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Evaluation of Mediterranean Tree Leaves as Valuable Biomass of Digestive Enzymes and Bacterial Inhibitors in the Concept of Circular Bioeconomy

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Abstract: This study aspires to evaluate the antibacterial and inhibitory effects of carbohydrate digestive enzymes in tree leaves that are widely distributed in the Mediterranean region. Leaves were sequentially extracted with solvents of increasing polarity. The results demonstrated a wide range of phenolic (3.5–770.7 mg gallic acid equivalent g^{-1}) and flavonoid (0.2–321.3 mg catechin equivalent g^{-1}) contents in leaf extracts. The minimum inhibitory and bactericidal concentration of leaf extracts was determined for six bacteria using the broth microdilution method. The polar extracts of carob, lentisk, and white mulberry leaves exerted strong antibacterial potency against Gram-positive bacteria, while the susceptibility of *Escherichia coli* on relative apolar extracts of carob, fig, and olive leaves was also observed. In parallel, the inhibitory effects of leaf extracts on carbohydrate digestive enzymes were evaluated. A robust inhibition of α-glucosidase was found for carob and lentisk leaf extracts, followed by extracts produced by white mulberry and olive leaves. Carob and lentisk leaves also act as a-amylase inhibitors at high concentrations. Overall, this study provides valuable data for the nutraceutical value of the "forgotten" treasure of Mediterranean tree leaves and assesses these plants as potential sources of antibacterial and carbohydrate digestive enzyme inhibitory agents for drug discovery.

Keywords: agricultural residues; antidiabetic activity; antimicrobial activity; phenolic compounds; α-glucosidase; α-amylase

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1. Introduction

Agricultural residues and food industry wastes are inexhaustive sources of bioactive compounds with diverse biological and health effects, attracting the interest of food and pharmaceutical sectors [1]. The cultivation of fruit crops generates an enormous amount of leaves that are discarded. Tree leaves are an underestimated treasure of boundless biomass that is rich in bioactive molecules. The exploitation of these agri-food waste materials as a renewable and inexpensive source of natural products is an attractive strategy from an economic and environmental point of view. The valorization of agri-food waste is also a sustainability goal that has been adopted in Europe. In particular, the circular economy model promotes sustainable and resource-efficient policies with multiple long-term benefits, adopting strategies to convert low-value side streams/residues/wastes into valuable products [2,3]. Considering that most new therapeutic drugs approved in the last forty years are natural products or are inspired by nature, the evaluation and utilization of discarded tree leaves is a promising challenge [4].

The need for new antimicrobial compounds is emanated from continuing global concerns about antimicrobial resistance. The World Health Organization's (WHO) Global Antimicrobial Resistance Surveillance program found high levels of antimicrobial resistance in an array of bacteria such as *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella* spp.,

Acinetobacter spp., *Staphylococcus aureus*, and *Streptococcus pneumoniae* [5]. The utilization of natural products to address global concerns is a successful strategy as numerous patents have been registered for the isolation and commercialization of natural products as antimicrobial agents [6–8]. In addition, a plethora of research articles recommend the use of pure phytochemicals and/or plant extracts as antimicrobial agents for the food industry, as natural products are able to extend the shelf life of foods [9,10].

Diabetes mellitus is the third leading cause of morbidity and mortality, after heart attack and cancer, imposing a heavy global burden on public health as well as socio-economic development [11]. Although its incidence has started to decrease in some countries, the prevalence of diabetes has increased in recent decades in the majority of developed and developing countries [12]. In the last two decades, plants have been used for the treatment and management of diabetes and associated conditions, having been adopted in various healthcare systems. The reduction in gastrointestinal glucose production and absorption through the inhibition of carbohydrate-digesting enzymes is one of the most potent therapeutic strategies for the amelioration of hyperglycemia. In particular, the inhibition of α-glucosidase and α-amylase remarkably reduces the postprandial increase in blood glucose, successfully contributing to its management following the consumption of carbohydrate foods [13]. In recent decades, our knowledge of the inhibitory effects of plant-derived substances on carbohydrate-digesting enzymes has increased, highlighting natural products as potential antidiabetic agents [14].

The utilization of unexploited tree leaves for the production of extracts with antimicrobial and/or antidiabetic effects could be an approach that opens new horizons in the discovery of new active agents and promotes sustainable agriculture. Previous studies reported that aqueous or alcoholic leaf extracts produced by carob tree, fig tree, mastic tree, Mediterranean medlar, mulberry tree, olive tree, and walnut tree contain inhibitors of digestive enzymes, namely α-glucosidase and α-amylase [15–21]. In addition, their polar extracts exert inhibitory effects against diverse pathogenic bacteria, although significant differences in bacterial resistance to extracts were found [22–28]. The present study aims to compare the antibacterial and enzyme-inhibitory effects of leaf extracts from common Mediterranean fruit-bearing trees, namely carob tree, fig tree, mastic tree, Mediterranean medlar, mulberry tree, olive tree, and walnut tree. All leaf samples were chosen due to their distribution in the Mediterranean basin, especially in Cyprus. Taking into consideration the impact of climatic conditions on the accumulation of bioactive compounds, the comparative study of tree leaves from Cyprus is really interesting since the island lies at the crossroads of Europe, Africa, and Asia. Furthermore, the serial exhaustive extraction method, using a solvent of increasing polarity, from non-polar (hexane) to polar (water), was used to prepare extracts, in contrast to previous studies in which polar solvents were used. This approach allows us to evaluate the inhibitory effects of a wide variety of phytoconstituents present in plant materials. The present study is expected to provide valuable data on the nutraceutical value of selected leaves and to promote tree leaves as potential antibacterial and inhibitory agents in carbohydrate digestive enzymes for the food and pharmaceutical industry.

2. Materials and Methods

2.1. Chemicals and Reagents

Hexane, dimethyl sulfoxide (DMSO), gallic acid, sodium nitrite ($NaNO_2$), aluminum chloride ($AlCl_3$), sodium dihydrogen phosphate anhydrous, disodium hydrogen phosphate dodecahydrate, and soluble starch were obtained from Scharlau Chemie (Barcelona, Spain). Acetone and methanol were acquired from Honeywell (Charlotte, NC, USA). Folin–Ciocalteu reagent, sodium carbonate, catechin, p-nitrophenyl-α-D-glucopyranoside (PNG), and 3,5-dinitrosalicylic acid (DNS) were obtained from Sigma-Aldrich (Steinheim, Germany). Sodium hydroxide and sodium chloride were purchased from Merck (Darmstadt, Germany). Finally, α-glucosidase from *Saccharomyces cerevisiae* and α-amylase from porcine pancreatic were acquired from Megazyme (Sydney, Australia).

2.2. Plant Materials

Mature leaves for seven fruit-bearing trees, namely carob tree (*Ceratonia siliqua* L.), fig tree (*Ficus carica* L.), lentisk (*Pistacia lentiscus* L.), Mediterranean medlar tree (*Crataegus azarolus* L), olive tree (*Olea europaea* L.), walnut tree (*Juglans regia* L.), and white mulberry tree (*Morus alba* L.) were harvested from Limassol District, Cyprus. More specifically, carob and lentisk leaves were collected from native trees in Ypsonas village (34.702521, 32.962417). Mediterranean medlar, olive, walnut, and white mulberry leaves were harvested from small-size orchards in Pachna village (34.777682, 32.786152). Approximately 1000 g of each plant material was cleaned with distilled water and oven-dried at 40 °C until a constant weight was obtained. Finally, dried leaves were pulverized using an electric grinder, Sage BCG820BSSUK (Breville Group Limited, Sydney, Australia). All plant materials were deposited in the department's herbarium.

2.3. Serial Extraction of Plant Material

Next, 5 g of dry powdered leaves was mixed with 30 mL of solvent in the following order: hexane, acetone, methanol, and water. The mixture was placed in an ultrasonic bath and sonicated for 60 min at 60 °C for hexane, methanol, and water and at 40 °C for acetone. After the ultrasound treatment, the mixture was allowed to cool at room temperature and then centrifuged for 10 min at 2500 rpm. The supernatant was collected and the remaining solid was extracted again with the next solvent, according to the solvent sequence described above. The solvent of extracts was evaporated using a rotary evaporator to obtain dry extracts, and these were stored at −20 °C until further analysis. Five replicates were performed for each extraction [29].

2.4. Phenolic and Flavonoid Contents of Leaf Extracts

The total phenolic content (TPC) of the plant extracts was determined using a 96-well microplate Folin–Ciocalteu method [30]. The extracts were re-dissolved in 20% (*v*/*v*) DMSO–water and filtered through a 0.45 µm membrane filter to remove any insoluble particles. Then, 20 µL of extract solution was mixed with 100 µL of Folin–Ciocalteu reagent (1:4 *v*/*v* diluted with water), and the mixture was shaken for 1 min in a 96-well microplate. The mixture was allowed to stand for 4 min, and then 75 µL of a saturated solution of sodium carbonate were added. The mixture was shaken for 1 min, and then allowed to stand in the dark at room temperature for 2 h. The absorbance of the reaction mixture was then measured at 750 nm using a Thermo Scientific Multiskan GO plate reader (ThermoFisher Scientific, Waltham, MA, USA). Gallic acid was used as a standard for calibration, and total phenolics were expressed as mg of gallic acid equivalent (GAE) g^{-1} extract.

The total flavonoid content (TFC) of the extracts was investigated using the aluminum chloride colorimetry method. Briefly, 25 µL of each extract was mixed with 100 µL of distilled water and 10 µL of a 50 g L^{-1} sodium nitrite solution in a 96-well microplate. After waiting for 5 min, 15 µL of $AlCl_3$ solution (100 g L^{-1}) was added to the reaction mixture. The mixture was allowed to stand for 6 min. Then, 50 µL of NaOH solution (1 mol L^{-1}) and 50 µL of distilled water were added and the reaction mixture was shaken for 30 s. The absorbance of the mixture was measured at 510 nm using a plate reader. Catechin was used as the reference standard, and TFC values were expressed as mg of catechin equivalent (CE) g^{-1} extract [31].

2.5. Inhibitory Effect of Leaf Extracts on Carbohydrate Digestive Enzymes

A mixture containing the extract solution (100 µL, 500 µg mL^{-1}) and 50 µL of 0.1 mM phosphate buffer (pH = 6.8) containing a-glucosidase (1.0 U mL^{-1}) was prepared and incubated at 37 °C. After 10 min of incubation, 50 µL of PNG (5 mM in 0.1 mM phosphate buffer, pH = 6.8) was added, and the reaction mixture was allowed to stand for 5 min. Finally, the absorbance of the mixture was measured at 405 nm against a blank solution where PNG was replaced with a buffer. The control, which represents 100% enzyme activity

was prepared by replacing the extract solution with 20% (v/v) DMSO–water. Results were expressed as the % inhibitory activity of the extracts compared to the control [32].

For the α-amylase assay, 100 µL of the extract solution (10 mg mL^{-1} in 20% v/v DMSO) was mixed with 100 µL of α-amylase solution (2 U mL^{-1} in 20 mM sodium phosphate containing 6.7 mM NaCl, pH 6.9), and the resulting mixture was incubated at 35 °C for 10 min. Then, 200 µL of soluble starch (1% w/v in the buffer) was added, and the reaction mixture was incubated at 35 °C for 20 min. Finally, the reaction was terminated by adding 200 µL of DNS reagent. The mixture was then boiled for 10 min, cooled down, and appropriately diluted (1:10 with water). The absorbance of the reaction mixture was measured against a blank sample at 540 nm. The results are expressed as the % inhibitory activity of the extracts compared to the control [33].

2.6. Bactericidal Effects of Leaf Extracts

For the determination of bactericidal effects of extracts, each bacterium was grown in suitable agar. More specifically, Listeria Agar, Baird Parker Agar, Mannitol Egg Yolk Polymyxin (MYP) Agar, Xylose Lysine Deoxycholate (XLD) Agar, Tryptone Bile Glucuronic (TBX) Agar, and Sakazakii Agar were used for the growth of *Listeria monocytogenes* ATCC 23074 (serotype 4b), *Staphylococcus aureus* ATCC 6538, *Bacillus cereus* ATCC 6089, *Enteritidis* NCTC 5188, *Escherichia coli* ATCC 11775 and *Cronobacter sakazakii* ATCC 29544, respectively. For the experiments, one colony of each of the bacteria was inoculated into 10 mL Brain Heart Infusion (BHI) Broth and incubated at 37 °C. Briefly, 50 µL of each plant extract was transferred, in triplicate, in a 96-well plate. An aliquot of 40 µL of BHI broth and 10 µL of microbial suspension were added to reach a final volume of 100 µL in each well. The final concentrations of the plant extracts in the wells were 2000, 1000, and 500 µg mL^{-1}. Microbial suspensions were adjusted so that the final concentration in the wells was 10^6 cfu mL^{-1}. Screening for the bactericidal activity of plant extracts was performed by adding 10 µL of each well in BHI agar plates, and the results were obtained following incubation for 24 h at 37 °C. Controls of 10%, 5%, and 2.5% v/v DMSO and microbial cultures were also tested. Stock solutions of 10 mg mL^{-1} for each plant extract were prepared using DMSO as a diluent. Aqueous and methanolic extracts were prepared in 50% v/v DMSO, while hexanic and acetonic extracts were prepared in pure DMSO. Stock solutions were further diluted using water to prepare working solutions [34,35].

2.7. Statistical Analysis

All measurements were performed in triplicate, and the obtained results are expressed as mean values ± standard deviation (SD). The means were compared, and statistically significant differences were determined through a one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (at a 95% confidence level). The differences between individual means were considered significant at $p < 0.05$. All statistical analyses were performed using RStudio statistical software (version 1.3.1073).

3. Results and Discussion

3.1. Phenolic and Flavonoid Contents of Leaf Extracts

Phenolics represent the largest category of bioactive phytochemicals and are the most widely distributed substances in the plant kingdom. Thus, the phenolic contents of all extracts were determined as they can be considered an index of the yield of extraction as well as potential inhibitors of digestive enzymes and bacteria growth [36]. The results show a great diversity of phenolic contents of leaf extracts, the TPCs ranged from 3.5 mg GAE g^{-1} to 770.7 mg GAE g^{-1} (Figure 1). Substantial differences were found between plant materials, although this is expected as they belong to six family plants. Undoubtedly, lentisk leaves are the richest source of phenolic compounds among studied leaves. Its phenolic content is at least two-fold higher than other plant materials. Previous research also demonstrated the high phenolic content of lentisk leaves compared to other plant materials [37]. On the other hand, fig leaves and white mulberry leaves contain the lowest amounts of phenolic

compounds. The results also demonstrate the significance of an extraction solvent as the phenolic contents are strongly affected by the solvent used. In general, polar solvents such as methanol and water are appropriate for the recovery of phenolics as they are usually found as glycosylated derivatives in plants. In the case of fig leaves, the acetone recovered the maximum amount of phenolics. The results can be a useful guide for the use of suitable solvents for the extraction of phenolics from the leaves studied. Furthermore, a distinct classification of leaves based on phenolic content is provided.

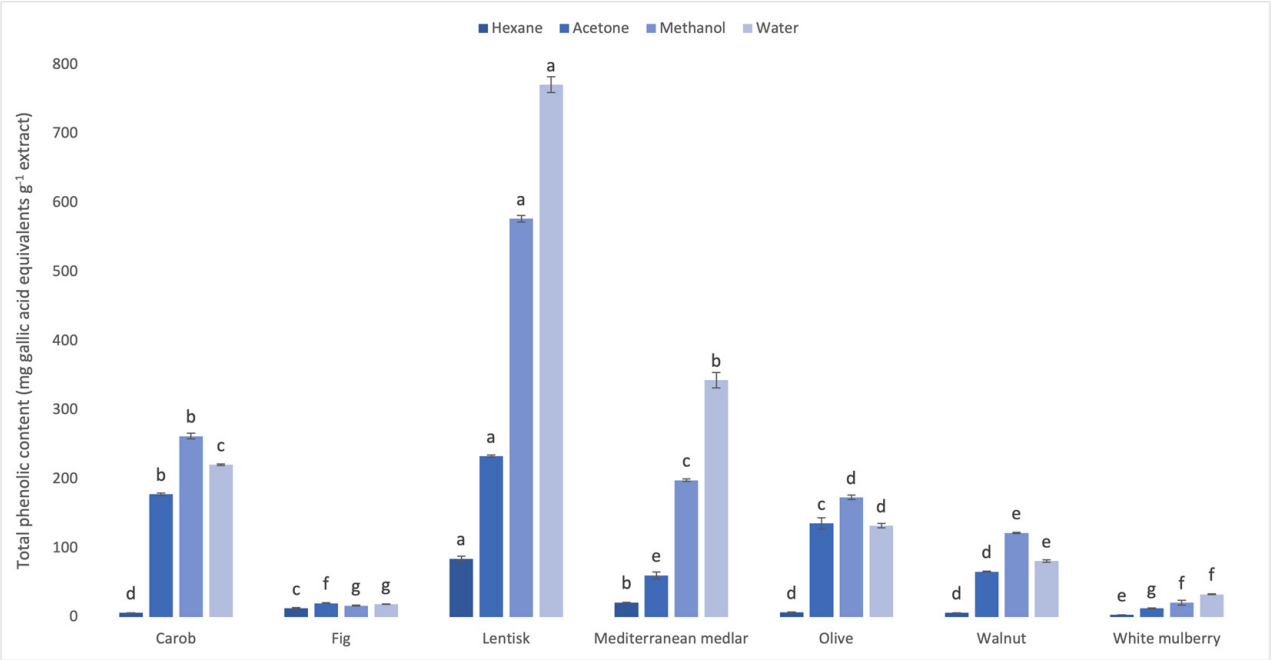

Figure 1. Total phenolic contents of Mediterranean tree leaf extracts. Results are expressed as mg gallic acid equivalent (GAE) g^{-1} material. The data are indicated as the mean ± SD. Different letters indicate statistically significant differences in contents ($p < 0.05$, Duncan's test).

The flavonoid contents of extracts were also assessed, as they present a group of phenolics with special antimicrobial and antidiabetic properties [38]. Similar to phenolic content, a notable fluctuation in the TFCs of extracts was observed (Figure 2). The latter can be attributed to genetic factors as well as the extraction medium. Lentisk leaves are also a valuable source of flavonoids, but the highest TFC was found in olive leaves. Mediterranean medlar leaves also contain significant amounts of flavonoids. The results demonstrate that fig leaves and white mulberry leaves had the lowest TFC among the studied leaves. According to findings, leaves harvested from lentisk, olive, and Mediterranean medlar trees can be considered biomass for the recovery of flavonoids. In addition, Figure 2 demonstrates interesting observations of the extraction solvent; acetone is the most efficient solvent for the recovery of flavonoids for the majority of leaves. However, the maximum yield of flavonoids from lentisk and Mediterranean medlar leaves was obtained with the use of water. This alteration may be correlated with structural differences in the extracted flavonoids. Olive leaves mainly contain luteolin, apigenin, and quercetin derivatives; lentisk leaves are rich in flavan-3-ols, myricetin, and quercetin derivatives; and Mediterranean medlar leaves comprise glycosylated derivatives of vitexin and quercetin [39–41].

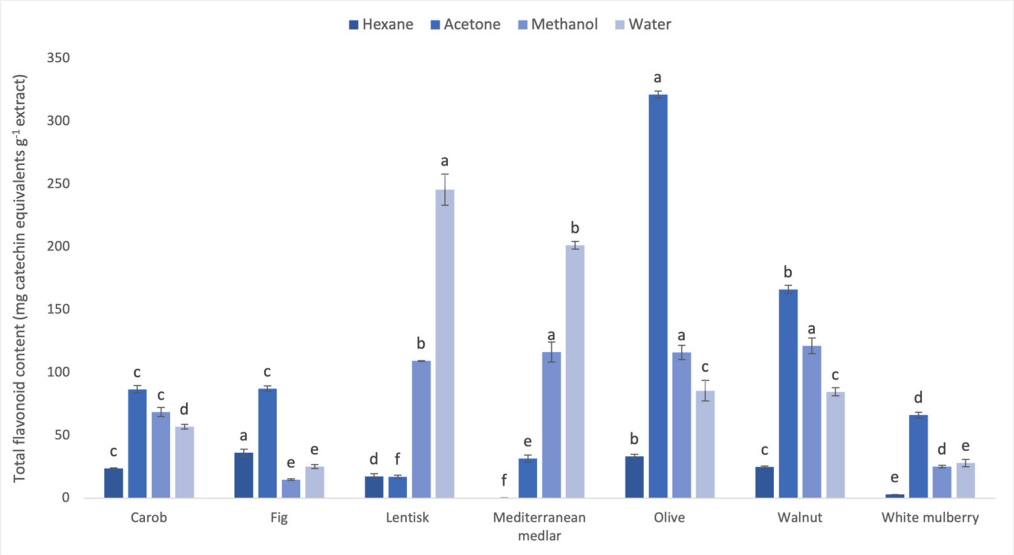

Figure 2. Total flavonoid contents of Mediterranean tree leaf extracts. Results are expressed as mg catechin equivalent (CE) g^{-1} material. The data are indicated as the mean ± SD. Different letters indicate statistically significant differences in contents ($p < 0.05$, Duncan's test).

3.2. Inhibitory Effect of Leaf Extracts on Carbohydrate Digestive Enzymes

The inhibitory effects of leaf extracts on a-glucosidase and a-amylase were determined as these enzyme assays per se are well-established biomarkers, which indicate possible antidiabetic effects. The digestive enzyme of a-amylase is responsible for transforming dietary starch to glucose prior to absorption. Its inhibition can lead to a reduction in post-prandial hyperglycemia in diabetic condition. Alpha-glucosidase is also a digestive enzyme, which is able to catalyze the cleavage of disaccharides to glucose. The inhibitors of a-glucosidase can retard the uptake of dietary carbohydrates and suppress postprandial hyperglycemia [11,14]. Therefore, the inhibition of α-glucosidase is considered an effective strategy to control diabetes. All extracts were more effective in inhibiting the activity of α-glucosidase than α-amylase since lower concentrations of extracts are required. Figure 3 summarizes the inhibitory activity of leaf extracts on the α-glucosidase enzyme. Lentisk and carob leaf extracts were the most potent inhibitors of a-glucosidase. In particular, all extracts of lentisk leaves demonstrated a percent a-glucosidase inhibitory activity of over 95%. Recently, Sehaki et al. (2023) correlated the strong a-glucosidase inhibitory effect of Algerian lentisk leaf extract with the presence of flavonoids, namely epigallocatechin and its derivatives [42]. However, the high enzyme imbibition by an apolar extract (hexane) and polar extracts (methanol and water) suggests that many compounds of different polarity contribute to this activity. A robust inhibitory effect was also found for polar extracts of carob leaves. According to a previous study, catechin and its derivatives as well as two hydroxybenzoic acids, namely gallic acid and gentsic acid, are the main phenolic constituents of methanol carob leaf extract [43]. Gallic acid has been approved as an a-glycosidase inhibitor; its activity is comparable with acarbose, a commercial active substance, for the management of type 2 diabetes [13]. Furthermore, catechins exhibit a favorable inhibitory effect on a-glucosidase depending on their conformational and substitution [44]. Olive leaves can also be considered as a potential a-glucosidase inhibitor since their acetonic and hexanic extracts have inhibitions of 84.9 ± 1.3 and 72.7 ± 1.0%. This effect may be correlated with the potent inhibitory effect of hydroxytyrosol, a characteristic compound of olive leaves, as well as the inhibitory effects of flavonoids such as luteolin, quercetin, and apigenin [45,46]. The hexanic leaf extract of white mulberry also had a strong inhibitory effect (85.1 ± 1.0%), although its phenolic and flavonoid contents were low. However, a bioassay-guided study manifests that the apolar types of moracin are the most active ingredients of these leaves, partially explaining the potent activity

of hexanic extract [47]. On the other hand, the extracts derived from fig, walnut, and Mediterranean medlar trees exhibit a weak-to-medium inhibitory activity. The inhibitory effect of Mediterranean medlar leaf extracts is surprising since they are rich in phenolic compounds (Figure 1). The conformational structure of Mediterranean medlar phenolics is perhaps responsible for the lack of inhibitory activity.

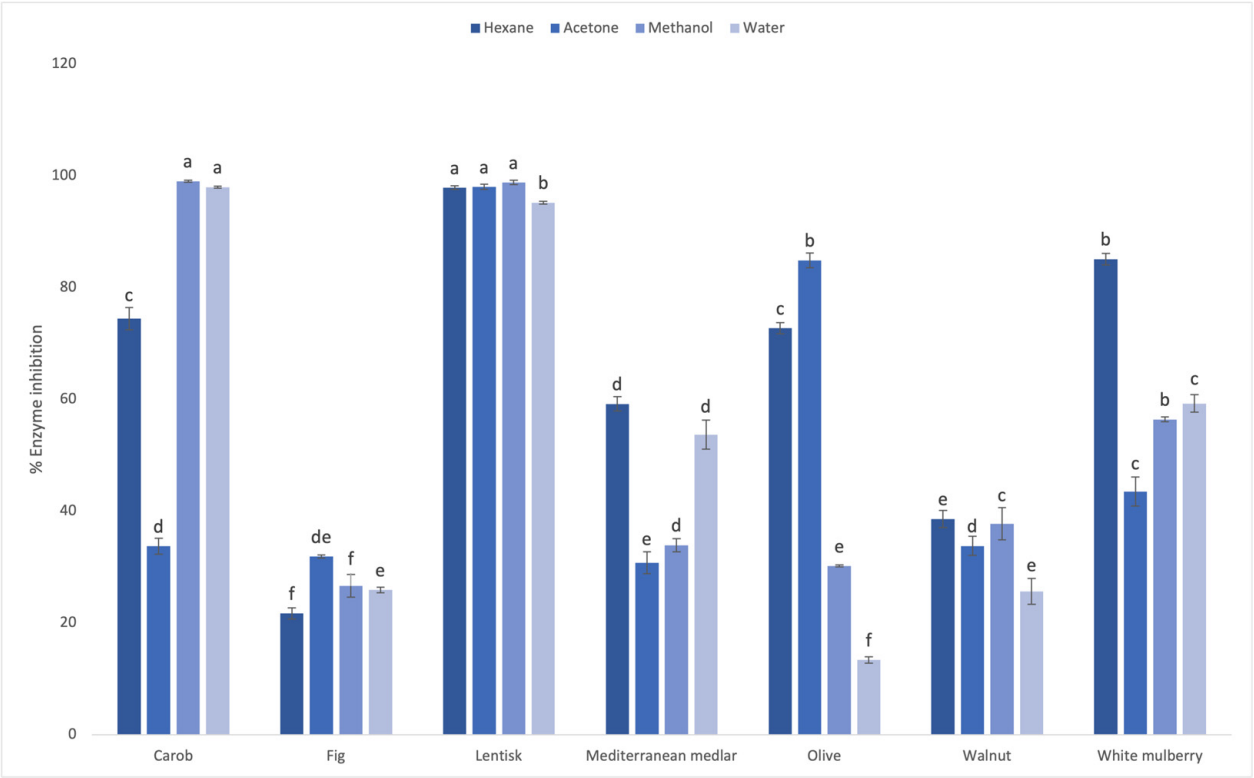

Figure 3. The percentage of α-glucosidase inhibition (%) of Mediterranean tree leaf extracts. The data are indicated as the mean ± SD. Different letters indicate statistically significant differences in contents ($p < 0.05$, Duncan's test).

The results show that higher concentrations of leaf extracts were needed to inhibit α-amylase since phenolic constituents are partially absorbed by starch during gelatinization, and different inhibition mechanisms occur [48]. Similar to the a-glucosidase assay, lentisk and carob leaf extracts present the highest a-amylase inhibition among the studied leaf extracts (Figure 4). Methanolic and aqueous extracts of both plants exhibited an inhibition of a-amylase of over 93%, whereas their hexanic and acetonic extracts had weak-to-moderate inhibitory effects. Regarding carob leaves, only information on their decoction is available, and their active constituents are unknown [20]. On the other hand, the inhibitory effect of lentisk leaves on a-amylase has been studied. This activity of lentisk leaves is linked with flavanone glycosides and luteolin. In addition, a dose-dependent increase in the percentage of inhibitory activity was observed, demonstrating that lentisk contains a substantial amount of a-amylase inhibitors [49]. The rest of the leaves produced extracts with a low inhibitory effect on a-amylase as their percentage inhibitions were lower than 50%, even though high concentrations of extracts were tested. In contrast to the glucosidase assay, a very weak a-amylase inhibitory activity was found for all hexanic extracts (<26%) and acetonic extracts (<37%), apart from the acetonic extract of lentisk leaves (62%). Overall, the polar extracts of lentisk and carob leaves can be considered potential inhibitors of both carbohydrate digestive enzymes.

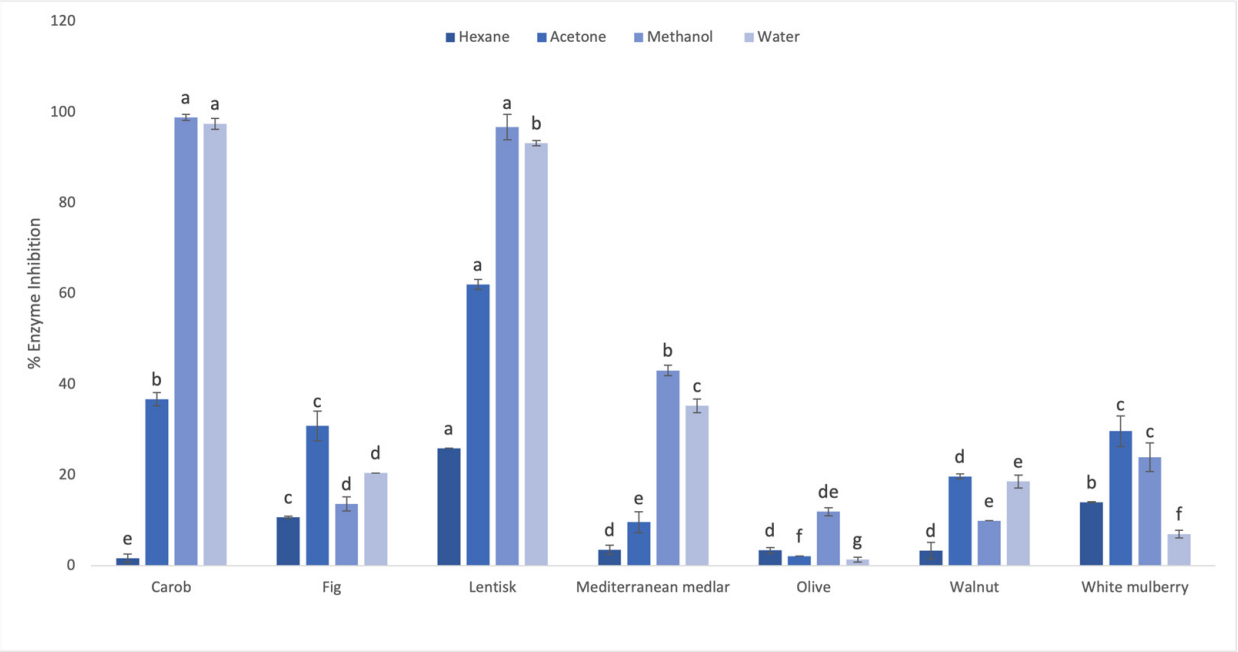

Figure 4. The percentage of α-amylase inhibition (%) of Mediterranean tree leaf extracts. The data are indicated as the mean ± SD. Different letters indicate statistically significant differences in contents ($p < 0.05$, Duncan's test).

3.3. Anti-Bacterial Potential of Leaf Extracts

The antibacterial potential of leaf extracts against Gram-positive bacteria is presented in Table 1. Leaf extracts were more efficient against *B. cereus* compared to other Gram-positive bacteria. All leaf material exhibited an inhibitory effect against *B. cereus* at a concentration of 250 μg g^{-1} or higher, whereas the required MBC values were 500 μg g^{-1} or higher. The strongest antibacterial activity was found for carob and lentisk leaves, followed by white mulberry and walnut leaves. This is the first evidence of the bactericidal effect of carob leaves against *B. cereus* since only the anti-bacillus potential of carob seeds was previously reported [50]. Furthermore, the MBC value of carob leaf extract was five-fold lower than the corresponding value of carob seeds. On the contrary, the efficacy of lentisk leaves in inhibiting the growth of *B. cereus* has been studied, but previous studies focused on a volatile fraction of leaves [51]. In the present study, the polar extracts were more active than the hexanic extract, for which the composition is similar to the volatile fraction. Walnut and white mulberry leaves also inhibited *B. cereus* growth, and MIC and MBC values were determined at 500 μg g^{-1} and 1000 μg g^{-1} as previously demonstrated [26,46]. The leaf extracts tested were also efficient against *S. aureus*, with results revealing the superiority of carob and white mulberry extracts to inhibit and potentially control *S. aureus*. Fig and lentisk leaves also exerted significant inhibitory and bactericidal activity against *S. aureus*. The anti-bacterial potency of polar extracts of these plant materials has been previously reported and is in line with our findings [22,52–54]. Their antimicrobial potential is mainly attributed to the presence of flavonoids and phenolic acids. Regarding *L. monocytogenes*, all leaf extracts demonstrated a weaker inhibitory effect, compared to the other Gram-positive bacteria tested. The most promising extracts for the control *L. monocytogenes* were the methanolic extracts of lentisk and white mulberry leaves. The inhibitory effect of lentisk leaf essential oils against *Listeria* strains was previously investigated [51,55], but the MIC and MBC of polar extracts are presented here for the first time. In contrast, the susceptibility of Listeria bacteria to alcoholic extracts of white mulberry leaves was previously reported and linked with the presence of flavonoids [56].

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of leaf extracts, against Gram-positive bacteria. MIC and MBC values were expressed as (μg mL^{-1}).

Plant	Solvent	*Bacillus cereus*		*Listeria monocytogenes*		*Staphylococcus aureus*	
		MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Carob	Hexane	1000	>2000	>2000	>2000	500	1000
	Acetone	1000	>2000	>2000	>2000	500	1000
	Methanol	250	500	>2000	>2000	250	500
	Water	250	500	>2000	>2000	250	500
Fig	Hexane	1000	>2000	>2000	>2000	500	1000
	Acetone	1000	1000	>2000	>2000	500	1000
	Methanol	>2000	>2000	>2000	>2000	>2000	>2000
	Water	>2000	>2000	>2000	>2000	>2000	>2000
Lentisk	Hexane	1000	>2000	>2000	>2000	500	1000
	Acetone	500	1000	>2000	>2000	500	1000
	Methanol	250	500	500	1000	500	1000
	Water	250	500	>2000	>2000	500	1000
Mediterranean medlar	Hexane	>2000	>2000	>2000	>2000	500	1000
	Acetone	>2000	>2000	>2000	>2000	500	1000
	Methanol	1000	1000	>2000	>2000	500	1000
	Water	>2000	>2000	>2000	>2000	>2000	>2000
Olive	Hexane	>2000	>2000	>2000	>2000	>2000	>2000
	Acetone	>2000	>2000	>2000	>2000	>2000	>2000
	Methanol	>2000	>2000	>2000	>2000	>2000	>2000
	Water	>2000	>2000	>2000	>2000	>2000	>2000
Walnut	Hexane	>2000	>2000	>2000	>2000	1000	>2000
	Acetone	500	1000	>2000	>2000	1000	>2000
	Methanol	>2000	>2000	>2000	>2000	>2000	>2000
	Water	>2000	>2000	>2000	>2000	>2000	>2000
White mulberry	Hexane	500	1000	>2000	>2000	250	500
	Acetone	500	1000	>2000	>2000	250	500
	Methanol	>2000	>2000	500	>2000	>2000	>2000
	Water	>2000	>2000	>2000	>2000	>2000	>2000

Noticeably, the investigated leaf extracts were inefficient in inhibiting the growth of Gram-negative bacteria (Table 2). The tested leaf materials had no remarkable bactericidal activity against *S. enterica* and *C. sakazakii* since MIC values were higher than 2000 μg mL^{-1} for all extracts. A moderate inhibitory effect against *E. coli* was observed in carob, fig, and olive leaf extracts. In contrast to Gram-positive bacteria, non- and medium polar extracts inhibited the growth of *E. coli*. Previous studies suggest that *E. coli* bacteria are sensitive to relative non-polar phytochemicals as they are able to pass through their cell membrane [57,58]. The susceptibility of *E. coli* on carob, fig, and olive leaf extracts is not unknown, but previous studies utilized their polar extracts at higher concentrations to effectively control the bacterium. The potent inhibitory effect of hexanic extract of carob leaves was demonstrated by Kivcak et al. (2002), but the MIC and MBC values were not determined [59].

Overall, MIC and MBC values provide valuable information for the antibacterial properties of leaf materials. It is clear that biomass can be utilized as a source of antibacterial agents for Gram-positive bacteria, although substantial differences were observed for the different bacteria tested. The most active plant materials were carob, lentisk, and white mulberry leaves. The results obtained from this study also highlight the impact of the extraction solvent, as relatively polar extracts better inhibited the growth of Gram-positive bacteria, namely *B. cereus*, *S. aureus*, and *L. monocytogenes*, whereas less polar extracts had potent inhibitory effects on *E. coli*, a Gram-negative bacterium.

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of leaf extracts, against Gram-negative bacteria. MIC and MBC values are expressed as (μg mL^{-1}).

Plant	Solvent	*Cronobacter sakazakii*		*Escherichia coli*		*Salmonella enterica*	
		MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Carob	Hexane	>2000	>2000	1000	>2000	>2000	>2000
	Acetone	>2000	>2000	>2000	>2000	>2000	>2000
	Methanol	>2000	>2000	>2000	>2000	>2000	>2000
	Water	>2000	>2000	>2000	>2000	>2000	>2000
Fig	Hexane	>2000	>2000	>2000	>2000	>2000	>2000
	Acetone	>2000	>2000	1000	>2000	>2000	>2000
	Methanol	>2000	>2000	>2000	>2000	>2000	>2000
	Water	>2000	>2000	>2000	>2000	>2000	>2000
Lentisk	Hexane	>2000	>2000	>2000	>2000	>2000	>2000
	Acetone	>2000	>2000	>2000	>2000	>2000	>2000
	Methanol	>2000	>2000	>2000	>2000	>2000	>2000
	Water	>2000	>2000	>2000	>2000	>2000	>2000
Mediterranean medlar	Hexane	>2000	>2000	1000	>2000	>2000	>2000
	Acetone	>2000	>2000	>2000	>2000	>2000	>2000
	Methanol	>2000	>2000	>2000	>2000	>2000	>2000
	Water	>2000	>2000	>2000	>2000	>2000	>2000
Olive	Hexane	>2000	>2000	1000	>2000	>2000	>2000
	Acetone	>2000	>2000	>2000	>2000	>2000	>2000
	Methanol	>2000	>2000	>2000	>2000	>2000	>2000
	Water	>2000	>2000	>2000	>2000	>2000	>2000
Walnut	Hexane	>2000	>2000	>2000	>2000	>2000	>2000
	Acetone	>2000	>2000	>2000	>2000	>2000	>2000
	Methanol	>2000	>2000	>2000	>2000	>2000	>2000
	Water	>2000	>2000	>2000	>2000	>2000	>2000
White mulberry	Hexane	>2000	>2000	>2000	>2000	>2000	>2000
	Acetone	>2000	>2000	>2000	>2000	>2000	>2000
	Methanol	>2000	>2000	>2000	>2000	>2000	>2000
	Water	>2000	>2000	>2000	>2000	>2000	>2000

4. Conclusions

The present study clearly demonstrated that Mediterranean tree leaves are an unexploited reservoir of inhibitors of bacteria and carbohydrate digestive enzymes. The results contribute to the ongoing scientific investigation of the application of leaf extracts as anti-bacterial and antidiabetic agents for the food and pharmaceutical industry. The results also classify Mediterranean tree leaves based on their ability to inhibit the activity of two carbohydrate digestive enzymes related to mellitus diabetes. More specifically, lentisk and carob leaves can be considered as valuable biomasses of α-glucosidase inhibitors, whereas their α-amylase inhibition is ineffective. Both leaves also exerted significant bactericidal potential against Gram-positive bacteria. Furthermore, fig and white mulberry can act as anti-bacterial agents at concentrations that are promising for their suitable application. Based on these findings, further investigation into effective plant materials is recommended to pinpoint the active ingredients responsible for the antibacterial and antidiabetic activity of these extracts and determine their possible mechanisms of action.

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