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Biological properties of *Cakile maritima* Scop. *(Brassicaceae)* extracts

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Abstract. – OBJECTIVE: ***Cakile maritima*** **scop. (CKM) is a herbaceous plant** ***(Brassicaceae)*** **growing also in high salinity environment. It is an annual plant growing in clumps or mounds in the sand on beaches and bluffs.**

MATERIALS AND METHODS: Stems, seeds, leaves and flowers of CKM were used to obtain 70% of ethanol extracts. The phenolic content of the different extracts was evaluated by the Folin-Ciocalteu method. The separation of phytochemical compounds was based on ultra-performance liquid chromatography coupled to mass spectrometry. Radical scavenging activity was determined by 1,1-diphenyl-2-picrylhydrazyl assay. The qualitative assay for the inhibition of α-glucosidase was quantified spectrophotometrically and the anti-inflammatory activity was determined in the U937 cell line by using gene expression of pro-inflammatory cytokines. Cell viability assay was done in U937, MM1S, and U266 cells by using the 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay. The antimicrobial activity was investigated by MIC determination, "double-triple combinations assay", and growth inhibition curves analysis, using the extracts individually or in various combination. Statistical analysis was performed by the Student's t-test and ANOVA.

RESULTS: All parts of the plant exhibited a high antioxidant capacity as measured by DPPH assay. Furthermore, all extracts reduced (about 10 folds) the expression of inflammatory cytokines in macrophage following LPS treatment. As regards the antibacterial activity, only the seeds extract was able to inhibit both Gram-negative and Gram-positive bacteria when tested alone, whereas dual combinations of different extracts (leaves, flowers, stems and seeds) caused bacterial inhibition exhibiting a synergic action. Finally, we showed that the extracts did not exhibit cytotoxic effects in normal cells and that, surprisingly, it exhibited an anti-proliferative effect (inhibition ≈80%) in multiple myeloma U266 cells.

CONCLUSIONS: Our study suggests that CKM possesses antioxidant, anti-inflammatory, antibacterial, anti-proliferative activities and such pleiotropic effects may be exploited under various pathological conditions.

Key Words:
Cakile maritima, Antimicrobial activity, α-glucosidase, Polyphenols determination, Anti-inflammatory activity.

Abbreviations

CKM: *Cakile Maritima;* PMA: Phorbol 12-myristate 13-acetate PMA; DPPH: 1,1-diphenyl-2-picrylhydrazyl; PBS: Phosphate-Buffered Saline; FBS: Fetal Bovine Serum; MIC: Minimal Inhibitory Concentration.

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Introduction

Cakile maritima Scop. (CKM) is an annual glabrous herbaceous plant known also as sea-rocket. It is present all over Europe, especially in high salinity locations, particularly on the coasts and its dunes[1,2]. The whole plant has been used in traditional medicine for its antiscorbutic properties, due to the high content of vitamin C, iron and iodine. The flowers are used to obtain a digestive, fluidifying and also diuretic herbal tea[1]; the flowering tops are used for the preparation of anti-dandruff infusions, while the seeds provide an essence that is used for aromatic sauces on an industrial level. The properties of this plant are dependent on its numerous active compounds such as mirosine and its substrate sinigrin[3], and other glucosinolates[4] including esters of phenolic choline[5], flavonol glycosides and phenolic acids, belonging to the benzoic and cinnamic series[6].

These compounds exhibit various biological properties such as reducing soil and plant pathogens[7,8] and antioxidant activity[9-11]. In addition, this plant possesses diuretic, antiscorbutic, digestive and purgative properties[1,6,12]. Various properties were traditionally attributed to the marine rocket plants[13]; indeed, their extracts were used for purifying the lungs of the harsh viscose phlegm, jaundice and dropsy, and finally, they were prescribed in scrupulous affections and lymphatic disorders[12,14]. Also other *Brassicaceae* showed biological properties, such as sea holly *E. maritimum,* which has numerous medicinal uses as diaphoretic, diuretic, stimulant, cystotonic, urethritis remedy, stone inhibitor, aphrodisiac, expectorant, and anthelmintic[13-15]. Therefore, natural crude extracts and their biologically active compounds may represent valuable sources for the research of new antibacterial molecules[16-18].

The aim of this study was to investigate the biological properties of CKM with particular regard to antimicrobial, anti-inflammatory, anti-oxidant and antitumor effects of its extracts.

In this work, we evaluated the biological properties of CKM, focusing on its possible antimicrobial, anti-inflammatory, anti-oxidant, and anti-tumor effects.

Materials and Methods

Stems, seeds, leaves and flowers of CKM were dried in a stove at 36°C (controlled temperature). The natural extract preparation has been performed through the crushing of plant material using pestle and mortar until obtaining a homogeneous sample. The samples were then extracted in 70% ethanol (v/v) for 30 min. Subsequently, they were filtered by a vacuum pump and stored in the dark at -20°C.

Polyphenols Determination

The phenolic content of the different extracts was evaluated by the Folin-Ciocalteu method[19]; as described below 1.25 mL of Folin-Ciocalteu reagent was mixed with 0.25 mL of each extract. After 3 min, 2.5 mL of a sodium carbonate solution (20%) was added to the mixture and the reaction was conducted in the dark for 1 h. The absorbance was spectrophotometrically measured at 725 nm, using a Perkin Elmer lambda 25 UV-VIS spectrometer. Gallic acid (Fluka, Honeywell International Inc., Morristown, NJ, USA) was used as reference standard for calibration curve (0.02-0.8 mg/mL; r2 = 0.9995). The total phenolic content was expressed as gallic acid equivalents in milligram per gram of dried sample.

UPLC-Ms/Ms

An analytical method was used to obtain a good separation of phytochemical compounds, based on ultra-performance liquid chromatography coupled to mass spectrometry (AB SCIEX API 2000™) instead of HPLC-Esi-Ms. The better separation was obtained using acetonitrile and water with 0.1% acetic acid as mobile phase (70:30 v/v at 300 µl/min) into a C18 column (Phenomenex Luna, 5 µm, 15x0.1 cm, Torrance, CA, USA). The following gradient elution program was carried out: from 0 to 2 min at 70:30 v/v; from 2 to 4 min acetonitrile was changed to 100% linearly; from 4 to 10 min the system was reconnected to the initial conditions. A total volume of 10 µl was injected. ESI-triple quadrupole mass was used with positive and negative polarities for a comprehensive assessment of the analyzed spectrum and the negative one, was selected for the detection of compounds. Other important polarities: Curtain gas 30, IonSpray Voltage (IS) -4500, Ion Source Gas1 (GS1) 30.0, Ion Source Gas2 (GS2) 60.0 and Interface Heater ON, Declustering Potential (DP) -50.0, Focusing Potential (FP) -400.0 and Entrance Potential (EP) -10.0, Temperature (TEM) 350°C. Initial analyses were carried out from 100 to 1000 Da. The phenolic component has also been evaluated through a column Phenomenex Kinetex 2.6u Biphenyl 100A 100 x 2.1 mm. The mass in full scan has been accomplished

in a range which covered from 100 to 1000 m/z; the atomizing gas was nitrogen. Each of the products was analyzed by a single analysis Q1 to Q3 and mass scanning. Data acquisition and peak integration were performed with Analyst® Software AB Sciex (Framingham, MA, USA).

Antioxidant Activity Measurement

The radical scavenging activity was determined by DPPH assay[20]. Briefly, a solution (100 μM) of 1,1-diphenyl-2-picrylhydrazyl DPPH • in methanol (Sigma-Aldrich, St. Louis, MO, USA) was used and 3 mL of solution were mixed with 70 μL of extract. The samples were incubated for 60 minutes at room temperature; then, the absorbance decrease at 515 nm (AE) was measured by spectrophotometry. The DPPH radicals have a maximum absorption at 515 nm, the peak disappears with a reduction caused by an antioxidant compound. A white sample was used as a reference, containing 70 μL of methanol solution in the DPPH solution. The data obtained were expressed as a percentage of DPPH radical inhibition.

Qualitative Assay for the Inhibition of α-Glucosidase

The α-glucosidase assay was conducted in accordance with Jabeen et al[21] with some modifications. α-glucosidase *S. cerevisiae* (E.C. 3.2.1.20) was prepared in potassium phosphate (0.1 mol/L, 3.2 mmol/L $MgCl_2$, pH 6.8). p-nitrophenyl-α-D-glucopyranoside was used as a substrate for the reaction and it was also dissolved in potassium phosphate buffer at 6 mmol/L. For the assay reaction, 282 μL of each plant extract and 200 μl substrate were mixed and incubated at 37°C for 5 min, and then, 200 μl of the enzyme solution was added. The enzyme reaction was carried out at 37°C for 15 min, the reaction was stopped by 1.2 mL of glycine buffer (pH 10); finally, the enzyme activity was quantified spectrophotometrically by measuring the absorbance at 410 nm. For the quantification of inhibition, the activity was compared with a negative and positive control using water and acarbose respectively instead of plant extract. The results were expressed as the sample concentration required to inhibit 50% of the enzyme activity (IC_{50})[22].

Anti-Inflammatory Activity in vitro

The U937 cell line was used to measure the anti-inflammatory activity of extracts. It is a type of human pro-monocytic myeloid leukemia cells isolated from a histiocytic lymphoma. This line shows many characteristics of monocytes and is used in the literature given its easy use. The cells were differentiated into macrophages through Phorbol 12-myristate 13-acetate (PMA) 200 nM for 72 hours. After incubation, the suspended cells were removed by aspiration and the adherent cells (U937Φ) were washed three times with Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA). To simulate the inflammation, the cells were further incubated with or without LPS at the concentration of 100 ng/ml for 2 hours with the same medium (Roswell Park Memorial Institute-1640 (RPMI-1640; Gibco, Grand Island, NY, USA) 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA). After the first 2 hours, the extracts of *Cakile maritima* L. were added at a dilution of 1 mg/ml for 4 h.

Cell Cultures and Cell Viability Assay

U937, MM cell lines such as MM1S and U266 (established from peripheral blood of a multiple myeloma patients in refractory and terminal stage) were cultured in a suspension using RPMI-1640 medium supplemented with 10% or 20% FBS and 1% penicillin/streptomycin at 37°C and 5% CO_2. After informed consent was obtained, human lymphocytes and monocytes were isolated from fresh buffy coat of healthy volunteers provided by the Transfusional Centre of E. Muscatello Hospital, Augusta, Italy. Then, monocytes and lymphocytes were purified from the lymphomonocytic population by positive isolation using magnetic beads coated with goat anti-mouse CD14+ IgG and anti-mouse CD3+ IgG respectively (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). After treatment of the above-mentioned cell types with CKM extracts at 0.5 and 1 mg/ml concentration, cell viability was assessed using the 3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay, according to the manufacturers' protocol. At the end of the treatment, the cells were incubated with MTT for 4 h; then, 100 μL of dimethyl sulfoxide was added and the absorbance was read at 590 nm, as previously described.

Antimicrobial Activity by "Double-Triple Combinations Assay"

Six type strains were used to evaluate the antibacterial activity of CKM extracts: *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Acinetobacter baumannii* ATCC 19606 and *Klebsiella pneumoniae* ATCC 700603. In agre-

ement with CLSI M100[23], to determine MIC values (minimum inhibitory concentration), a microdilution test was performed in a 96-well polystyrene plates (Thermo Fisher Scientific™ Nunc™ Cell Culture Dishes, Waltham, MA, USA) with Cationic Adjusted Muller Hinton Broth (CAMHB, Merck KGaA, Darmstadt, Germany) as medium. Extracts from 4 different parts of the plant (leaves, flowers, seeds, stems) were tested. Moreover, each strain was tested using double and triple combinations of extracts: leaves and seeds, leaves and stems, seeds and stems, and finally leaves, seeds and stems together. This evaluation was performed in a 96-well polystyrene plates as described by El-Azizi et al[24]. Plates were placed at 37°C overnight. Then, MIC values of the combinations were evaluated with respect to the most potent extract, single or in double-triple combinations. As described by El-Azizi et al[24], to assess the antibiotic combinations therapy *in vitro*, an interaction code (IC) was created for each combination: for any 2-fold increase or decrease in the MICs values a numerical value was assigned. Based on this value, the result (interaction type, IT) is defined as Antagonism if IC is -2 or less, Indifferent if -1<IC<+1, Synergism IC≥+ 2. Triple combination follows the same rules considering the most potent double combination.

Growth Bacterial Inhibition Curves Analysis

Growth inhibition curves analysis was tested for the pathogens where double combinations resulted synergic. Growth inhibition analysis was performed to reveal the impact of double combinations on sensitive bacteria[25]. A bacterial suspension of 0.5 McFarland ($1.5x10^8$ CFU/mL) was prepared after an overnight subculture in Mueller-Hinton broth (MH broth, Merck KGaA, Darmstadt, Germany) and a series of dilutions were prepared to obtain a final concentration of $7.5x10^5$ CFU/mL in MH broth. The range concentration tested of combined extracts used for each organism was chosen based on the previously determined MIC values. Inoculated 96-well polystyrene plates (Thermo Fisher Scientific™ Nunc™ Cell Culture Dishes, Waltham, MA, USA) were incubated aerobically with shaking at 37°C for 24 hours and OD measurements at 600 nm (Model 680 Microplate Reader, Bio-Rad, Hercules, CA, USA) were made any 30 minutes. All measurements were repeated eight different times.

Gene Expression Analysis

RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). First strand cDNA was then synthesized with an Applied Biosystems reverse transcription reagent (Foster City, CA, USA). Tumor necrosis factor alpha (TNF-α), interleukin 6 (IL6), and heme oxygenase 1 (HO-1) mRNA expressions were assessed by the TaqMan Gene Expression (Applied Biosystems; Foster City, CA, USA) and quantified using a fluorescence-based Real Time detection method by 7900HT Fast Real Time-Polymerase Chain Reaction System (RT-PCR; Life Technologies, Gaithersburg, MD, USA). For each sample, the relative expression mRNA levels mRNA was normalized using Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an invariant control.

Statistical Analysis

Statistical significance between the two groups was analyzed by the Student's *t*-test. One-way and two-way analysis of variance (ANOVA), followed by the Tukey's post-hoc test, were used for multiple comparisons. p-values < 0.05 were considered statistically significant. All the results and graphs were generated using a GraphPad® Prism version 6 software (La Jolla, CA, USA).

Results

Polyphenols Determination

The concentration of polyphenols fluctuated depending on the part of plant considered (Table I); leaves and stems extracts possessed a very higher content of polyphenols compared to other parts. Indeed, the content in leaves and stems were about 14.54±0.2 mg/g and 14.37±0.2 mg/g, respectively. Instead, the polyphenol content in seeds was only 7.79±0.5 mg/g, while in flowers was 3.97±0.5 mg/g.

UPLC-MS/MS

A separation of phytochemical compounds was obtained by ultra-performance liquid chro-

Table I. Content of total polyphenols in different parts of the plant (leaves, flowers, seeds and stem).

Plant part	mg/g
Leaves	14.54±0.2
Flowers	3.97±0.5
Seeds	7.79±0.5
Stems	14.37±0.2

Table II. Concentration of major components revealed by ultra-performance liquid chromatography couplet to mass spectrometry.

Component	% Relative		
	Leaf	Stem	Seed
Cumaric acid	0.387	0.258	0.19
Bergaptene	0.184	0.071	0.0254
Esculetine	0.191	0.559	0.169
Prenylnarigenine	0.191	0.559	0.169
Isosakuranetin	0.082	0.021	0.175
Kaempferol	0.075	-	0.140
Scutellarein	0.054	-	-
Caffeic acid	0.020	-	0.646
Resveratrol	0.010	-	-
Gallic acid	0.004	-	-
Ellagic acid	0.001	-	0.815
Quercetin	0.002	-	-
Ferulic acid	0.001	-	-
Chlorogenic acid	0.007	0.037	0.938
Cinnamic acid	0.003	-	0.185
Fertaric acid	0.059	0.360	0.318

-: not detected

matography coupled to mass spectrometry. The major phenol compounds detected were reported in Table II.

Anti-Radical Activity: DPPH

The scavenger and antioxidant activities of CKM were evaluated by performing the DPPH test. Data obtained showed a great antioxidant effect of leaves and stems (Figure 1); consistently, the polyphenol content correlates with this antioxidant power.

CKM Inhibits α-Glucosidase Activity

Table III showed IC_{50} values obtained on the inhibition of the α-glucosidase activity by the different parts of the plant. The results showed a higher inhibition by stem and seed extracts compared to the other parts of the plant, respect to acarbose (12.7±0.1 μmol/L).

In vitro Anti-Inflammatory Activity

To investigate the possible anti-inflammatory effect of the extracts, we evaluated the gene expression of pro-inflammatory cytokines TNFα, IL-1β, and IL-6, known to be involved in inflammation processes. As previously mentioned, to simulate an inflammation condition *in vitro*, the treatment with CKM was carried out on macrophages subjected in turn to pretreatment with LPS. Figure 2 shows that the expression of TNFα, IL-1β, and IL-6 gene levels after treatment of cells with LPS were significantly increased compared to untreated cells. Conversely, the combined treatment of the extracts with LPS shows a marked decrease in the expressed cytokines levels confirming an anti-inflammatory effect of all extracts. In addition, we evaluated the induction of oxidative stress induced by LPS treatment. LPS treatment induced HO-1 gene expression when compared to control cells, while combined treatment with the CKM extracts showed a significant decrease in HO-1 expression suggesting the antioxidant potential of tested extract.

Cytotoxicity and Anti-Proliferative Effect on Human Cells

To evaluate a possible cytotoxicity effect in healthy cells and/or anti-proliferative and antitumor effect in cancer cells, we analyzed the cell viability in lymphocyte cells from healthy donor patients and two different cell lines of MM, MM1S, and U266. The results showed that all extracts not exhibited cytotoxicity effects in healthy human cells and MM1S (Figure 3 A, B, C and E). Sur-

Table III. I_{c50} Values for α-glucosidase inhibition in different parts of the plant (leaves, flowers, seeds and stems).

Plant part	IC50 (μmol/L)
Leaves	8.163265306
Flowers	48.97959184
Seeds	69.3877551
Stems	77.12244898

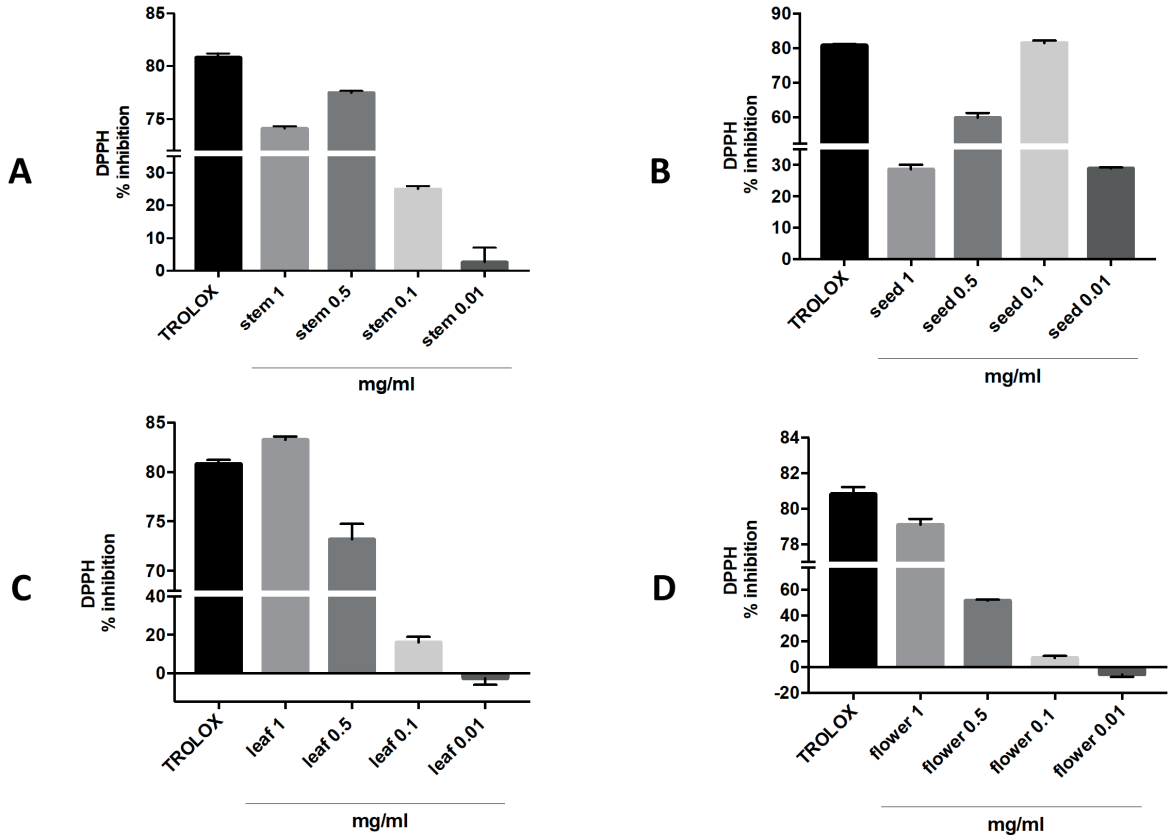

Figure 1. *in vitro* antioxidant activity measured through percentage of DPPH Inhibition. A = stem extract; B = seed extract; C = leaf extract; D = Flower extract. Trolox was used as positive controls.

prisingly, in U266 cell line, the leaves extract of CKM showed a remarkable decrease in cell proliferation in a dose-dependent manner (Figure 3D).

Antimicrobial Activity and Evaluation of "Double-Triple Combinations"

Antimicrobial susceptibility test on six type strains (*Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii,* and *Klebsiella pneumoniae*) showed a variable susceptibility to the plant extracts. In particular, only seeds extract has shown antibacterial activity; instead all bacteria tested were resistant to leaves, flowers, and stems which have not shown any activity (Table IV). Seeds extract was effective against all tested strains with MIC range of 150-75 µg/mL.

To evaluate the double and triple combinations of the extracts, three different double combinations and one triple combination were tested

Table IV. MIC values of microdilution assay of Cakile maritima extracts.

	Drugs alone (µg/mL)			
Strains	**Leaves**	**Flowers**	**Seeds**	**Stems**
Escherichia coli ATCC 25922	-	-	75 µg/mL	-
Staphylococcus aureus ATCC 25923	-	-	150 µg/mL	-
Enterococcus faecalis ATCC 29212	-	-	150 µg/mL	-
Pseudomonas aeruginosa ATCC 27853	-	-	75 µg/mL	-
Acinetobacter baumannii ATCC 19606	-	-	150 µg/mL	-
Klebsiella pneumoniae ATCC 700603	-	-	150 µg/mL	-

-: no activity

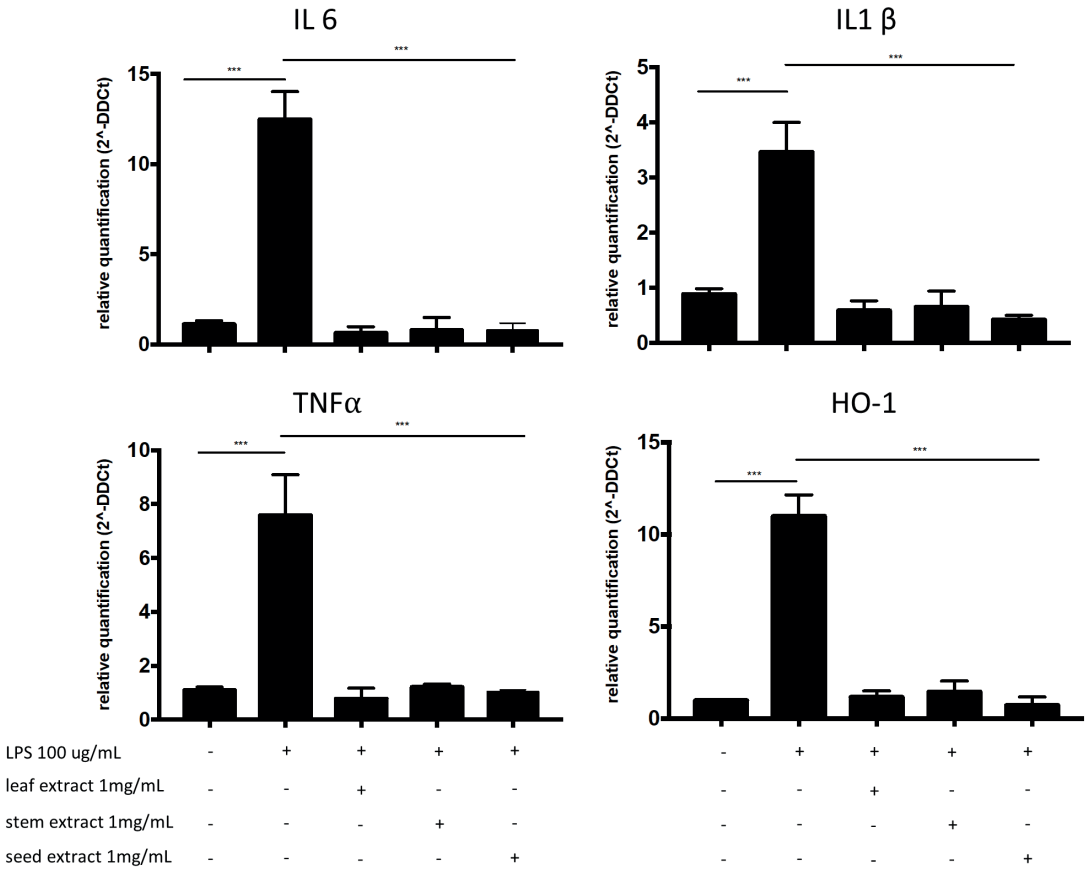

Figure 2. Gene expression of inflammatory cytokines TNFα, IL-1β, IL-6 and antioxidant enzyme HO1. Bars represent the mean ± SEM of six independent experiments. (Calculated value of $2^{-\Delta\Delta Ct}$ *vs.* untreated was 1).

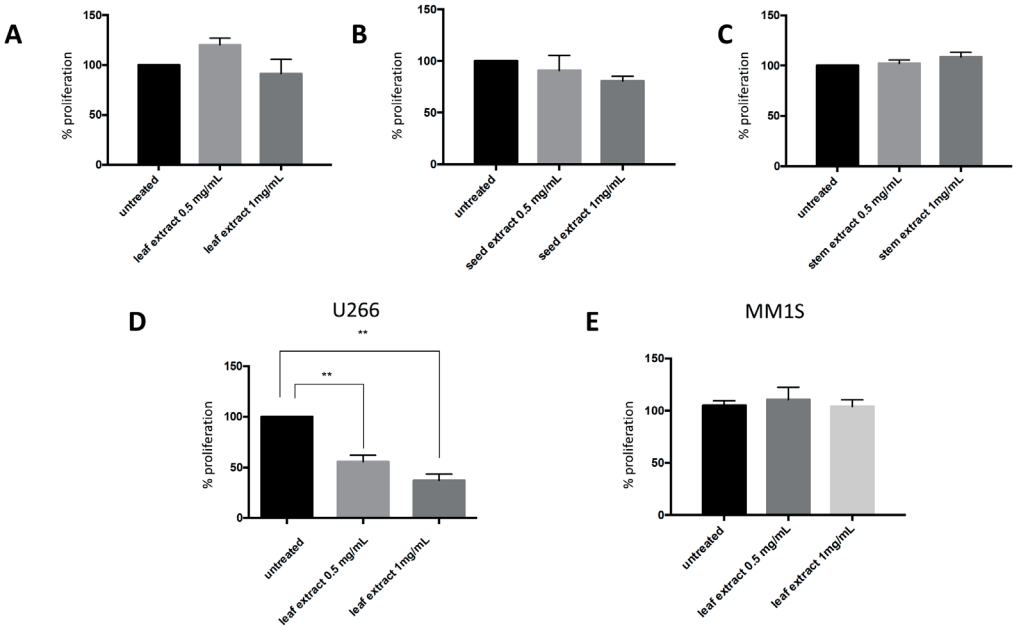

Figure 3. The survival assay in healthy primary human lymphocytes ***(A, B, C)*** and in myeloma cell line U266 cells ***(D)***, MM1 cells ***(E)*** with CKM. Bars represent the mean ± SEM of six independent experiments. ** $p < 0.001$ *vs.* untreated cells.

Table V. Double-triple combinations assay of *Cakile Maritima* extracts against bacterial strains.

Strains	Double combination (µg/mL)									Triple combination (µg/mL)		
	leaves + seeds			leaves+ stems			seeds + stems			leaves + seeds + stems		
	MIC	IC	IT	MIC	IC	IT	MIC	IC	IT	MIC	IC	IT
Escherichia coli ATCC 25922	18.75	+2	S	18.75	+2	S	18.75	+2	S	18.75	0	I
Staphylococcus aureus ATCC 25923	150	0	I	150	0	I	150	0	I	150	0	I
Enterococcus faecalis ATCC 29212	75	+1	I	75	+1	I	75	+1	I	75	0	I
Pseudomonas aeruginosa ATCC 27853	18.75	+2	S	18.75	+2	S	18.75	+2	S	18.75	0	I
Klebsiella pneumoniae ATCC 700603	75	+1	I	37.5	+2	S	75	+1	I	75	-1	I
Acinetobacter baumannii ATCC 19606	18.75	+3	S	18.75	+3	S	18.75	+3	S	18.75	0	I

against the same strains. In ten cases, the double combinations were synergic, whereas in 8 cases double combinations showed indifference activity. Moreover, no antagonism was observed in all interactions (Table V). The extracts of leaves and stems have not shown activity against bacteria when tested alone, but when they were included in double combinations some strains became susceptible. In particular, *Escherichia coli* and *Pseudomonas aeruginosa* became more susceptible with an increase in two folds respect the MIC values of seeds tested alone. *Klebsiella pneumoniae* showed an increase in two-fold when tested with the double combination of leaves and stems. Moreover, the best results were obtained for all three double combinations against *Acinetobacter baumannii* with an increase in three folds (Table V). Unfortunately, the triple combination did not show synergism for all bacteria tested.

Growth Bacterial Inhibition Curves Analysis

To evaluate the real power of double combinations, growth inhibition curves analysis was done. Growth inhibition curves analysis was tested for the pathogens where double combinations resulted synergic: *Escherichia coli*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa,* and *Klebsiella pneumoniae*. The growth rates of strains compared to that of control are shown in Figures 4 to 7. Our results have shown that bacteria resulted sensitive to the double combinations compared to the extracts tested alone: in particular, the percentage of inhibition depends on the concentrations tested, but bacterial growth is inhibited more than 90% in almost all the conditions tested.

Discussion

In this work, we evaluated the possible antimicrobial, antioxidant, anti-inflammatory, and anti-proliferative effects of extracts of CKM. Our data showed for the first time the biological properties of the single parts of the plant extracts (stem, seed, leave, and flower). We observed that the extracts were able to inhibit the formation of reactive oxygen species (ROS) as observed by DPPH assay in a concentration-dependent manner. In other studies[2,26], the authors showed that the total extract of CKM presented the antioxidant properties.

In particular, we analyzed the extracts of different parts of the plant including leaves, flowers, stems, and seeds. Several studies[27,28] showed a correlation between the phenolic content and antioxidant activity, since phenolic compounds contribute directly to antioxidant and antimicrobial activities.

The phenolic compounds in plants are produced through the phenylpropanoid pathway, and they can be induced by environmental stresses[29]. The phenolic compounds in plants can be changed by salt stress, but this is critically dependent on the salt sensitivity of plants[29]. Interestingly, some studies[2,30,31] showed that the total phenolic content of

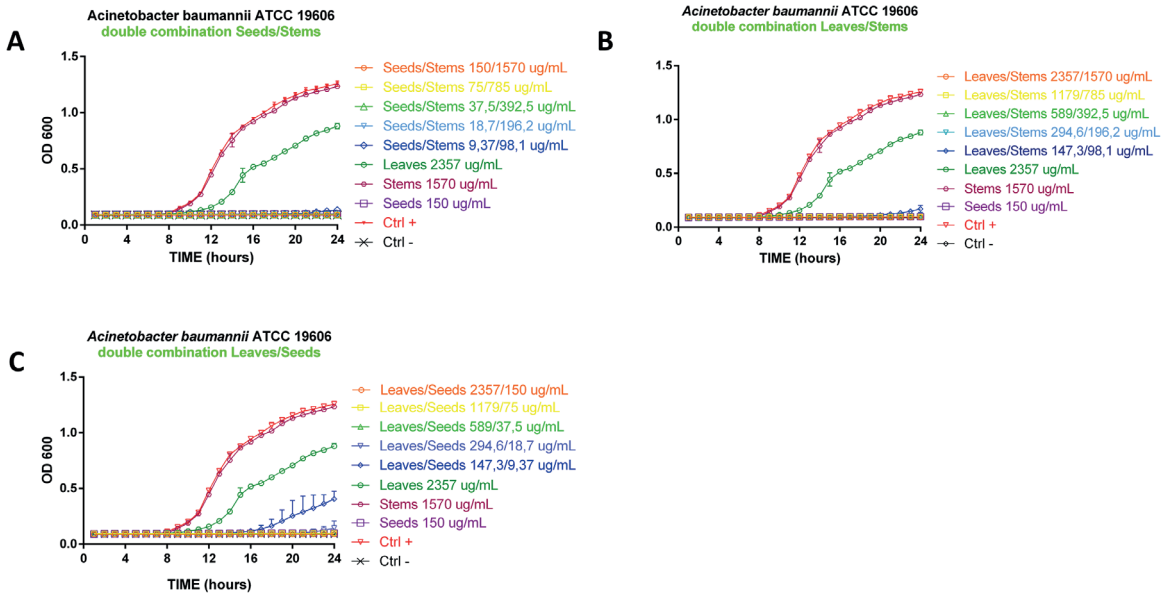

Figure 4. Growth inhibition curves analysis of *Acinetobacter baumannii* ATCC 19606 for synergic double combinations "seeds + stems" (MIC value equal to 9.37/98.1 µg/mL), "leaves + stems" (MIC value equal to 147.3/98.1 µg/mL) and "leaves + seeds" (growth inhibition 65.76% at 147.3/9.37 µg/mL). All experimental data are expressed as mean ± standard error (SD). Significance was assessed by ANOVA or Student's *t*-test: $p < 0.05$.

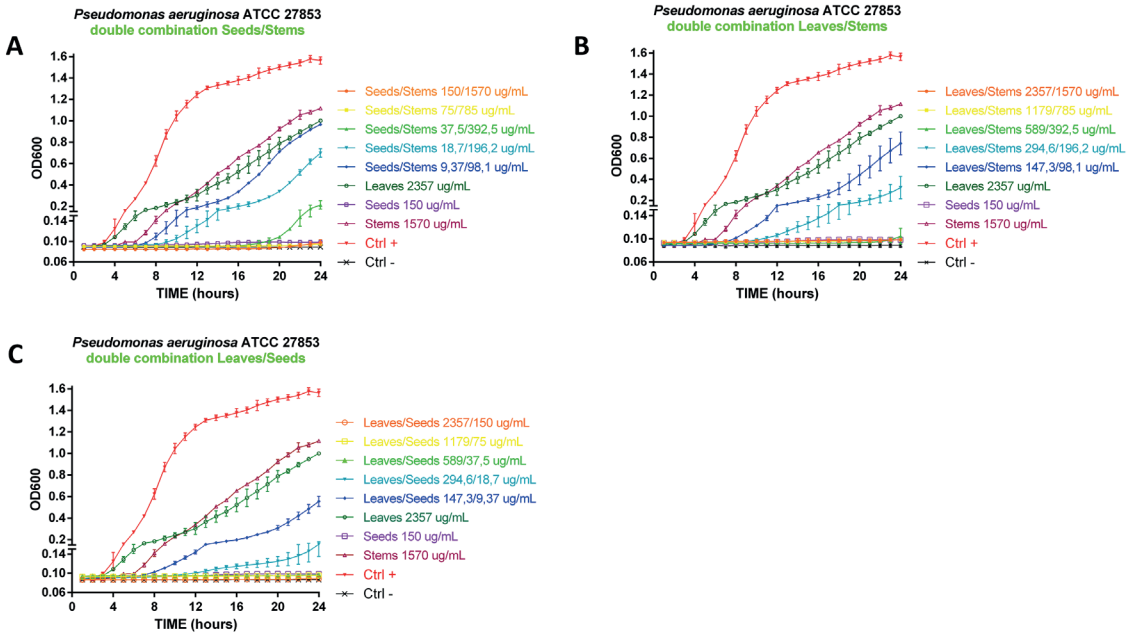

Figure 5. Growth inhibition curves analysis of *Pseudomonas aeruginosa* ATCC 27853 for synergic double combinations "seeds + stems" (growth inhibition 99.98% at 37.5/392.5 µg/mL), "leaves + stems" (growth inhibition 99.97% at 294.6/196.2 µg/mL) and "leaves + seeds" (growth inhibition 99.96% at 147.3/9.37 µg/mL). All experimental data are expressed as mean ± standard error (SD). Significance was assessed by ANOVA or Student's *t*-test: $p < 0.05$.

radish sprouts treated with different concentration of NaCl was significantly increased. Therefore, the property to grow in extreme environments makes such class of plants valuable because enriched with biologically active molecules to adapt to extreme growing conditions. To this regard, salt-induced growth variations correlated with parallel variations in antioxidant (phenols) accumulation and antioxi-

dative ability, and with opposite changes in lipid peroxidation. Thus, as expected, the salt stress is effective in augmenting extractable, active antioxidants from CKM, and there is an intraspecific variability for the accumulation of these compounds in response to salt[2]. The polyphenolic components contained in the various extracts were analyzed and the results revealed that a higher content of polyphenols was found in the leaves and stems; furthermore, our results showed that polyphenolic content correlated with antioxidant activity. We also evaluated the potential of these extracts to inhibit α-glucosidase (E.C. 3.2.1.20), an enzyme capable of hydrolyzing the α-1,4 bond at the non-reducing end and α-1,6, of starch with glucose release as the final product. The inhibition of its activity leads to a delay in the absorption of glucose and therefore to a decrease in its levels in the blood. The inhibition of these enzymes in the intestinal villi membrane slows the digestion of carbohydrates[22,32]. Notably, the results showed that the extract of the stem of CKM was a potent inhibitor of α-glucosidase. CKM plants from other parts of the world, such as Brittany and Tunisia, already showed antimicrobial activity[2,26]. In those contests, the extract of the entire plants was studied for the overall activity. The antibacterial activity of CKM is conceivably due to the active molecules contained in the extracts, but although the content in polyphenols in stems and leaves was twice that of the seeds, these did not show inhibitory activity when tested alone. Indeed, only seeds extract is able to inhibit both Gram-negative and Gram-positive bacteria. The latter finding can be due to the major content of well knows active molecules[33-35]. Therefore, double and triple combinations of the extracts were tested to evaluate the possible synergic action among the different compounds of the plant parts[24]. The double combination of leaves and stems, stems and seeds, as well as seeds and leaves, resulted in bacte-

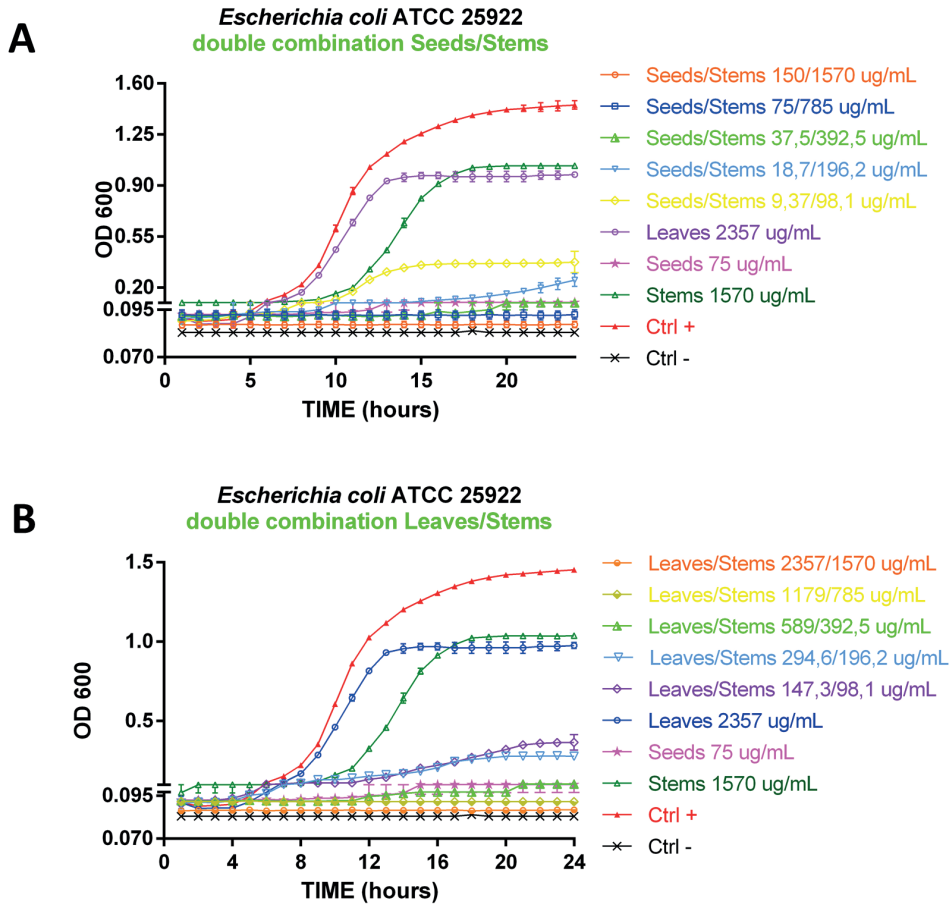

Figure 6. Growth inhibition curves analysis of *Escherichia coli* ATCC 25922 for synergic double combinations "leaves + stems" (growth inhibition 99.98% at 294.6/196.2 µg/mL) and "seeds + stems" (growth inhibition 99.98% at 18.7/196.2 µg/mL). All experimental data are expressed as mean ± standard error (SD). Significance was assessed by ANOVA or Student's *t*-test: $p < 0.05$.

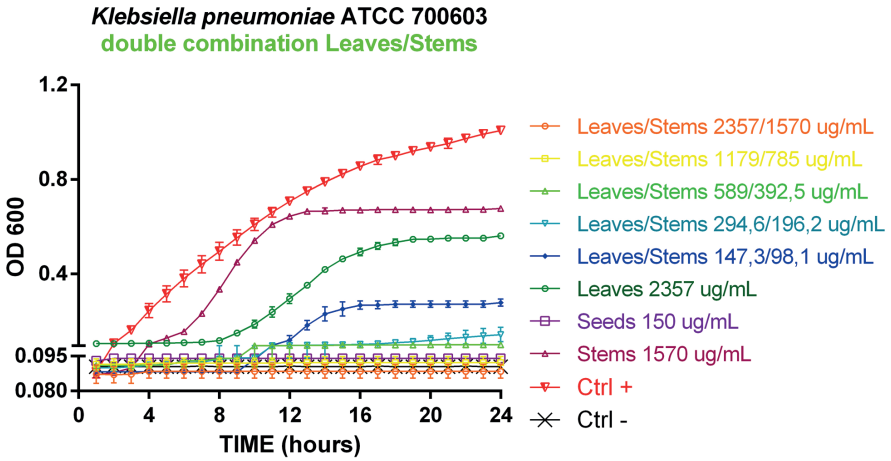

Figure 7. Growth inhibition curves analysis of *Klebsiella pneumoniae* ATCC 700603 for synergic double combination "leaves + stems" (growth inhibition 99.98% at 294.6/196.2 µg/mL). All experimental data are expressed as mean ± standard error (SD). Significance was assessed by ANOVA or Student's *t*-test: $p < 0.05$.

rial inhibition confirming synergic action between the two parts. Unfortunately, triple combination (leaves, stems, and seeds) showed no synergism, indicating that the antibacterial activity doesn't change by adding one of the three elements, indiscriminately. Based on these results, the following strains were the most sensitive: *Acinetobacter baumannii* and *Pseudomonas aeruginosa* for all three "synergic double combinations", *Escherichia coli* for "leaves + stems" (growth inhibition 99.98% at 294.6/196.2 µg/mL) and "seeds + stems" (growth inhibition 99.98% at 18.7/196.2 µg/mL); instead *Klebsiella pneumoniae* only for "leaves + stems" combination (growth inhibition 99.98% at 294.6/196.2 µg/mL).

Furthermore, to analyze the anti-inflammatory effect of the extracts of CKM, we evaluated the gene expression of inflammatory and oxidative stress markers *in vitro* inflammation model consisting of macrophages exposed to LPS.

Following treatment with LPS alone, all the marker genes of the inflammatory response, IL1β, IL-6, and TNFα, and of a marker of oxidative stress HO-1, were upregulated, whereas the extracts of CKM was able to downregulate the aforementioned markers conferring an anti-inflammatory and antioxidant activity to the extracts.

Finally, our results showed that all extracts did not present a cytotoxic effect on human cells from healthy donors while surprising only the leaf extract of CKM displayed an anti-proliferative effect in U266 cell lines (multiple myeloma cells).

Conclusions

Our study showed that extracts of different parts of the CKM plant have antioxidant, antimicrobial, anti-inflammatory and anti-proliferative effects. These beneficial properties could be exploited in the prevention or as therapeutic support of many diseases.

Conflict of interest

The authors declare no conflicts of interest.

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