimental group of C-4 I cell (Figure 12). The difference between the experimental groups and the control groups was significant ($p < 0.01$).

DISCUSSION

In this study, it has been shown that at 1, 0.1, and 0.01 mg/mL concentration of *C. umbrosum* plant extract applied on Vero and C-4 I cells during 24 hours caused a further decrease in the viability of cancer cells compared to normal cells. It has been determined that the IC50 concentration, which caused the death of approximately half of the cells of the plant extract at 1 mg/mL in Vero cells and 0.01 mg/mL in C-4 I cells, between these three concentration, is the optimal concentration to be used at other stages of the study. According to this results, the plant extract more antiproliferative in C-4 I cells according to Vero cells.

MTT, known as one of the viability tests, is a rapid method used to determine the antiproliferative effect of agents on cells (23).

It was shown that PAC, commonly used in cancer chemotherapy, applied to Vero and C-4 I cells over 24 hours, causes a decrease in the viability of both normal cells and cancer cells at 7.5 nM, 15 nM and 30 nM doses. Of these three doses, the 15 nM

dose appears to be the optimal dose to be used in other stages of the study (called the IC50 dose), which causes the death of half of both cells.

In a study conducted, the antiproliferative effect of PAC on HeLa cells was determined. The optimal dose determination was found to be nM dose of the IC50 value of PAC and related viability values (23).

The *Colchicum* genus of the Liliaceae (Colchicaceae) family has been used for therapeutic purposes since ancient times. As a result of studies on *Colchicum* species, the substance named Colchicine was found in the structure. Colchicine shows anticancer properties because it can stop cell division in the metaphase. However, although it is known that colchicine, which is highly toxic at the desired doses for cancer treatment, has a potent antiproliferative effect, the therapeutic index is too low to be used therapeutically for this purpose. Derivatives of colchicine such as Demecolchine, N-deacetylcolchine, and trimethyl colchicinic acid, are less toxic derivatives and have been shown to be more promising in cancer treatment (13).

In one study, the chemical constituents of the different parts of *C. baytopiorum* plant extract and their antiproliferative effects in K562 and HL60 cells were reported. In this study, colchicine, 2-demethylcolchicine, 3-demethylcolchicine, 3-demethyl-N-formyl-N-deacetylcolchicine, colchifoline, 2-demethylcolchifoline, demecolchine, 2-demethyl demecolchine and 2-demethyl- -lumicolchicine 12 were isolated from all the separated parts of *C. baytopiorum*. Additionally, the antiproliferative effects of different concentrations (500, 100, 50, 5, 1, and 0.1 $\mu\text{g/mL}$) of all extracts prepared from the different parts of *C. baytopiorum* in K562 and HL60 cells were investigated. IC50 values were calculated and the results were compared with each other and with those for colchicine and demecolchine standards. According to this study, all extracts were more active than the demecolchine standard, and they also showed a similar activity to colchicine (13).

In a second report, the apoptotic effect of 0.1 mg/ml of *C. baytopiorum* plant extract on HeLa cells by using qRT-PCR was researched. According to this report, the regulatory genes' expression of apoptosis was significantly increased with the plant extract in the treatment group compared to the control group in HeLa cells (24). In other similar studies it was shown that colchicine may be more effective by interacting with other substances found in plant extracts (25-27).

Nowadays, anticancer therapies promote apoptosis using a variety of cellular signaling pathways. By elucidating the details of the apoptotic mechanism, new targets can be created in tumor treatment and it will be possible to increase the treatment efficiency. In this study, the apoptotic effect of the IC50 concentration of *C. umbrosum* plant extract in normal and cancer cells was investigated.

In attempts to improve the outcome of traditional cancer therapies, intensive investigations have been directed at various models of regulation of P53 and other key molecules involved

in DNA damage network processes. In one model; the main role of the P53 pathway in determining cell destiny has been suggested either to activate the control points in the cell cycle or facilitate repair, thereby increasing survival or, inducing apoptotic cell death (28-30).

In a cell that enters the apoptotic death process, loss of volume, loss of contact with neighboring cells, chromatin condensation and orientation to the nucleus membrane, formation of recesses in the plasma membrane, and disruption of the apoptotic body of the cell are the main causes (31).

Previous studies regarding the *C. umbrosum* extract have determined that the plant extract has apoptotic effects on HeLa cells. In this report, it was shown that 0.1, 0.5, and 1 mg/mL concentrations of the plant extract had increased compared to the control group at 24 hours ($p < 0.01$) (22).

Goldstein et al. investigated apoptosis triggered in high and low dose UV irradiated HeLa cells by accelerated microscopy. It has been reported that the apoptotic process is of a certain duration and apoptosis is a process that progresses all or nothing, regardless of the amount of UV dose (32).

The determination of AI in malignant growth is accepted as a prognostic marker (19).

In this study, the formation of recesses and apoptotic bodies in the plasma membrane, which are morphologic changes in apoptosis induced by IC₅₀ doses of C-4 I and Vero cells during 24 and 48 hour periods, were detected by fluorescence microscopy using DAPI application.

In this study, 0.01 mg/mL concentration of *C. umbrosum* plant extract caused an AI increase due to progression in C-4 I cells. It was determined that plant extract applied to Vero cells at a 1 mg/mL concentration of plant showed a time-dependent decrease in the cells. In this context, it has been shown that the plant extract used induces more cell death in cancer cells than in normal cells.

CONCLUSION

It has been shown that *C. umbrosum* plant extract applied on normal and cancer cells cause a decrease in mitochondrial dehydrogenase enzyme activity from cell kinetics parameters of cancer cells, resulting in an increase in AI. In normal cells, the cell death rate is less than in cancer cells and AI decreases with time. It was found that PAC caused more cell death in normal cells compared to the plant extract application. The strategy applied in cancer treatment is to reduce chemotherapy drugs in cancer cells while increasing survival in normal cells. In this context, it is thought that the use of plant extract in the treatment of cancer according to PAC, which is the chemotherapy drug used in treatment, is more promising.

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“Small Protected Areas” for Conservation Priorities in South Anatolia (Başkonuş Mountain-Kahramanmaraş)

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ABSTRACT

Objective: The Başkonuş Mountain (Kahramanmaraş) is located in the transition zone of Irano-Turan and Mediterranean plant geographical regions and in the southern part of the Anatolian Diagonal. A specific region in the East Mediterranean basin consists of an area that holds great biological importance and is a world hotspot. In this study, the small protected areas of the Başkonuş Mountain were determined.

Materials and Methods: A total of 17 endemic and 9 rare taxa were selected based on their IUCN statutes, altitudes, and habitats. Their GPS coordinates were loaded on a computer and digitized in a map projection system. Distributions of the selected taxa were marked on a topographic map in the scale of 1/25.000. The 26 maps were overlapped so that the areas of plant biodiversity could be observed on the map.

Results: Six small protected areas were defined on the Başkonuş Mountain. Several threats and conservation suggestions were presented in this study.

Conclusion: Local data about biodiversity sources are limited. Thus, conservation biologists and government offices should play active roles in formal educational settings and employ alternative methods to the community in Kahramanmaraş.

Keywords: Başkonuş Mountain, small protected areas, biodiversity, Turkey

INTRODUCTION

The “biodiversity hotspots” term is one of the most successful methods developed to preserve the richest and most endangered areas on the world (1). The Mediterranean Basin is one of the most important area for plant diversity (2,3). The plant diversity is excessive, with approximately 30.000 plant species with 13.000 endemics and many of the endemics plants are local (4). The flora of Turkey has approximately 10.000 vascular plants, so it is the richest flora in the temperate zone (5,6). Also, it has special habitats. But, the incomparable flora and habitats of Turkey have been threatened and have diminished rapidly over the last four decades; Turkey has 144 Important Plant Areas (7) which have contributed much to the origin of many cultivated plant species.

The aim of this study is to reveal the biodiversity of the Başkonuş Mountain and to determine alternatives for the protection of the Small Protected Areas (SPAs).

Kahramanmaraş is a region that is comprised of plains in mountains which is located in the South-East Anatolia region of Turkey (8,9) (Figure 1). The flora of Kahramanmaraş is very rich, almost 2500 taxa and many endemic species (20%) (10-16).

The main reasons for this richness are due to the transition zone of Irano-Turan and Mediterranean plant geography regions, which began in the South of the Anatolian Diagonal and extend to north-eastern Anatolia to the Mediterranean Taurus mountains, this region has been critical for plant diversification (10). The Başkonuş



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Mountain, Nature Conservation Area, and is in Kahramanmaraş province. The highest point of the examined area is Başkonuş Mountain (1775 m). The research area is located within the Mediterranean phytogeographical region. The three types vegetation basically can be observed in the area are; forest, macchie, and steppe vegetation (13-16).

MATERIALS AND METHODS

Study Area

The study area is located in Kahramanmaraş province. According to Equator 37°38'35"- 37°28' 32" north latitudes, according to Greenwich 36 028'48"- 36° 41' 54" east meridians on the topographic map with in scaled to 1/25.000 (Figure 2). This area has a surface area of 203.084 km² (118.258 km² forest, 84.826 km² opened forest) and varies in height from 345 m to the peak of the Başkonuş mountain (1779 m). The Başkonuş Mountain is studied because;

1) Some observations on the vegetation in the Başkonuş Mountain (13-16), and detailed floristic studies in this area during the Project of KANBK (Conservation for Rare and Endemic Plants of Kahramanmaraş and Ardahan was carried out in 2007-2011 and funded by BTC-UNDP (SGP) (10-12).

2) The study area is a promenade area.



Figure 1. Map of the study area; Başkonuş Mountain

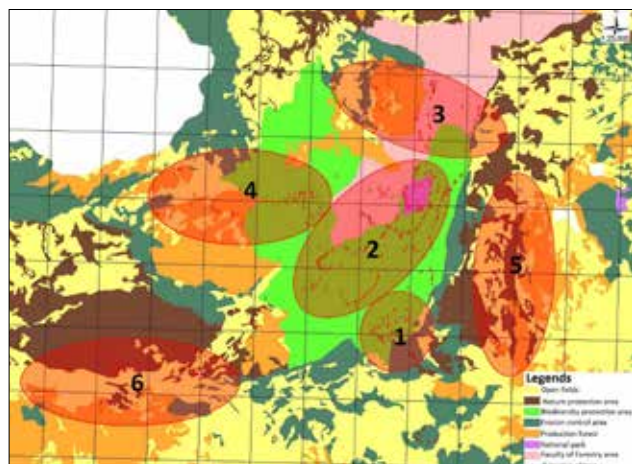


Figure 2. Small Protected Areas of Başkonuş Mountain: 1. Tower of fire observation and around, 2. Locality of Yediardic, 3. Pond and castle around, 4. Ziyaret Hill, 5. Locality of Rahmacılar, 6. Locality of Topcalı and Sersem

3) Phytogeographic location of Başkonuş Mountain and Anatolian Diagonal (17).

Selection of Taxa

The Flora of Başkonuş Mountain consists of 66 endemic taxa (18). In the study, 17 endemic and 9 rare taxa (17 threatened endemic taxa and 9 national threatened rare taxa) were selected according to their IUCN (International Union for Conservation of Nature) statutes (19), altitudes and habitats as listed in Table 1. Endemic which includes endemic taxa found solely in Kahramanmaraş and in neighbouring areas (e.g., Ahır Mountain, Binboğa Mountain, Çimen Mountain) (13-16).

Field Investigations

The field work was carried out in 2011 and information of various data such as GPS (Global Positioning System), altitude, habitat, density of plant population and selected taxa for each 10 km, were recorded. Some newly recorded plants for the Flora of Başkonuş Mountain were collected during the field studies; the plants were identified and kept in ISTE (The Herbarium of Istanbul University Faculty of Pharmacy).

In this study, 13 habitats types were defined and presented in a simplified list as Table 2, where the endemics and rare taxa of the Başkonuş Mountain grow. The numbers of endemic and rare plants in the habitats were presented by a chart. Additionally altitudinal distributions of the selected taxa were showed with a chart. During the field work, 610 GPS were loaded on the computer and digitized in Map Projection System; ED 1950 UTM Zone 37 N which is a GIS program (Geographic Information Systems). Distributions of the selected taxa were marked on the topographic map in scaled to 1/25.000. Afterwards the 26 maps were overlapped so that the areas of plant biodiversity were occurred on the last map could be seen on Figure 3. As the results of there, six SPAs were defined in Başkonuş Mountain.

RESULTS

The results identified six sites which were based on one or more of the following criteria: 1. Presence of rare species, 2. Presence of botanical diversity, 3. Presence of threatened habitats. The six determined SPAs were described using altitudes, size (km²), habitats, selected taxa and other plants. Descriptions of the SPAs were presented in Table 3.

Habitat Types and Altitude Differentiation

Habitat types were grouped as rocks, grasslands, meadows, shrubs, woods, cultivated area, waste and then 13 habitats types were defined. *Pinus brutia*, *P. nigra*, *Cedrus libani*, *Abies cilicica*, *Taxus baccata*, *Alnus glutinosa* are the main taxa of forest vegetation. Additionally *Quercus coccifera*, *Styrax officinalis*, *Arbutus unedo*, *Pistacia terebinthus* and *Cotinus coggyria* are the main taxa of shrubby vegetation.

Some endemics and rare plants have been found in several different habitats, these being: *Lathyrus laxiflorus* subsp. *angustifolius*, *Helleborus vesicarius* –in shrubs, scree, cliffs and rocks. Some endemics have been found only in special habitats,

Table 1. List of selected taxa and their IUCN statutes (Ekim et al.2000), altitudes and habitats

Taxa	IUCN	Altitudes	Habitats
Threatened Endemic Taxa (17 taxa)			
<i>Acer monspessulanum</i> L. subsp. <i>oksalianum</i> Yalt.	VU	1000-1200	W3, R3, S2
<i>Allium glumaceum</i> Boiss. & Hausskn.	LR (cd)	1500-2200	R2
<i>Astragalus distinctissimus</i> Eig	EN	1200-1520	W1
<i>Astragalus leporinus</i> Boiss. var. <i>hirsutus</i> (Post) D.F.Chamb.	LR (lc)	600-1500	W2, C1
<i>Centaurea lycopifolia</i> Boiss. & Kotschy	LR (nt)	50-2000	R1, S1
<i>Colchicum davisii</i> C.D. Brickell	EN	1000-1950	R2, G1
<i>Crataegus aronia</i> (L.) Bosc. ex DC. var. <i>minuta</i> Browicz	LR (lc)	600-1300	G1
<i>Cyclamen pseudoibericum</i> Hildebr.	EN	550-1500	G1, W1, W2, W3
<i>Fritillaria alfredae</i> Post subsp. <i>glaucoviridis</i> (Turrill) Rix	VU	500-1600	S1
<i>Helleborus vesicarius</i> Aucher	LR (nt)	550-1300	S1, W4, G2
<i>Kitaibelia balansae</i> Boiss.	EN	1900-2000	R1, G1, C2
<i>Lathyrus laxiflorus</i> (Desf.) Kuzntze subsp. <i>angustifolius</i> (Post ex Dinsm.) P.H.Davis	VU	1200-1400	R1, S1, W3
<i>Michauxia tchihatchewii</i> Fisch. et Mey.	LR (nt)	500-1800	R1
<i>Rhamnus nitidus</i> P.H.Davis	LR (cd)	700-1300	W1
<i>Verbascum amanum</i> Boiss.	VU	400-2000	R1, S1, W1
<i>Verbascum pinetorum</i> (Boiss.) Kuntze	VU	370-1000	S1, W1
<i>Hyacinthus orientalis</i> L. subsp. <i>chionophilus</i> Wendelbo	LR (nt)	1900-2000	R1, R3, G1
National Threatened Rare Taxa, nonendemic (9 taxa)			
<i>Anemone blanda</i> Schott & Kotschy	VU	150-2600	R1, S1
<i>Allium opacum</i> Rech. fil.	EN	800-1700	R1, R2
<i>Allium dodecanasii</i> Karavokyrou & Tzanoud.	EN	400-1000	R3, W1
<i>Anacamptis pyramidalis</i> (L.) Rich.	LR (lc)	200-1500	W4, G1, C1, C2
<i>Cephalanthera rubra</i> (L.) Rich.	LR (lc)	200-1500	W1, W2, S1
<i>Comperia comperiana</i> (Steven) Asch. & Graebn.	EN	500-1600	W1, W2, W3
<i>Epipactis condensata</i> Boiss.	LR (lc)	600-1600	W1
<i>Eranthis hyemalis</i> (L.) Salisb.	VU	1300-1800	G2
<i>Lathyrus variabilis</i> (Boiss. & Ky.) Maly	VU	1000-1700	R1, S1, W3
IUCN statutes: VU: Vulnerable; EN: Endangered; LR (cd): Lower Risk/conservation dependent; LR (lc): Lower Risk/least concern; LR (nt): Lower Risk/near threatened. Grasslands and meadows (G), Shrubs (S), Woods (W), Cultivated and waste (C)			

these being: *Allium glumaceum*- in screes, *Fritillaria alfredae* subsp. *glaucoviridis* - in shrubs.

plants of Başkonuş Mountain develop densely in 1200-1400 m and in 600-1000 m.

The relationship between the selected taxa and altitudinal zones has been reported (Figure 3). The endemics and rare

Ziyaret Hill has maximum habitats although it is the smallest SPA. Its altitude zone and the richness of the habitat provide a

Table 2. Habitat types of the selected plants from the Başkonuş Mountain

Habitats	Habitats	Number of endemics	Number of rare plants	Total	%
Rocks (R)				31	23.66
R1 High and Mid altitued cliffs and rocks	R1	12	2	14	11.69
R2 Screes	R2	10	2	12	9.16
R3 Moist rocks	R3	4	1	5	3.82
Grasslands and meadows (G)				13	9.92
G1 Calcareous rocky grasslands at mid and high altitude	G1	2	1	3	2.29
G2 Alpine grasslands, snow beds	G2	9	1	10	7.63
Shrubs (S)				10	7.63
S1 Shrubby places	S1	4	1	5	3.82
S2 River bed shrubs	S2	4	1	5	3.82
Woods (W)				61	46.56
W1 Low and mid altitude needle-leaved forest	W1	16	5	21	16.03
W2 Low and mid altitude broad-leaved forest	W2	7	2	9	6.87
W3 Low and mid altitude mixed forest	W3	12	2	14	10.69
W4 Forest edges	W4	14	3	17	12.98
Cultivated and waste (C)				16	12.21
C1 Traditionally cultivated fields	C1	6	1	7	5.34
C2 Fallows, roadsides	C2	8	1	9	6.87
TOTAL		108	23	131	100

high level biodiversity, so the smallest size SPA competes with the largest size SPA (Locality of Yediardıç) about in number of contained endemic and rare taxa.

Endemism

The flora of Başkonuş Mountain has 565 taxa of which 66 are endemics with an overall endemism of 11.68%. According distributions of the selected endemic and rare taxa, Small Protected Area 2 (Locality of Yediardıç) has the highest number of selected endemic and rare taxa (Figure 4). Firstly, the selected endemic and rare plants were found in a woody habitat (W1 and W4) with a rate of 29.01%. Secondly, they were found in a rock habitat (R1 and R3) with a rate of 20.85% (Figure 5).

Threats and Conservation State

The main factor responsible for the threats to plants in the Başkonuş Mountain is human pressure. Intensive grazing of sheep and goats in the meadows and grasslands, uncontrolled plant collection, especially some orchids (*Anacamptis pyramidalis*, *Comperia comperiana*), and working on road and forestry activities (uncontrolled tree cutting or silviculture) are the most important threats to the flora of Başkonuş Mountain.

According to IUCN categories, 7 taxa is EN (endangered), 8 taxa is VU (vulnerable), 4 taxa is LR (nt) (near threatened), 2 taxa is LR (cd) (conservation dependent) and 5 taxa is LR (lc). The endangered and vulnerable taxa are distributed in the second SPA (Figure 6). This is not surprising due to the fact that the most numerous endemic taxa observed are in this area.

The Başkonuş Mountain has various types of rock slides and screes. This habitat is a biotope of most numerous endemic geophytes (*Allium glumaceum*, *Colchicum davisii*, *Fritillaria alfredae* subsp. *glaucoviridis*, *Cyclamen pseudoibericum*, *Hyacinthus orientalis* subsp. *chionophilus*).

DISCUSSION

Başkonuş Mountain has specific habitat such as medium-height steep slopes and forest edges. This habitat is also a refuge for endemics. Habitat loss is thought to lead to endemic species extinctions. Many studies use species-area relationships and predict substantial extinctions when levels of habitat loss exceed approximately 70-80% (16).

Table 3. Descriptions of the Small Protected Areas

SPA 1: Tower of fire observation and around

Coordination:	37°33'00"N 36°35'00"E	Size:	0.5 km ²	Altitude:	1700-1800 m
Habitat types:	Cliffs and rocks, scree, calcareous rocky grasslands, alpine grasslands, snowbeds and needle leaved forest				
Threatened endemic taxa:	<i>Centaurea lycopifolia</i> , <i>Colchicum davisii</i> , <i>Crataegus aronia</i> var. <i>minuta</i> , <i>Cyclamen pseudoibericum</i> , <i>Helleborus vesicarius</i> , <i>Lathyrus laxiflorus</i> subsp. <i>angustifolius</i> , <i>Verbascum amanum</i> , <i>Verbascum pinetorum</i> ,				
Threatened rare taxa:	<i>Allium opacum</i> , <i>Anemone blanda</i>				
Threats:	Working on forest road, uncontrolled tree cutting, and plant collection.				

SPA 2: Locality of Yediardic

Coordination:	37°34'00"N 36°35.5'00"E	Size:	1 km ²	Altitude:	1400-1500 m
Habitat types:	Forest, shrubby places, scree and alpine grasslands				
Threatened endemic taxa:	<i>Acer monspessulanum</i> subsp. <i>oksalianum</i> , <i>Allium glumaceum</i> , <i>Astragalus distinctissimus</i> , <i>Astragalus leporinus</i> var. <i>hirsutus</i> , <i>Centaurea lycopifolia</i> , <i>Colchicum davisii</i> , <i>Crataegus aronia</i> var. <i>minuta</i> , <i>Cyclamen pseudoibericum</i> , <i>Fritillaria alfredae</i> subsp. <i>glaucoviridis</i> , <i>Helleborus vesicarius</i> , <i>Lathyrus laxiflorus</i> subsp. <i>angustifolius</i> , <i>Rhamnus nitidus</i> , <i>Verbascum amanum</i> , <i>Verbascum pinetorum</i> ,				
Threatened rare taxa:	<i>Anemone blanda</i> , <i>Cephalanthera rubra</i> , <i>Comperia comperiana</i> , <i>Lathyrus variabilis</i> , <i>Anacamptis pyramidalis</i> , <i>Epipactis condensata</i>				
Threats:	human pressure, uncontrolled grazing, and construction				

SPA 3: Pond and Castle Around

Coordination:	37°34.5'00"N 36°35'00"E	Size:	0.83 km ²	Altitude:	1300-1400 m
Habitat types:	Cliffs and rocks, moist rocks, river bed shrubs, forest				
Threatened endemic taxa:	<i>Acer monspessulanum</i> subsp. <i>oksalianum</i> , <i>Allium glumaceum</i> , <i>Astragalus distinctissimus</i> , <i>Centaurea lycopifolia</i> , <i>Crataegus aronia</i> var. <i>minuta</i> , <i>Cyclamen pseudoibericum</i> , <i>Helleborus vesicarius</i> , <i>Kitaibelia balansae</i> , <i>Lathyrus laxiflorus</i> subsp. <i>angustifolius</i> , <i>Verbascum amanum</i> , <i>Verbascum pinetorum</i> ,				
Threatened rare taxa:	<i>Cephalanthera rubra</i> , <i>Comperia comperiana</i> , <i>Lathyrus variabilis</i> , <i>Eranthis hyemalis</i>				
Threats:	Working on highway, uncontrolled grazing, and plant collection.				

SPA 4: Ziyaret Hill

Coordination:	37°34.5'00"N 36°35'00"E	Size:	0.4 km ²	Altitude:	1200-1300 m
Habitat types:	Cliffs and rocks, moist rocks, river bed shrubs, alpine grasslands, forest				
Threatened endemic taxa:	<i>Acer monspessulanum</i> subsp. <i>oksalianum</i> , <i>Astragalus distinctissimus</i> , <i>Centaurea lycopifolia</i> , <i>Colchicum davisii</i> , <i>Crataegus aronia</i> var. <i>minuta</i> , <i>Fritillaria alfredae</i> subsp. <i>glaucoviridis</i> , <i>Helleborus vesicarius</i> , <i>Lathyrus laxiflorus</i> subsp. <i>angustifolius</i> , <i>Rhamnus nitidus</i> , <i>Verbascum amanum</i> , <i>Verbascum pinetorum</i>				
Threatened rare taxa:	<i>Comperia comperiana</i> , <i>Lathyrus variabilis</i> , <i>Epipactis condensata</i>				
Threats:	Uncontrolled grazing and plant collection. human pressure				

SPA 5: Locality of Rahmacılar

Coordination:	37°33'00"N 36°35.5'00"E	Size	0.7 km ²	Altitude	1200-1300 m
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Table 3. Descriptions of the Small Protected Areas (Continue)

Habitat types:	Shrubby places and forest				
Threatened endemic taxa:	<i>Acer monspessulanum</i> subsp. <i>oksalianum</i> , <i>Allium glumaceum</i> , <i>Centaurea lycopifolia</i> , <i>Crataegus aronia</i> var. <i>minuta</i> , <i>Cyclamen pseudoibericum</i> , <i>Fritillaria alfredae</i> subsp. <i>glaucoviridis</i> , <i>Helleborus vesicarius</i> , <i>Hyacinthus orientalis</i> subsp. <i>chionopphilus</i> , <i>Lathyrus laxiflorus</i> subsp. <i>angustifolius</i> , <i>Rhamnus nitidus</i> , <i>Verbascum amanum</i>				
Threatened rare taxa:	<i>Anemone blanda</i> , <i>Lathyrus variabilis</i> .				
Threats:	Working on road, uncontrolled plant collection. human pressure,				
SPA 6: Locality of Topcalı and Sersem					
Coordination:	37°33'00"N 36°35.5'00"E	Size:	0.79km ²	Altitude:	1300-1400 m
Habitat types:	Shrubby places, alpine grasslands, snowbeds, screes and forest				
Threatened endemic taxa:	<i>Acer monspessulanum</i> subsp. <i>oksalianum</i> , <i>Allium glumaceum</i> , <i>Centaurea lycopifolia</i> , <i>Crataegus aronia</i> var. <i>minuta</i> , <i>Helleborus vesicarius</i> , <i>Lathyrus laxiflorus</i> subsp. <i>angustifolius</i> , <i>Michauxia tchihatchewii</i> , <i>Rhamnus nitidus</i> , <i>Verbascum amanum</i>				
Threatened rare taxa:	<i>Allium dodecanesii</i> , <i>Epipactis condensata</i> , <i>Lathyrus variabilis</i> .				
Threats:	Opening agricultural area, working on road, uncontrolled plant collection. human pressure,				

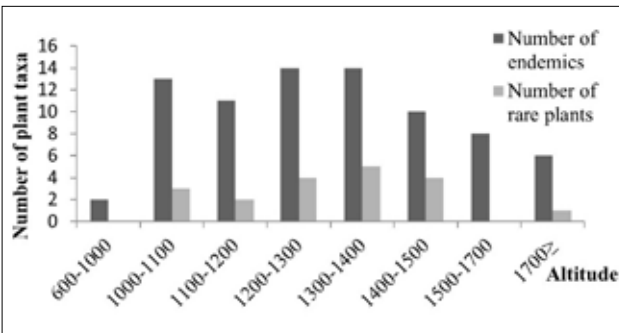


Figure 3. Altitudinal distribution of endemic and rare vascular plant taxa in Başkonuş Mountain.

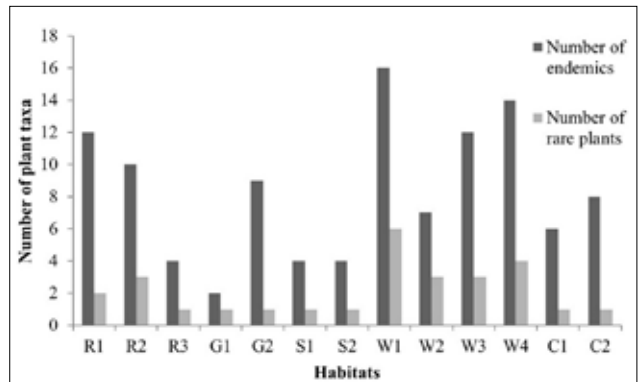


Figure 5. Number of endemic and rare vascular plant taxa according to habitats in Başkonuş Mountain.

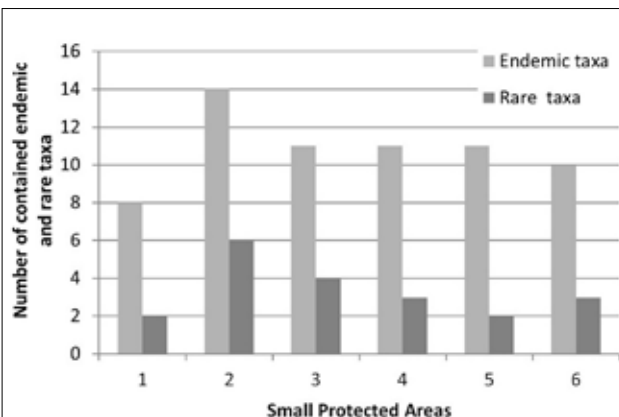


Figure 4. Numbers of endemic and rare taxa in the Small Protected Areas.

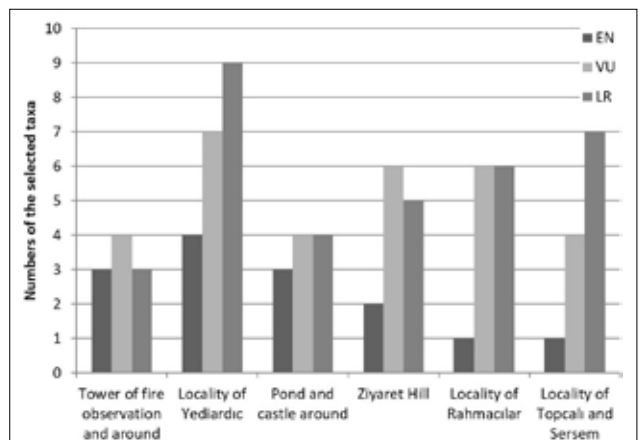


Figure 6. IUCN categories of selected taxa

Table 4. Comparison of endemism from Europe and Mediterranean areas

Region	Number of endemics	Endemism	Total number of taxa	Total area (km ²)	Endemic taxa/area rate	References
Italian Maritime Alps	107	3.40	3137	5000	0.627	Casazza et al. 2005
French Maritime Alps	108	3.80	2867	4500	0.637	Casazza et al. 2005
Maritime Alps (southwestern France)	115	3.20	3605	9500	0.379	Casazza et al. 2005
Pyrenees (France - Spain)	174	5.00	3480	94300	0.037	Villar and Garcia 1989
Andalucía Mountains (Spain)	125	36.00	347	82800	0.004	Favarger 1972
Peloponnesus (Greece)	300	12.50	2400	5425	0.442	Iatrou 1986
Başkonuş Mountain (Turkey)	66	10.62	565	203	0.325	The present study

Selected endemic taxa which include endemic plants found only in Kahramanmaraş and in neighbouring areas (e.g., Ahır, Binboğa, Çimen Mountains) (17-20). In fact most endemism grow in high stress level populations, such as screes (R2), cliffs and rocks high and mid altitude (R1) and alpine grasslands (G2) (16,21), but it should be emphasized that endemics and rare plants are found in woody habitats, especially coniferous habitats in the study.

The flora of the Başkonuş Mountain is comparable with data from the literature for mountainous areas in the Mediterranean recognized as one of the most important centers of endemism on Earth (22,23). Besides, the relationship is considered biodiversity, habitats and endemic plants is remarkable compared to other Mediterranean hotspots (24-26). For example, although the Başkonuş Mountain has a smaller area than the Alps and the Pyrenees, its endemism rate is higher than them. Additionally, the ratio of endemic species per unit area is higher than Pyrenees and the Andalucía Mountains could be seen on Table 4.

Ecologists recognize that the Earth's biota is now experiencing the sixth great extinction wave (27). The conversion of native forest and grasslands, to plantation crops is one of the largest threats to terrestrial biodiversity and a key of the global extinction crisis (28). Additionally, intensive grazing causes transformation of the vegetation in scrub communities and in the woods (21).

While people have become more aware of environmental problems recently, most do not get actively involved in movements that support a more liveable future (3). Many people reach limited data about environmental from sources that may be reached, especially the media. Thus ecologists should be more active in educational settings and alternative methods of biodiversity in the community of Kahramanmaraş. In 2010, the tenth meeting of the Conference of Parties to the Convention on Biological Diversity led to the adoption of a global strategic plan for biodiversity for the period of 2011-2020. The EU follows a similar policy. The new Biodiversity Strategy purposes to stop the loss of biodiver-

sity and the degeneracy of ecosystem services in the EU by 2020 (29,30). The strategy should be carried out in Turkey.

An agreement among politicians and managers of the local administrations and parks (City Councils, Government and National Parks) is necessary in Kahramanmaraş and Turkey.

Major threats to biodiversity include human-associated factors, such as habitat destruction and degradation, over profiteering, invasive species, incidental mortality, pollution, climate change diseases and human population growth (31).

CONCLUSION

Six SPAs were defined in Başkonuş Mountain. Some threats and conservation suggestions were presented in this study. Local people reach limited information about biodiversity issues from sources that may be biased. Thus conservation biologists and government offices should be much more active in formal educational settings and via alternative methods to a diversity of audiences in Kahramanmaraş.

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