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Research Article

Investigation of Sterol structures and biological activities in Cochineal and *Hibiscus sabdariffa* extracts

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Abstract: In the future, it is necessary to discover natural resources with no or less toxicity and side effects instead of synthetic drugs. Therefore, it is crucial to recognize, isolate, measure, and reveal the biological activities of substances in the structure of natural resources. In this study, the two extracts prepared from the plant (*Hibiscus sabdariffa)* and the insect (*Dactylopius coccus)* were examined. Sampling of the released substances was performed using Gas-Chromatography-Mass Spectrometry (GC-MS). In addition, viability, apoptosis, and oxidative stress of the derivatized samples were determined. Due to the biological differences between the samples, the chemical structures observed in the GC-MS analysis were not the same. It was observed that stigmasterol and its derivatives were found in the pomegranate sample, whereas naphthol and its derivatives were more abundant in the Cochineal insect sample. The cell viability increased with increasing concentrations of stigmasterol, which is present in large amounts in the structure of the pomegranate flower. However, the cell viability decreased with the Cochineal insect sample. Apoptosis and oxidative stress test results were also found to be different and contrasted for both samples. Therefore, the present study presents a new, natural, and different source that can be used as an antiviral and anticancer agent.

1. INTRODUCTION

Natural substances come from three different sources, namely plants, animals, and minerals. Dyes obtained from plants are particularly environmentally friendly and biodegradable (Geetha, 2013; Saxena, 2014; Kumar, 2020). Phytochemicals, natural, biologically active chemical derivatives found in plants, can be divided into three basic categories: The first category is terpenoids, which contain aliphatic alkenes and have cyclic structures. The second category includes flavonoids, which mostly contain an aromatic benzene ring and its derivatives. The third category comprises alkaloids which share structural similarities with flavonoids (Belay, 2014; Ogunyemi, 2020). Another natural source of matter is insects, as there are many organic

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substances in the structure of insects. Similar to flavonoids in plants, insects, such as kermes, cochineal, and lac insects, contain dyes in vibrant colors, particularly red (Singh, 2017). Especially in the textile field in Europe, the red color of the American Cochineal (*Dactylopius coccus*) is proposed to be superior to other insect reds and is preferred. The dye obtained is known as carmine in the form of carminic acid and is preferred due to its relatively lower cost compared to the kermes insect dye (Gonzalez, 2002; Deveoglu, 2020; Baaka, 2022). Various methods have been tested to dissolve the dye and to observe the dye content and the performance of the solvent (Borges, 2012; Dapson, 2007; Gabrielli, 2018; Nallar, 2021; Serrano, 2011; Yousaf, 2018).

Furthermore, studies have been conducted to determine the antioxidant activity of carminic acid (CA). CA exhibits a crucial antiradical activity like that of known antioxidants such as ascorbic acid quercetin and Trolox (Gonzalez, 2010). Excessive fructose intake poses a risk in kidney patients. In this case, according to the results of a study investigating the positive effect of carminic acid, CA significantly decreases inflammation and oxidative stress response in the kidneys of mice through regulating Nuclear factor kappa B (NF-κB) and Nuclear factor erythroid2-related factor 2 (Nrf-2) signaling pathways. Carminic acid supplementation protects against inflammation and oxidative stress by inducing Nrf-2 signaling (Li, 2021). A study was conducted to increase the role of natural hydroxyanthraquinones in oxidative stress in comparison with the cytotoxic effect of carminic acid (Nemeikate-Ceniene, 2002). Another investigation revealed that carminic acid has free radical scavenging activity, providing a food additive (Guo, 2010). Apoptosis, or programmed cell death, is a well-known phenomenon in many cellular systems and determines cell biological activity. Apoptosis is characterized by several changes in the cell, including the degradation of nuclear chromatin into internucleosomal fragments, and cell membrane degradation, presumably by the activation of an endogenous endonuclease (Obeng, 2020).

Colorectal cancer, a type of colon cancer formed by the growth of tissues in these parts, is more common in developing countries, and the death rate is the fourth among other cancer types (Marmol, 2017). Some compounds used in cancer treatment have been obtained from some naturally occurring organisms because they have been used in traditional treatments for many years (Esmeeta, 2022). Emodin (6-methyl-1,3,8-trihydroxyanthraquinone), isolated from the *Rheum palmatum* plant, is an active ingredient and has been widely used in traditional treatments in China (Chun-Guang, 2010; Srinivas, 2003). It is one of the best-known remedies in Chinese herbal medicine and is used for the soothing treatment of constipation, gastrointestinal bleeding, and ulcers.

Therefore, this study aimed to investigate the biological activities by determining and comparing the chemical contents of cochineal and roselle extracts obtained from two different natural sources.

2. MATERIAL and METHODS

Dried cochineal insects were obtained from a Natural Dye company in İstanbul. Other chemicals and solutions used were of analytical grade and locally manufactured.

2.1. Sample 1 Preparation

Dried Roselle (*Hibiscus sabdariffa*) obtained from a market in the Talas region of Kayseri province was pounded in a mortar. A 5 g sample was added to 100 mL of distilled water (dH_2O), and it was boiled for 30-35 minutes. It was kept covered at room temperature and then filtered the next day. The water in the filtrate was poured into an Eppendorf tube to evaporate, and the remaining solid was used for derivatization.

2.2. Sample 2 Preparation

The cochineal was bought from a company that sells natural dyes in İstanbul and dried in an oven at 60 degrees (NUVE 500) for 6 hours. After grinding it in a hand mill, 5 g was placed into a beaker with precision weighing. 100 mL of dH_2O was added, boiled for 30 minutes, and kept covered overnight. The next day, it was filtered with Whatman paper. The water in the filtrate was poured into an Eppendorf tube to evaporate and the remaining solid was used for derivatization.

2.3. Derivatization of Samples

0.5 g was taken from each of the solids separated from Sample 1 and Sample 2. After dissolving it with methyl alcohol, derivatization with *N*, *O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was applied for 4 hours at 100°C. After the derivatization process, the sample was filtered and was ready for injection and the analysis was started.

2.4. Cell Culture

DLD-1 colorectal carcinoma cells were used for this study. The cells were cultured in Dulbeccos's Modified Eagle's Medium (DMEM) with 10% Fetal Bovine Serum (FBS), 1% penicillin-streptomycin, 1% L-glutamine, and in a humidified 37°C 5% CO_2 incubator. When the cells reached 85-90% confluence, they were detached from the flasks by using a trypsin-EDTA solution. For adequate cell numbers, the cells were cultured in the same conditions.

2.5. MTT Assay

For the MTT assay, $3x10^5$ cells were seeded in 96 well plates for test cell proliferation. After 24 hours, both Cochineal extracts were added to the cells as 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78 and 0.39 µL. After 48 hours 0.5 mg/mL of the last concentration of MTT reagent was added to the wells and 3 hours later all the well contents were aspirated and 100 µL of Dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The purple color was formed by dissolving the formazan crystals and was read by using an ELISA reader at 560nm (Promega, Glomax, USA).

2.6. Apoptosis and ROS Activity Assays

For Apoptosis and ROS Activity Assays, $4.5x10^5$ cells were seeded in the 6 well plates. After 24 hours, both Cochineal extracts were added to the cells as 200 µL and 25 µL (correlated with 12.5µL and 1.56µL). Annexin V and Oxidative Stress assays (Luminex, USA) were performed according to the manufacturer's instructions. The Annexin V kit presents the percentage of live cells: early, late, and total apoptotic cells. The Oxidative stress assay presents ROS (+) and ROS (-) cell percentages.

2.7. Immunocytochemistry

For the immunocytochemistry (ICC) staining, 5 x105 cells were seeded on the sterile 1 cm^2 glass microscope slides in 24 well plates, and after 24 hours both Cochineal extracts were added to the cells at 12.5 and 1.56 µL. After 48 hours cells were fixed by 4% paraformaldehyde for 12 min. The cells were then rinsed 3x5 times and mounted with propidium iodide (PI) and 4',6-diamidio-2-phenylindole (DAPI) containing mounting medium for 15 minutes. The cells were washed again and were imagined by using a fluorescence microscope (Nikon, Ti-Eclipse).

2. 8. GC-MS Analysis

GC-MS analysis of the sample 1 and sample 2 extracts was performed on a SHIMADZU QP2010 ULTRA GC System fitted with a Rtx-5MS capillary column (30 m 0.25 mm inner diameter, 0.25 µm film thickness, max. temperature, 350 °C) coupled to a SHIMADZU GC-MS. Pure, ultra-high helium (99.99%) was used at a sustained flow rate of 1.0 mL/ min. Ion source temperatures and transfer line injection were all 290 °C. The ionizing energy was 70 eV. The electron multiplier voltage was obtained from an auto-tuning. The oven temperature was

programmed from 60°C (hold for 2 minutes) to 280°C at a rate of 3°C/min. The samples were diluted with a convenient solution (1/100, v/v) and filtered. The particle-free diluted extracts (1 µL) were aspirated into a syringe and injected into the injector at a split ratio of 50:1. All data were obtained from the full-scan mass spectra within the scan range of 40-850 amu. The percentage composition of the sample extracts was expressed as a percentage by peak area. The characterization and identification of the chemical compounds in various sample extracts were based on the GC retention time. The mass spectra were computer-matched with those of standards available in mass spectrum libraries.

2.9. Statistical Analysis

T-test was used for statistical analysis. *p* values less than 0.05 were considered statistically significant.

3. RESULTS

3.1. Cell Culture

Cells were grown in culture plates without any contamination and by keeping their epithelial-like cell morphology.

3.2. MTT Assay

Cell viability decreased when both samples were compared with the control. However, in Sample 1, cell proliferation and viability increased in the right proportion with sample concentration. In Sample 2, the viability and proliferation decreased as the sample increased. In other words, two samples showed adverse effects on cells in terms of viability and proliferation. Sample 1 showed the highest concentration and Sample 2 the lowest concentration values closest to the control, and unlike all other data, there was no statistically significant difference as they gave a value close to the control when compared to the control ($p > 0.05$), which is shown in Figures 1a and 1b.

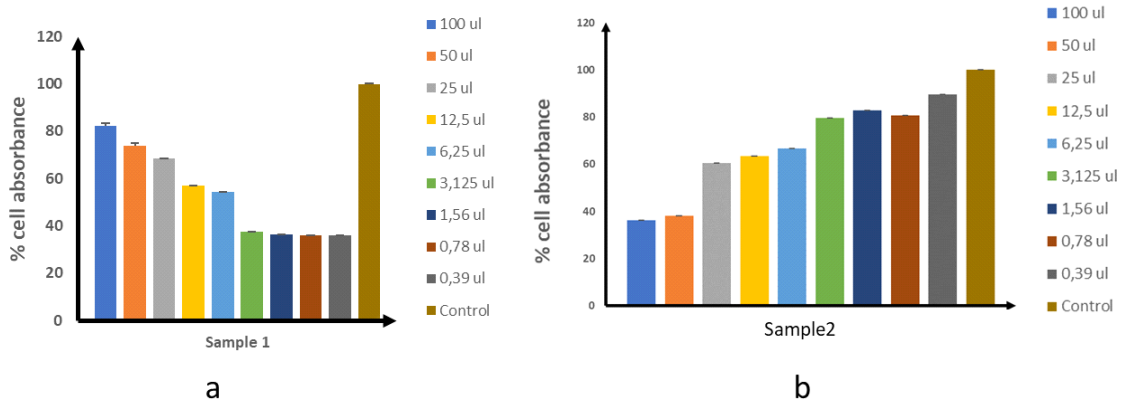

Figure 1. MTT Assay on DLD-1 cells with incubation. a) Sample 1, b) Sample 2.

3.3. Apoptosis Assay

Apoptosis was generally increased compared to the control, however, the high dose of Example 1 (200 µl) increased cell proliferation and viability, decreased late and total apoptosis and statistically decreased early apoptosis ($p < 0.003$). These are shown in the graphs in Figure 2.

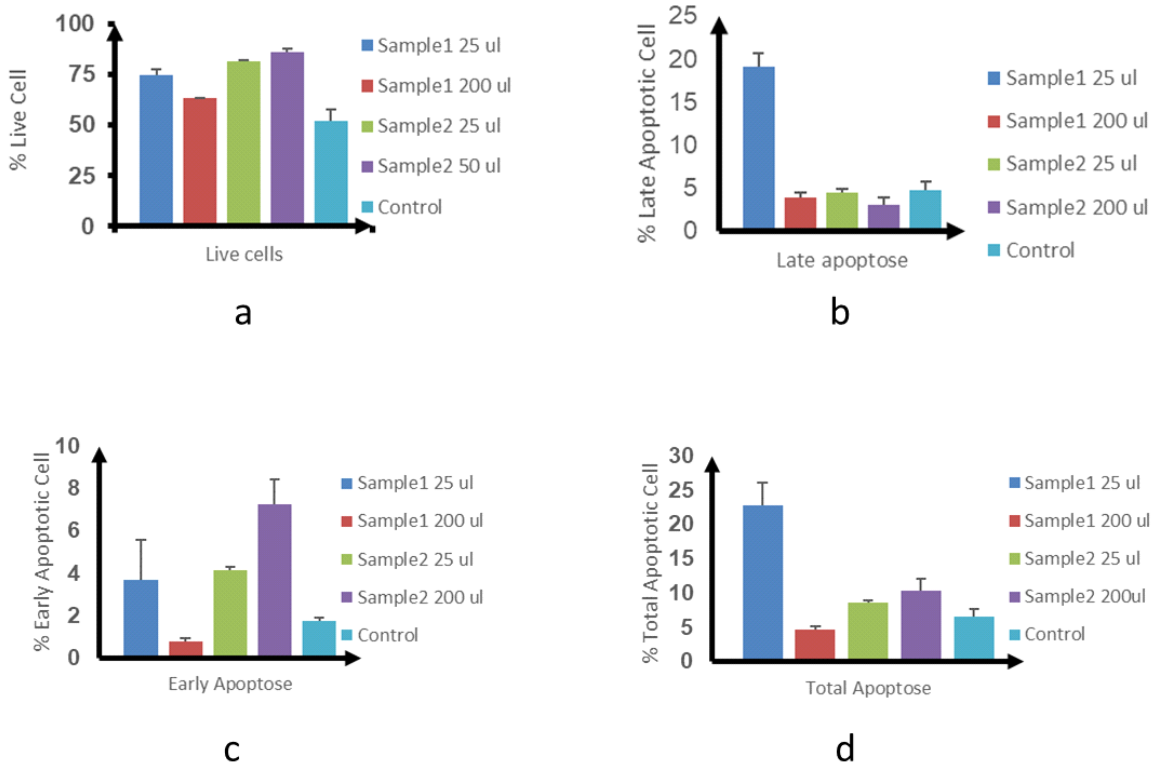

Figure 2. Demonstration of; a) % live cell profile, b) % late apoptotic cell profile, c) % early apoptotic cell profile, d) % total apoptotic cell profile.

3.4. ROS Activity Assay

As for the oxidative stress, Figure 3 shows that Sample 2 does not affect ROS activity when compared to the control, while Sample 1 has a statistically significant increase in ROS activity in cancer cells ($p_{25\mu L} < 0.002$; $p_{200\mu L} < 0.014$).

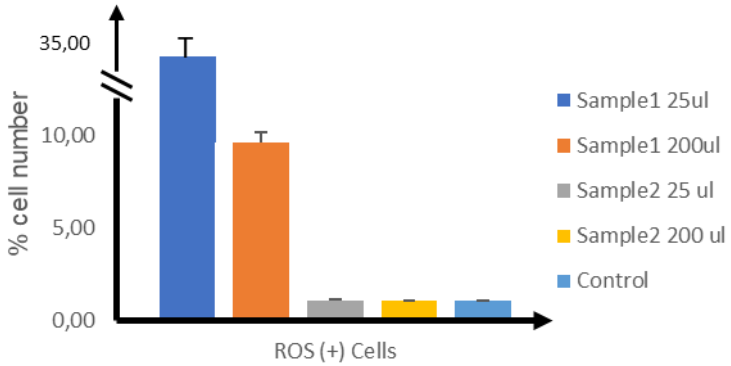

Figure 3. Demonstration of % cell number at ROS (+) cells.

3.5. Immunostaining

The immunocytochemical staining with the nuclear dye DAPI and the apoptosis indicator PI is shown in Figure 4, which shows that there were few cells stained with PI in the control group and that the cells treated with the samples and the cells treated with Cis-platin as a positive control were stained with PI like this.

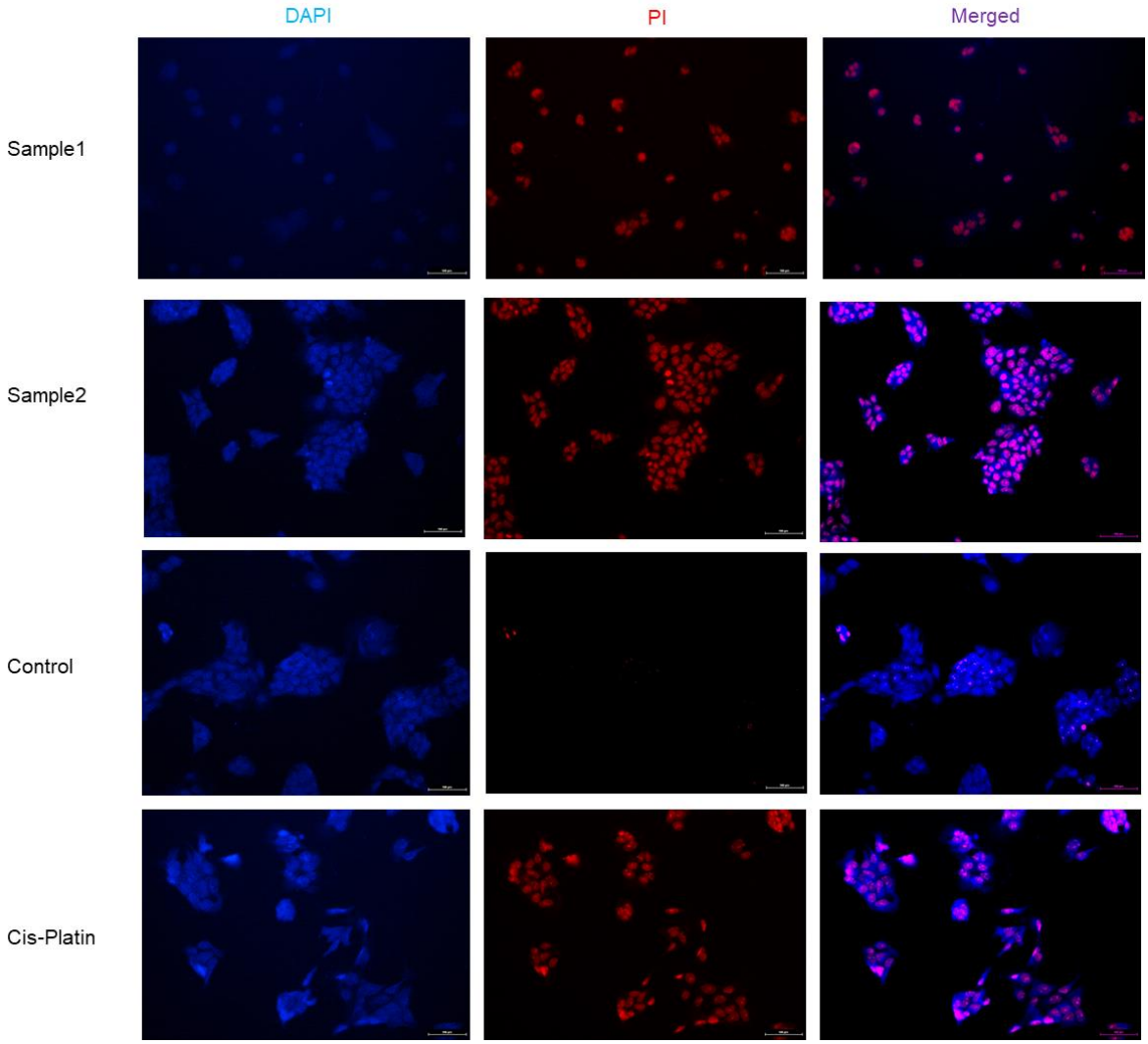

Figure 4. Experimental groups and positive and negative control groups stained with PI and DAPI (microscope magnification 10x, scale bar 100um).

3.6. GC-MS Analysis Results of Samples

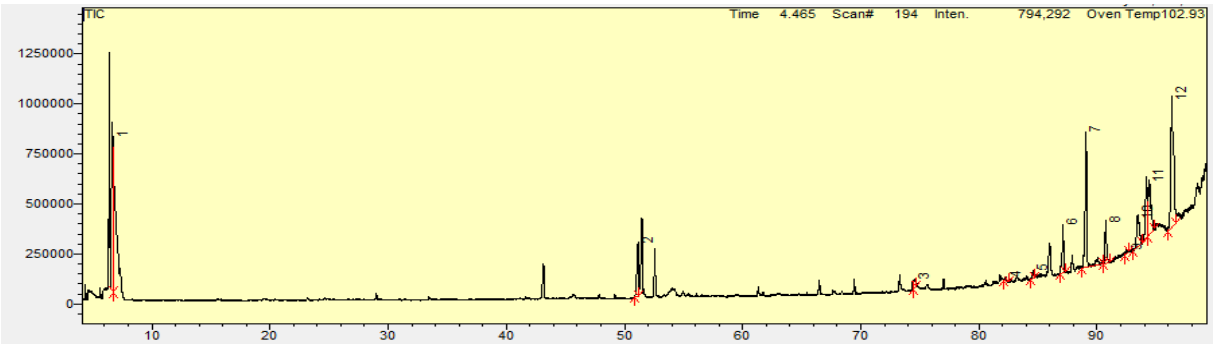

Figure 5. GC-MS spectrum of Sample 1.

The GC-MS spectrum confirmed the presence of various components with different retention times as shown in Figure 5. Large chunks of the compound are broken down into smaller compounds, which cause peaks at different m/z ratios. These mass spectra are the fingerprints of this compound that can be identified from the data library. As a result of scanning databases such as NIST11 and W9N11, the presence of 12 types of esters, alcohols, acids, ketones, and

an especially high percentage of steroidal and steroidal esters with high molecular weight were obtained and are shown in Table 1.

Table 1. GC-MS analysis results of sample 1.

No	Compound Names	% Area	Ret. Index	Molecular Weight	Molecular Formula
1	Methanesulfonamide, N-[4-(2-thienylcarbonyl) phenyl]-	6.38	2422	492.6	$C_{25}H_{20}N_2O_3S_3$
2	Benzoic acid, 2-methoxy-3-(4-methoxy-2-methyl-4-oxobutyl)-6-methyl	7.37	2076	294	$C_{16}H_{22}O_5$
3	Carbamic acid, N-[1-(3-acetylaminophenyl) ethylidenamino]-, ethyl ester	1.17	2363	263	$C_{13}H_{17}N_3O_3$
4	Cholest-5-en-3-ol (3.beta.)-, trifluoroacetate	0.66	2570	482	$C_{29}H_{45}F_3O_2$
5	Stigmastan-3-ol, 5-chloro-, acetate, (3.beta.,5.alpha.)-	0.96	2993	492	$C_{31}H_{53}ClO_2$
6	Stigmasta-5,22-dien-3-ol, acetate, (3.beta.,22Z)-	6.57	2879	454	$C_{31}H_{50}O_2$
7	Stigmast-5-en-3-ol, oleate	19.84	4469	678	$C_{47}H_{82}O_2$
8	dl-.alpha.-Tocopherol	5.80	4149	430	$C_{29}H_{50}O_2$
9	Stigmasta-5,22-diene, 3-methoxy-, (3.beta.,22E)-	0.60	2688	426	$C_{30}H_{50}O$
10	Ergost-5-en-3-ol, (3.beta.,24R)- (CAS)	7.33	2632	400	$C_{28}H_{48}O$
11	Stigmasterol	12.25	2739	412	$C_{29}H_{48}O$
12	Gamma-Sitosterol	31.07	2731	414	$C_{29}H_{50}O$

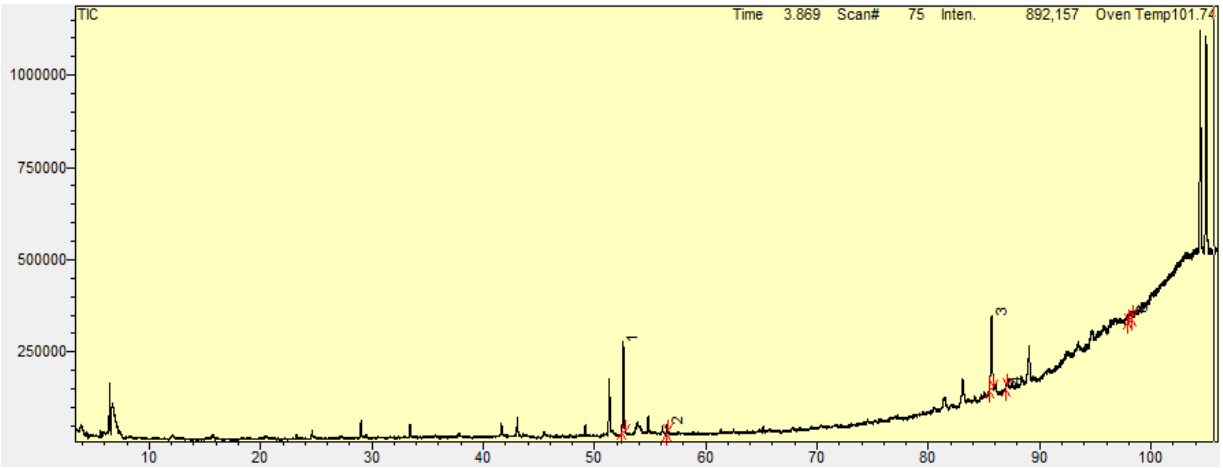

Figure 6. GC-MS spectrum of Sample 2.

The GC-MS spectrum of Sample 2 was shown in Figure 6. The presence of 6 different molecules such as amino esters, phosphonic alcohols, and naphthyls was obtained and they are shown in Table 2.

Table 2. GC-MS analysis results of Sample 2.

No	Compound Names	% Area	Molecular Weight	Molecular Formula
1	N-1-ol-8-dıphenylphosphınyl (Naphthol)	42.32	328	$C_{20}H_{23}O_2P$
2	6-Amino-5-cyano-4-isobutyl-2-phenyl-4H-pyran-3-carboxylic acid ethyl ester	2.70	326.4	$C_{19}H_{22}N_2O_3$
3	2-(1-Hydroxycyclohexylmethyl)-2'-sulphanylmethyl-1,1'-binaphthyl (Naphthyl)	49.95	412	$C_{28}H_{28}OS$
4	3-(1,2,2,6,6-Pentamethyl-1,2,3,6-tetrahydro-4-pyridinyl)-2-(2-methyl-2-aminopropyl)-1-methylindole	1.71	353	$C_{23}H_{35}N_3$
5	11-HYDROXY-3-HYDROXYMETHYLACRONINE	1.63	353	$C_{20}H_{19}NO_5$
6	1H-Pyrazole-1-acetic acid, 3-methyl-5-[[(2-oxo-2H-1-benzopyran-3-yl) carbonyl]amino]-, ethyl ester	1.69	355	$C_{18}H_{17}N_3O_5$

4. DISCUSSION and CONCLUSION

In this study, two different samples of plant origin and animal origin are discussed. The same procedures and analyses were applied to their extracts. For the determination of their biological activities, cell viability by MTT test, apoptotic cell profiles, and oxidative stress status were determined. Since the biomaterial samples used in the study contain different chemical structures, different analysis results were obtained even though the same procedures were applied to analyze the samples. Thus, the biological activity studies of the two samples were found with different contents and contrasts. In the relevant literature, the GC-MS analysis and derivatization of two samples using the method followed in this study have not been performed previously. After derivatization, red dyes were obtained with naphthol and naphthyl structures in the cochineal. The stigmasterol and sitosterol structures obtained from *Hibiscus sabdariffa* had a light color and less flavonoid content.

There have been many studies on the HPLC analysis of dried cochineal. In an article by Serrano *et al.* (2011), 7 cochineal species and 63 historical cochineal insect specimens were analyzed using the two methods. The results, mild extraction methods, and HPLC-DAD/MSn analysis produced distinctive profiles, that, in combination with a PCA reference database, provide a powerful tool for the identification of red insect dyes. New hydrophobic compounds of carminic acid, mainly used as a food dye, have been synthesized and concluded in polymeric hydrophobic matrices. Additionally, the computational investigations allowed the rationalizing of experimental data.

In a study, the degradation status in the chromatin structure was based on the results of apoptosis to explain the binding (Telford, 1992). It can be thought that substances that prevent apoptosis in cancer cells interact with the chromatin structure or DNA structure in the cell nucleus, which was observed in the immunohistochemical staining study in our specific with the growth and shape change in nuclei stained with DAPI dye. Although it is thought that the DNA in the nuclei of cancer cells incubated with Cochineal extract interacts with the extract, DNA binding and cleavage studies are required to clarify. However, substances that increase apoptosis may have therapeutic effects that support a decrease in the number of cancerous cells. It may be recommended to carry out other studies to support the use of cochineal extract as an anticancer agent. This is the first study using cochineal and *Hibiscus extracts* on DLD-1 colorectal cancer cells to our knowledge while there are many studies about Hibiscus sabdariffa extract mostly on other types of cancer (Sunkara, 2015; Adeyemi, 2014; Nguyen, 2019) as well as studies of this extract with HT-29 colon cancer cells (Czerwonka, 2017; Hsu, 2017; Kim, 2005; Li, 2013; Mata, 2016; Rouhollahi, 2015). However, there is no study of cochineal extract on DLD-1 colorectal

cancer cells. Therefore, this study fills this gap in the literature and presents a new, natural, and different source that can be used as an anticancer agent.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Dilek Bahar: Investigation, Resources, Visualization, Software, Formal Analysis, and Writing - original draft. **Nilgün Kuşçulu**: Resources, Visualization **Mehmet Çadır**: Resources, Visualization

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