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ORIGINAL ARTICLE / ÖZGÜN MAKALE

CISTUS CRETICUS L.: ANTIOBESITY, ANTIMICROBIAL AND ANTIBIOFILM PROPERTIES

CİSTUS CRETİCUS L.: ANTİOBEZİTE, ANTİMİKROBİYAL VE ANTİBİYOFİLM ÖZELLİKLERİ

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ABSTRACT

Objective: *Cistus creticus L. is widespread in the coastal regions of Türkiye. In this study, we investigated the cytotoxic, antiobesity, antimicrobial and antibiofilm properties as well as the total phenolic and flavonoid content of both aqueous and ethanolic extracts in vitro.*
Material and Method: *Two different extracts were prepared from the flowering aerial parts of Cistus creticus using ethanol and water. The total phenolic content and total flavonoids were determined by the Folin-Ciocalteu method and the aluminum chloride colorimetry, respectively. The effect of extracts on the cell viability of 3T3-L1 was determined by methyl thiazole tetrazolium (MTT), and the evaluation of differentiation and the effects of the plant extracts on lipid accumulation in 3T3-L1 adipocytes was performed by Oil-Red O staining. In addition, MIC values and antibiofilm activities were also investigated.*
Result and Discussion: *The total phenol content of the EtOH and water extract was determined to be 134.2849 mg GAE/g and 96.1803 mg GAE/g, respectively. The total flavonoids in the water and EtOH extracts were found to be 33.1942 mgQE/g and 22.8338 mgQE/g, respectively. The lowest MIC values were determined for the strains Bacillus subtilis DSM 1971, Bacillus licheniformis DSM 13 and Bacillus amyloliquefaciens DSM 7, while the highest MIC concentration was found for the strains Escherichia coli and Eenterococcus gallinarum. The MIC/16 concentration of Pseudomonas aeruginosa ATCC 27853 and Escherichia coli ATCC 25922 also proved to be effective in inhibiting biofilm formation. We observed that noticeable but not strong effects on lipid accumulation were observed in 3T3-L1 adipocytes treated with EtOH extract.*
Keywords: *Antibiofilm property, antimicrobial activity, antiobesity activity, Cistus creticus, total flavonoid content, total phenol content*

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ÖZ

Amaç: *Cistus creticus L. Türkiye'nin kıyı bölgelerinde yaygın olarak bulunur. Bu çalışmada, sulu ve etanollü ekstrelerin sitotoksik, antiobezite, antimikrobiyal ve antibiyofilm özelliklerinin yanı sıra toplam fenolik ve flavonoit içeriği in vitro olarak araştırılmıştır.*

Gereç ve Yöntem: *Cistus creticus'un çiçekli toprak üstü kısımlarından hareketle etanol ve su kullanılarak iki farklı ekstre hazırlanmıştır. Toplam fenolik içerik ve toplam flavonoitler sırasıyla Folin-Ciocalteu ve alüminyum klorür kolorimetrik yöntemleriyle belirlenmiştir. Cistus creticus'un toprak üstü kısımlarının etanollü ve sulu ekstrelerinin 3T3-L1 hücre canlılığı üzerindeki etkisi metil tiyazol tetrazolyum (MTT) ile belirlenmiş ve bitki ekstrelerinin 3T3-L1 adipositlerinde farklılaşma ve lipit birikimi üzerindeki etkileri Yağ-Kırmızı O Boyama ile değerlendirilmiştir. Ayrıca, MIC değerleri ve antibiyofilm aktiviteleri de incelenmiştir.*

Sonuç ve Tartışma: *EtOH ve sulu ekstrelerin toplam fenol içeriği sırasıyla 134.2849 mg GAE/g ve 96,1803 mg GAE/g olarak belirlenmiştir. Su ve EtOH ekstrelerindeki toplam flavonoitlerin sırasıyla 33.1942 mgKE/g ve 22.8338 mgKE/g olduğu bulunmuştur. En düşük MİK değerleri Bacillus subtilis DSM 1971, Bacillus licheniformis DSM 13 ve Bacillus amyloliquefaciens DSM 7 suşları için belirlenirken, en yüksek MİK konsantrasyonu Escherichia coli ve Enteroccus gallinarum suşları için bulunmuştur. Pseudomonas aeruginosa ATCC 27853 ve Escherichia coli ATCC 25922'nin MIC/16 konsantrasyonunun da biyofilm oluşumunu engellemede etkili olduğu kanıtlanmıştır. EtOH ekstresi ile muamele edilen 3T3-L1 adipositlerinde lipid birikimi üzerinde belirgin ancak güçlü olmayan etkiler gözlemledik.*

Anahtar Kelimeler: *Antibiofilm özellik, antimikrobiyal aktivite, antiobezite aktivite, Cistus creticus, total fenol içeriği, toplam flavonoit içeriği*

INTRODUCTION

The genus *Cistus* (known as rockrose), a member of the *Cistaceae* family, is native to Türkiye. Among them, *Cistus creticus* L. is widespread in the coastal regions of Türkiye [1]. *Cistus* species are used in Turkish folk medicine to treat rheumatism, stomach pain, hemorrhoids, inflammation of the urinary tract and diabetes mellitus [2,3]. They have been also employed in traditional folk medicine for their anti-inflammatory, antiulcerogenic, wound healing, antibacterial, cytotoxic and vasodilatory effects [4,5]. In Morocco, *Cistus creticus* is recognized and employed as a remedy for rheumatism [6].

The World Health Organization (WHO) defines obesity as a chronic and complicated disease characterized by an excess of stored fat, which can have a negative impact on health. It is also mentioned that obesity is not only a significant risk factor for type 2 diabetes and heart disease, bone health issues, reproductive problems, and some types of cancers but also has a negative impact on quality of life. WHO notes that antibiotic resistance and overweight/obesity are becoming increasingly serious health problems. Considering the traditional applications of different *Cistus* species in Türkiye, including their use for weight loss and antibacterial activity, the aim was to evaluate aqueous and ethanolic plant extracts concerning these two biological activities [7].

As the development of resistance to antibiotics complicates the treatment of infectious diseases, the search for new phytochemicals with potential antimicrobial and antibiofilm effects continues. In this context, the species of the genus *Cistus* L. occupy a prominent place in the literature. The species of *Cistus* L. exhibit remarkable biological activities, including antiviral, antiparasitic, antifungal and antibacterial effects [8]. Although there are many studies in the literature in which the antimicrobial activities of crude extracts of *Cistus* L. species have been tested, there are very few studies in which extracts obtained with different solvents have been comparatively tested against a large number of Gram-negative and Gram-positive strains, including multidrug-resistant strains such as methycillin-resistant *Staphylococcus aureus* ATCC 43300 and *Enterococcus faecalis* ATCC 29212.

Biofilms are microbial communities formed by microorganisms adhering to a surface and embedded in extracellular polymeric components [9]. The exopolymer matrix that encloses biofilm cells reduces the diffusion and efficacy of antimicrobial agents [10]. As multidrug resistance spreads and biofilms complicate the treatment of infectious diseases, the discovery of new antimicrobial agents that can prevent the formation of biofilms or remove existing biofilms is important. Due to their potential antimicrobial or antibiofilm properties, natural plant substances have been the subject of numerous

studies to discover novel antibiofilm agents in recent years [11].

Only a few studies in the literature evaluate the potential antibiofilm activity of *Cistus creticus.* Erdoğmuş et al. [12] emphasized in their studies that the aqueous extract of *Cistus creticus* showed remarkable biofilm activity against some important Gram-negative and Gram-positive strains. Interestingly, another study suggests that an 80% ethanol extract of *Cistus creticus* has no antibiotic effect on *Staphylococcus aureus* strains but stimulates biofilm production of some strains of *Enterococcus faecalis*, *Bacillus subtilis* and *Pseudomonas aeruginosa* [13]. Conversely, Mocan et al. found that an 80% ethanol extract of *Cistus creticus ssp. creticus* significantly reduced biofilm production of the *P. aeruginosa* PA01 strain even at sub-inhibitory concentrations [14]. There is also evidence that the extract from *C. creticus* suppresses the microbial growth of some oral pathogens and the biofilm production of *Streptococcus mutans* [15].

In this study, we investigated the cytotoxic, anti-obesity and antimicrobial properties as well as the total phenolic and flavonoid content of the aqueous and ethanolic extracts from the flowering aerial parts of *Cistus creticus in vitro*. Considering the limited number of studies on the potential antibacterial effects of *Cistus creticus*, we also wanted to evaluate the potential antibiofilm activities of the extracts comparatively.

MATERIAL AND METHOD

Plant Material

The flowering aerial parts of *Cistus criticus* were collected in May 2021 in Adatepe, Çanakkale. The voucher specimen was registered in the herbarium of the Faculty of Pharmacy of Ankara University under the number AEF 30753.

Extraction Method

10 g of the air-dried and powdered plant were weighed separately. Two different extracts were prepared from the plant material by using ethanol and water. Each sample was extracted in an ultrasonic bath (Bandelin, Sonorex, Germany) at a frequency of 35 kHz for 60 min. The ethanolic extract was filtered and evaporated to dryness. The aqueous extract was filtered and freeze-dried.

Total Phenolic Content

The total phenolic content of the plant extracts was determined using Singleton and Rossi's method [16]. In each well, 30 µL of the sample was added, along with 15 µL of Folin reagent, and left to incubate for 3 min in the dark. The wells were then filled with 5% Na_2CO_3 and distilled water, they were subjected to incubation for 60 min. The absorbance value of the samples was obtained at 725 nm. The experiments were done in triplicate. The same procedure was performed with gallic acid and the outcomes were stated as mg of gallic acid equivalent (GAE) per g of crude extract for each sample.

Total Flavonoid Content

The total flavonoid content of the plant extracts was determined according to the method of Zhishen et al. [17]. Briefly, plant extract samples, Na-acetate and distilled water were added to each well. Following 5 min of incubation, 10% aluminum chloride was added and kept in the dark for 30 min. At 425 nm, the absorbance was recorded. The tests were carried out in triplicate. The same procedure was performed with quercetin and the outcomes were stated as mg of quercetin equivalent (QE) per g of crude extract for each sample.

Antimicrobial Activity

The list of strains evaluated in the antimicrobial activity tests is shown in the Supplementary file (Table 1).

Determination of Antimicrobial Activity by Disc Diffusion Method

The microorganisms listed in Table 1 are stored with 60% glycerol at -86 °C. First, the stock cultures were inoculated onto Tryptic Soy Agar (TSA, Merck, Darmstadt, Germany) plates using the

streak plate technique and the cultures were incubated at 37 °C for 18 h. The antimicrobial activity tests were performed with these subcultures. From the cultures developed on TSA plates, individual colonies were taken from each culture under aseptic conditions using a sterile loop and inoculated onto Mueller Hinton Agar (MHA, Merck, Darmstadt, Germany) plates. After incubation at 37 ℃ for 18 h, a single colony was selected from the grown cultures and the colonies were suspended in tubes containing 5 ml of sterile saline (0.9% NaCl) and culture densities were adjusted according to the 0.5 McFarland standard. The prepared suspensions were inoculated onto the surfaces of the MHA plates using sterile swabs and allowed to dry for a while under room conditions.

To test the antimicrobial efficacy of the ethanolic and aqueous extracts, previously prepared stocks of 10 and 5 mg/ml were used. 10 µl of the extracts were dropped onto sterile 6-mm discs. As a control group, absolute ethanol, which is used in the preparation of ethanolic extracts, was dropped onto the discs. The discs were placed on MHA plates and then the plates were incubated at 37 °C for 18 h. After incubation, the diameter of the inhibition zones was measured.

Minimum Inhibition Concentration (MIC) Test

In this experiment, the MIC values for the microorganisms susceptible to the ethanolic extract of *Cistus* sp. were determined taking into account the results of the disc diffusion method. In this context, the cultures of the susceptible microorganisms and the culture suspensions were prepared as previously described. First, 100 µl of Mueller Hinton Broth (MHB) medium was added to each well of the microdilution plates using a multichannel micropipette. Then a series of dilutions with 10 mg/ml ethanolic extract were prepared using the microdilution method. The wells containing only the medium and inoculum were designed as positive control wells and the wells containing only the medium were designed as negative control wells.

The microdilution plates were incubated at 37° C for 18 h. The concentration values of the first wells, in which no microbial growth was observed at the end of the incubation, were considered as MIC values. All tests for antimicrobial activity were carried out following the recommendations of the Clinical and Laboratory Standards Institute [18].

Antibiofilm Activity of Ethanolic Extract

The strains *P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922, MRSA ATCC 43300 and *S. aureus* ATCC 25923 were used for the biofilm studies as they represent important Gram-negative and Gram-positive models. Modified media and incubation conditions were favored for each strain to promote biofilm production. In this context, TSB medium with 1% glucose was used for the *P. aeruginosa* ATCC 27853 strain, LB (Luria-Bertani) without NaCl for the *E. coli* ATCC 25922 strain and TSB medium with 3% NaCl for the MRSA ATCC 43300 and *S. aureus* ATCC 25923 strains [19,20].

In the first phase, the active cultures of all strains were prepared as described above. Single colonies were taken from the culture of each strain and inoculated into appropriate media (5 ml). Liquid cultures were incubated overnight at 37 ℃ (28 ℃ for *E. coli* ATCC 25922) to promote biofilm production [21]. At the end of incubation, the active culture of each strain was diluted 1:100 in the appropriate media. After the suspensions were prepared, 140 µl of the modified media were added to the wells of the 96-well U-bottom microtiter plates (LP Italiana, Italy). At this point, a serial dilution was performed for each strain taking into account the previously determined MIC values (2-fold, MIC, MIC/2, MIC/4, MIC/8…).

10 µl of each culture suspension was taken and inoculated into the wells of the microtiter plate. The wells containing only media and inoculum served as positive controls, and the wells containing only media served as negative controls. The plates were incubated under static conditions at 37 ℃ (28℃ for *E. coli* ATCC 25922) for 24 h. At the end of incubation, a crystal violet binding assay was conducted. The wells were emptied and washed three times with sterile physiological saline to remove planktonic cells. The plates were dried at room temperature for 10 min and then 140 µl of a 95% methanol solution was added to the wells. The plates were again incubated at room temperature for 10 min and the plate wells were emptied. After fixation, 140 µl of a 0.1% crystal violet solution was added to the wells and the plates were incubated at room temperature for 30 min. At the end of the incubation, the wells were washed with sterile distilled water. 140 µl of an acetone-ethanol solution (30:70) was added to each

well. The plates were incubated for 15 mins at room temperature and the dissolved dyes were read in a microplate reader set to 595 nm [22].

Antiobesity Activity

Cell Culture

3T3-L1 (CRL-173) preadipocytes were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The cell culture studies were based on Longo et al. [23].

The Effect of Ethanolic and Aqueous Extracts of Aerial Parts of *Cistus creticus* on Cell Viability of 3T3-L1 by Methyl Thiazole Tetrazolium (MTT)

To investigate cell viability and cytotoxicity, 3T3-L1 preadipocytes were treated with different concentrations of EtOH and aqueous extracts for 48 h and the MTT assay was performed according to the method described by Lee et al. [24]. Absorbance (Abs) in the wells was measured at 570 nm using a spectrophotometer (Uvmini-1240, Shimadzu, Kyoto, Japan) [25]. Cytotoxicity was calculated using the following formula. The half (50%) maximum inhibitory concentration (IC_{50}) of the plant extracts was calculated using a non-linear regression model.

Cell viability (%) = (Mean Abs_{570} nm value of treated cells / Mean Abs_{570} nm value of control cells) × 100.

Differentiation of 3T3-L1 Preadipocytes into Mature Adipocytes

Differentiation induction medium (DMI) was used to differentiate 3T3-L1 preadipocytes into mature adipocytes. For the experiments, 3T3-L1 preadipocytes were cultured and then differentiated into mature adipocytes in the absence or presence of EtOH or aqueous (at a concentration that maintains cell viability ≥90%) extracts of the plant for 8 days. Differentiated, untreated mature 3T3-L1 adipocytes were used as controls [26,27].

Evaluation of Differentiation and The Effect of Ethanolic and Aqueous Extracts of Aerial Parts of *Cistus creticus* on Lipid Accumulation in 3T3-L1 Adipocytes by Oil-Red O Staining

Evaluation of differentiation and effects of the plant extracts on lipid accumulation in 3T3-L1 adipocytes by Oil-Red O staining was performed according to previous reports [27-29].

The Genotoxic Effect of Ethanolic and Aqueous Extracts Aerial Parts of *Cistus creticus* on 3T3-L1 Adipocytes by Agarose Gel Electrophoresis and DNA Fragmentation Assay

Agarose gel electrophoresis and DNA fragmentation assays were applied to determine the genotoxic effect of water and EtOH extracts of *Cistus creticus* on 3T3-L1 adipocytes [30].

DNA Extraction

At the end of the maturation period, a commercial kit was used according to the manufacturer's protocol for the extraction of genomic DNA from 3T3-L1 adipocytes [31].

DNA Gel Electrophoresis

For gel electrophoresis, the concentration of isolated DNA was measured using a spectrophotometer (Nanoprop 2000c, Thermo Scientific, USA) at 260 nm. DNA was then taken from each DNA sample with 15 µl and mixed with 4 µl of loading dye, loaded onto a 1.5% agarose gel and run at 110 Vot for 40 min. DNA was stained with 1.5 µl of SYBR-safe dye. The 1 kb DNA marker was used and the DNA fragments, and gels were visualized and photographed using an imaging system (BioSpectrum 810, UVP, USA) [32,33].

DNA Fragmentation Assay

For the diphenylamine method, the DNA sample was centrifuged at 10.000 rpm for 20 min at 4°C. The pellet and supernatant were mixed with 1/4 (v/v) trichloroacetic acid (TCA) and incubated at 4°C for 24 h. The samples were centrifuged for 20 min at 10.000 rpm at 4°C and the supernatant was

suspended in 5% TCA (v/v). It was incubated at 90°C for 15 min. Then, DPA solution (prepared with 50 µl of acetaldehyde, 10 ml of acetic acid, and 1.5 g of diphenylamine in 150 µl of sulfuric acid) was added to each sample and incubated at room temperature for 24 h. DNA was then stained with diphenylamine and Abs was measured at 600 nm using a spectrophotometer. DNA fragmentation was calculated using the following formula [34].

Fragmented DNA (%) = [Abs_{600} nm (Supernatant) / (Abs_{600} nm (Supernatant) + Abs_{600} nm (Pellet)] × 100

Statistical Analysis

Statistical Package for Social Science (SPSS) v24.0 version (Inc., Chicago, IL, USA) package program was used for statistical analysis of the data. All measurements made in the study were repeated three times. Data were expressed as mean ± SD. The One-ANOVA test (with Tamhane correction for post-hoc testing) was used to compare data between more than two independent groups for the data were normally distributed. Statistically, a p-value of <0.05 was considered significant. IC_{50} values were analyzed using Graphpad Prism (Graphpad Software Inc.) software and calculated using the non-linear regression analysis method.

RESULT AND DISCUSSION

In the present study, total phenolic content and total flavonoids were determined in both EtOH and aqueous extracts of the flowering aerial part of *Cistus creticus* to investigate the relationship between antiobesity and antimicrobial activity. According to the results of this study, extraction with water gave a yield of 12.90%. The yield of extraction with EtOH was determined to be 8.10%. The total phenolic content of the EtOH and water extract of the plant sample was determined to be 134.2849 mg GAE/g and 96.1803 mg GAE/g, respectively. It was found that the total flavonoid content in the water and EtOH extract was 33.1942 mgQE/g and 22.8338 mgQE/g, respectively (Table 1). Our results are in agreement with those of Gedikoğlu et al. who determined a total phenolic content in methanol and water extracts of 135.24 mg GAE/g and 114.35 mg GAE/g, respectively [35]. The total flavonoid content was higher in the methanol (34.34 mg QE/g) than in the water extract (19.78 mg QE/g), which is consistent with our results.

As natural compounds are regarded to be significant sources of various pharmacological properties, studies on different biological activities of plant materials are growing day by day. Especially, there are major concerns about treating bacterial infections caused by microorganisms that exhibit antibiotic resistance, increasing the demand for novel antimicrobial agents.

Phytochemical studies have shown that *Cistus* species contain a variety of secondary metabolites, especially phenolic compounds and terpene derivatives [8]. *Cistus creticus* showed remarkable variability in the total amount of water-soluble chemicals, flavonol glycosides and derivatives of punicalagin. Two chemovariations were reported depending on whether punicalagin derivatives were present or not. It was also mentioned that the specific composition of polyphenolic compounds of *Cistus* species seems to be related to evolutionary events [36].

The broad spectrum of antimicrobial activity of *Cistus* essential oil and resin, which contain terpenic chemicals, has been demonstrated by earlier reports [37,38]. The disk diffusion method was used to examine the *in vitro* antimicrobial activity of extracts of water, methanol, chloroform, ethyl acetate, and buthanol made from the dried and powdered leaves and fruits of five different *Cistus* species against the following microorganisms: *Streptococcus faecalis*, *Bacillus subtilis*, *Bacillus cereus*, *Pseudomanas aeruginosa*, *Escherichia coli*, *Candida albicans* and reported that butanol extract of *C. creticus* demonstrated antimicrobial activity against *S. aureus* while extracts of all *Cistus* species did not show any activity against *P. aeruginosa* and *C. albicans* [39].

Our findings on the diameters of the inhibition zones and the MIC values from the disc diffusion and MIC tests are listed in Table 2. None of the microbial strains tested showed susceptibility to the aqueous extract. However, the ethanolic extract showed antimicrobial activity, except for the strains *C. albicans* ATCC 26555 and *K. quasipneumoniae* subsp s*imilipneumoniae* ATCC 7006003. The inhibition zones were also observed in control groups including absolute ethanol seeded discs among

Gram-negative strains except *K. quasipneumoniae* subsp *similipneumoniae* ATCC 7006003 and *Shigella* sp. It was clearly established that the ethanolic extract content was superior to the control groups in terms of antimicrobial activity. While the concentration values from the MIC test were between 625 and 5000 µg/ml, the MIC value for the tested concentrations for strain *K. quasipneumoniae* subsp. *similipneumoniae* ATCC 7006003 could not be determined. This is because the maximum solubility of *Cistus* sp. in ethanol is 10 mg/ml. The lowest MIC values of 625 µg/mL were determined for the strains *B. subtilis* DSM 1971, *B. licheniformis* DSM 13 and *B. amyloliquefaciens* DSM 7, while the highest MIC concentration was found for the strains *E. coli* and *E. gallinarum*.

The antibiofilm activity of the extract against MRSA ATCC 43300, *S. aureus* ATCC 25923, P. *aeruginos*a ATCC 27853 and *E. coli* ATCC 25922 was shown in Figure 1. Each strain was treated with its MIC and sub-MIC concentrations for the antibiofilm activity of the ethanolic extract. The concentrations MIC, MIC/2, MIC/4 and MIC/8 of each strain proved to be very effective in inhibiting biofilm formation. The MIC/16 concentration of *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 also proved to be effective in inhibiting biofilm formation. An earlier report by Erdoğmuş et al. showed varying degrees of antibiofilm efficacy against various bacteria at different doses and agreed with our findings [12].

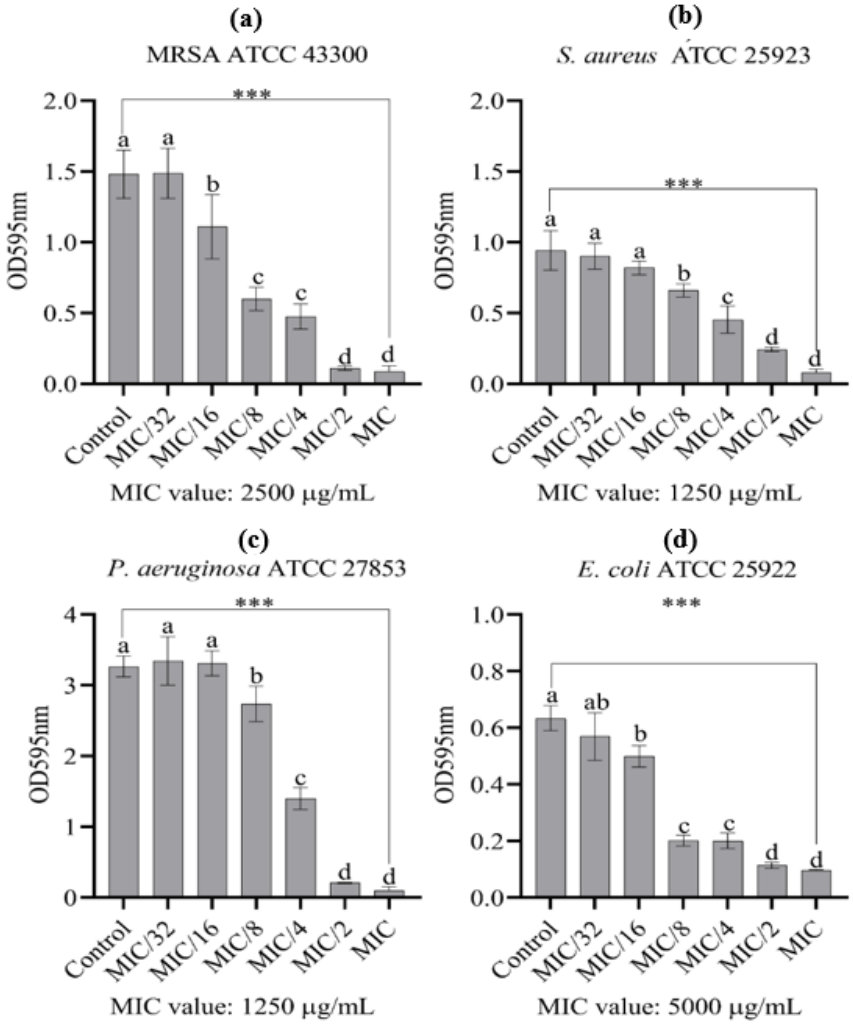

Figure 1. Antibiofilm effects of the ethanolic extract on the biofilm formation of the tested strains. (a) MRSA ATCC 43300 (b) *S. aureus* ATCC 25923 (c) *P. aeruginosa* ATCC 27853 (d) *E. coli* ATCC 25922. Different letters show statistical differences (One-Way ANOVA Test, Tukey's Test, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

We have also investigated the relationship between the anti-obesity activity and the extracts of *Cistus creticus*. Previous reports have mentioned the folkloric use of another species, *Cistus laurifolius*, as a weight-loss and antidiabetic agent [40,41]. We started by determining the effect of the plant parts on the viability of 3T3-L1 preadipocytes exposed to different concentrations of EtOH and aqueous extracts (0-2500 µg/ml) for 48 h. The results are shown in Figure 2.

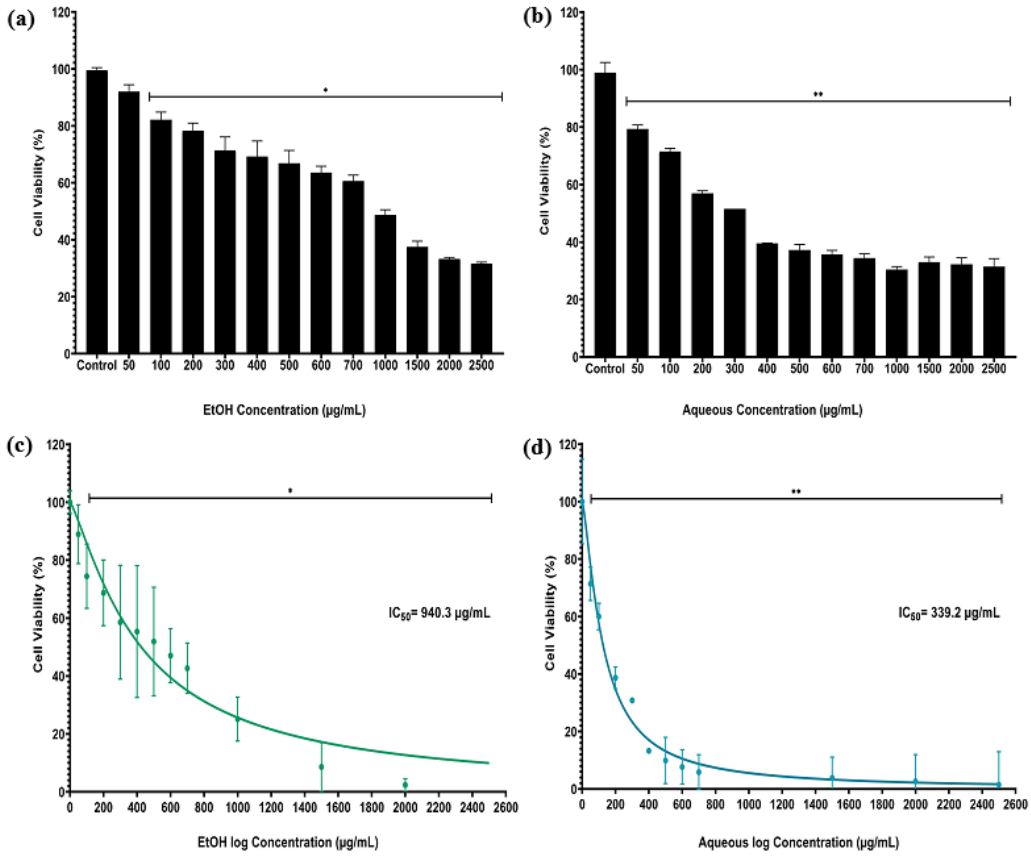

Figure 2. Effect of plant extracts on cell viability. (a) and (b): MTT assay data of extracts. 3T3-L1 preadipocytes cultured in 96-well plate were subjected to treatment with increasing concentration of EtOH or aqueous extracts of *C. creticus* for 48 hours. Cytotoxicity was then determined by the MTT test. In addition, the cell in the medium without plant extract was used as the control, and the cell in the medium containing 0.01% DMSO without the plant extract was used as vehicle control. (c) and (d): IC_{50} value of the extracts. IC_{50} was calculated by nonlinear regression model. Data were expressed as the mean ± SD from three independent experiments. One-way ANOVA test was used for statistical analysis. *$p < 0.005$; and**$p < 0.005$ significantly different from untreated 3T3-L1 preadipocyte. MTT: Methylthiazole tetrazolium; EtOH: Ethanol; DMSO: Dimethyl sulfoxide; IC_{50}: half (50%) maximum inhibition concentration; SD: Standard deviation

Cells exposed to a concentration of 50 µg/ml *Cistus creticus* EtOH extract showed a 10% reduction in viability compared to the control, which continued significantly at higher concentrations, so these concentrations were used in subsequent experiments (Figure 2a). The aqueous extract significantly reduced cell viability even at lower doses of 20 µg/ml (Figure 2b). Therefore, concentrations of 50 µg/ml EtOH extract and 20 µg/ml aqueous extract were used in subsequent experiments.

The extract concentration (IC_{50}) that caused a 50% reduction in cell viability was determined to be 940.3 ± 3.062 µg/ml and 339.2 ± 1.850 µg/ml for EtOH and aqueous extract, respectively (Figure 2c, 2d). At the end of the treatment, oil red staining was performed to confirm terminal adipocyte

differentiation and to evaluate the effect of *Cistus creticus* extracts on lipid accumulation of mature adipocytes (Figure 3). Under the inverted microscope, a reduction in Oil-Red-O staining was observed in the treated group of mature 3T3-L1 adipocytes treated with EtOH and aqueous extracts of *Cistus creticus* compared to the untreated adipocyte controls (Figure 3a). Compared to the untreated adipocyte controls, a clear and significant effect on lipid accumulation was observed in the 3T3-L1 adipocytes treated with EtOH extract, with a value of 32.10%. The percentage value of lipid accumulation of the aqueous extracts was reported to be 16.78% (Table 3). These data indicate that *Cistus creticus* inhibits adipogenesis in mature 3T3-L1 adipocytes by reducing lipid droplets.

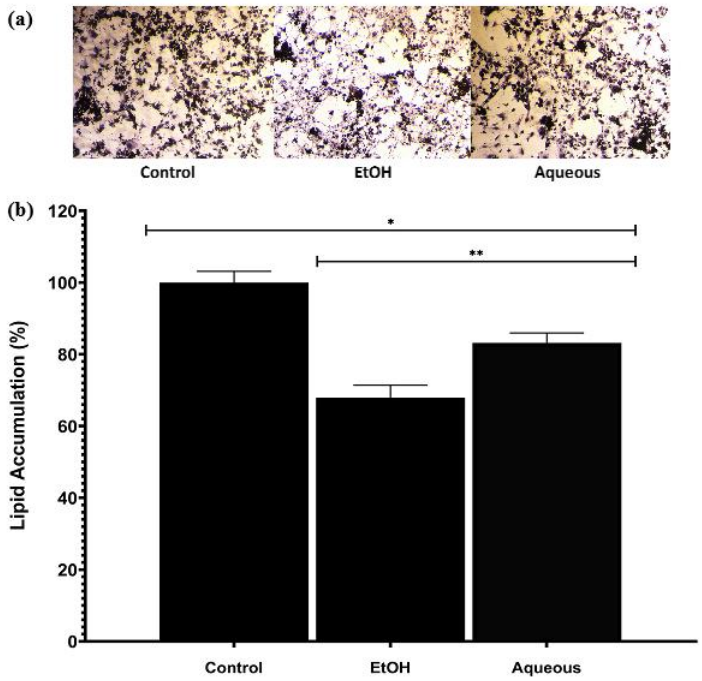

Figure 3. The effect of aerial parts of the extracts of *C.creticus* on adipogenesis and lipid accumulation. (a) Preadipocytes were differentiated into mature adipocytes for 8 days in the absence of extract treatment of *C.creticus* in DM or in the presence of EtOH (50 µg/ml) or Aqueous extract (20 µg/ml). Differentiated and untreated adipocytes were used as controls. Microscopic images of cells stained with Oil-Red O at 10X magnification. (b) Quantitative data of Oil-Red O staining. The data with relative change of OD490 obtained from three independent experiments were presented as mean ± SD and expressed as floor change compared to the control. A one-way ANOVA test was used for statistical analysis. *$p < 0.005$ is significantly different from untreated 3T3-L1 adipocytes. **$p < 0.005$ is significantly different from the EtOH extract. EtOH: Ethanol; DM: Differentiation medium; OD: Optical density; SD: Standard deviation

At the end of the treatment, the genotoxic activity of the flowering plant parts of *C. creticus* on mature adipocytes was evaluated by agarose gel electrophoresis, DNA fragmentation assay and diphenylamine method (Figure 4). No significant laddering was observed in the DNA samples isolated from 3T3L-1 cells treated with extracts of *C. creticus* by agarose gel electrophoresis (Figure 4a). DNA fragmentation in the cells treated with EtOH or aqueous extract of the plant increased by 6.02% and 8.6%, respectively, compared to the control group using the diphenylamine method, but this increase was not statistically significant ($p = 0.1148$ and $p = 0.0112$, respectively). These data show that treatment with *Cistus creticus* has no genotoxic effect on 3T3-L1 cells.

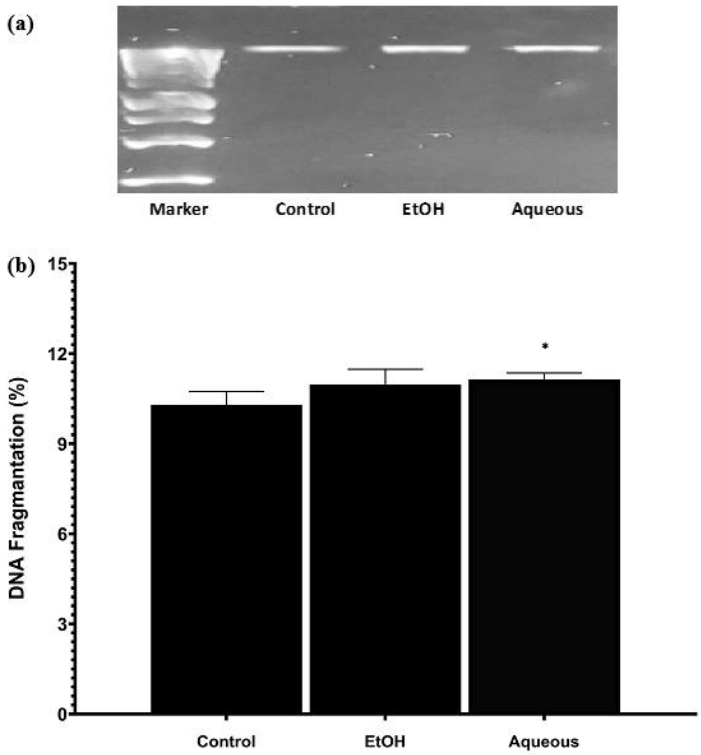

Figure 4. The effect of the aerial parts of *Cistus creticus* extracts on genotoxicity. (a) DNA gel electrophoresis data of the plant extracts. Preadipocytes were differentiated into mature adipocytes for 8 days in the absence of extract treatment of *Cistus creticus* in DM or in the presence of EtOH (50 µg/ml) or aqueous extract (20 µg/ml). Differentiated and untreated adipocytes were used as controls. Representative image of the electrophoretic pattern of DNA isolated from 3T3-L1 cells in agarose gel (20 µl/band). 1 bp DNA was used as a ladder marker. (b) Quantitative data of DNA fragmentation by diphenylamine method. OD600 data obtained from three independent experiments were presented as mean ± SD. A one-way ANOVA test was used for statistical analysis. $p < 0.005$, treated and untreated 3T3-L1 adipocytes are similar. EtOH: Ethanol; DM: Differentiation medium; OD: Optical density; SD: Standard deviation

Obesity is a growing problem with limited therapeutic options. It may be useful to evaluate the therapeutic benefits of plants based on their traditional use through experimental designs. To this end, we investigated the anti-obesity, antimicrobial and antibiofilm activities of the flowering aerial parts of *Cistus creticus* and found that a noticeable, but not strong, effect on lipid accumulation was observed in 3T3-L1 adipose cells treated with an EtOH extract. In addition, antimicrobial and antibiofilm properties were also observed and the conclusions should be useful for future research.

Table 1. The total phenolic and total flavonoid content of *Cistus creticus* extracts

	Total phenolic content mg GAE/g extract	**Total flavonoids mg QE/g extract**
EtOH extract	134.285 ± 0.052	33.194 ± 0.034
Aqueous extract	96.180 ± 0.035	22.834 ± 0.003

Table 2. The diameters of the inhibition zones and the MIC values from the disc diffusion and MIC tests for the extracts of *Cistus creticus*

Microorganisms	Zone of Inhibition (mm)			MIC Concentration (µg/ml)
	Ethanolic extract	Aqueous extract	Control	
***B. subtilis* DSM 1971 (ATCC 21394)**	14.33 ± 0.58	-*	-	625
***B. subtilis* ATCC 6633**	11.67 ± 0.58	-	-	2500
***B. licheniformis* DSM 13 (ATCC 14580)**	13.50 ± 0.58	-	-	625
***B. amyloliquefaciens* DSM 7**	17.67 ± 0.58	-	-	625
***C. albicans* ATCC 26555**	-	-	-	-
***E. coli* ATCC 25922**	14 ± 0.00	-	7 ± 0.00	5000
***E. coli* ATCC 11230**	14.33 ± 0.58	-	7.33 ± 0.58	5000
***E. faecalis* ATCC 29212**	13 ± 0.00	-	-	2500
E. gallinarum	14.67 ± 0.29	-	-	5000
***K. quasipneumoniae* subsp. *similipneumoniae* ATCC 700603**	-	-	-	5000 <**
***L. monocytogenes* NCTC 5348**	20.33 ± 1.15	-	-	2500
***P. aeruginosa* ATCC 27853**	14 ± 0.87	-	8.83 ± 0.29	1250
***S. enterica* subsp. *enterica* serovar Infantis DMC12**	13.83 ± 1.04	-	7 ± 0.00	2500
***S. enterica* subsp. *enterica* serovar Typhimurium DMC4**	15 ± 0.00	-	6.67 ± 0.29	2500
***S. enterica* subsp. *enterica* serovar Montevideo**	11 ± 0.00	-	8 ± 0.00	2500
***S. enterica* subsp. *enterica* serovar Typhimurium ATCC 13311**	14 ± 0.50	-	11.33 ± 0.29	1250
***Shigella* sp.**	13 ± 0.00	-	-	2500
***S. aureus* ATCC 25923**	15 ± 0.00	-	-	1250
***S. aureus* ATCC 29213**	15 ± 1.00	-	-	2500
Methicillin-resistant *S. aureus* ATCC 43300	13 ± 0.00	-	-	2500

* "-" indicates that there was no antimicrobial activity
**MIC value cannot be detected for tested concentrations
"±" indicates standard deviation

Table 3. Effects of EtOH and aqueous extracts of the plant on cell viability, lipid accumulation and DNA fragmentation

	Cell viability (µg/ml)	Lipid accumulation (%)
EtOH extract	940.3 ± 3.062	32.10
Aqueous extract	339.2 ± 1.850	16.78

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AUTHOR CONTRIBUTIONS

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CONFLICT OF INTEREST

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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