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ORIGINAL ARTICLE

Bioactivities of Ethanolic Extract and its Fractions of *Cistus laurifolius* L. (Cistaceae) and *Salvia wiedemannii* Boiss. (Lamiaceae) Species

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ABSTRACT

Background: *Cistus laurifolius* L. (Cistaceae) and *Salvia wiedemannii* Boiss. (Lamiaceae) have been used for treatment of some illnesses in Turkish folk medicine. In the present study, the ethanolic extract and its fractions obtained using re-extraction by hexane (Hx), chloroform ($CHCl_3$), butanol, and remaining-water (*r*-H_2O) of *C. laurifolius* were screened for their *in vitro* bioactivities. **Materials and Methods:** Activities were determined against both standard and the isolated strains of *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Acinetobacter baumannii, Staphylococcus aureus, Enterococcus faecalis*, as well as yeasts such as *Candida albicans* and *Candida parapsilosis* by microdilution method. Also, antiviral activity of *C. laurifolius* and *S. wiedemannii* extracts were tested on *herpes simplex virus*-1 (HSV-1) and *parainfluenza*-3 (PI-3) using Madin-Darby bovine kidney and vero cell lines. **Results:** Tested extracts of *C. laurifolius* (minimum inhibitory concentration 32 µg/mL) exerted a strong antimicrobial activity against Gram-negative bacteria of *E. coli, P. mirabilis, K. pneumoniae*, and *A. baumannii*. **Conclusion:** The Hx extract of *C. laurifolius* (cytopathogenic effect of 32–8 µg/mL) had antiviral activity on PI-3. Also, the *r*-H_2O, $CHCl_3$, and ethanol extracts (16–<0.25 µg/mL) of *S. wiedemannii* had significant antiviral activity on HSV-1, same as control.

Key words: Antibacterial activity, antifungal activity, antiviral activity, *Cistus laurifolius*, cytotoxicity, *Salvia wiedemannii*

SUMMARY

- The objective of this study was to evaluate the bioactivity of plant extracts used in folk medicine
- Ethanolic extract and its fractions obtained using re-extraction by hexane (Hx), chloroform ($CHCl_3$), butanol, and remaining-water (r-H_2O) of *Cistus laurifolius* L. (Cistaceae) and *Salvia wiedemannii* Boiss. (Lamiaceae) were screened against both standard and the isolated strains of *E. coli, P. aeruginosa, P. mirabilis, K. pneumoniae, A. baumannii, S. aureus, E.faecalis, C. albicans* and *C. parapsilosis* by microdilution method
- Antiviral activity were tested on HSV-1 and PI-3 using MDBK and Vero cell lines
- Extracts of *C. laurifolius* exerted a strong antimicrobial activity against *E. coli, P. mirabilis, K. pneumoniae,* and *A. baumannii*
- (MIC; 32 µg/mL)
- The Hx extract of *C. laurifolius* had antiviral activity on PI-3 (CPE; 32–8 µg/mL). Also, the r-H_2O, $CHCl_3$, and ethanol extracts (16–<0.25 µg/mL) of *S. wiedemannii* had significant antiviral activity

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INTRODUCTION

Resistances to current antimicrobiological agents have been increasing. Therefore, there is a need for developing new antimicrobial drugs. Plants can be a source of these new medications given the fact that plants have been used as active substance of numerous antibiotics by industry[1,2] and as "anti-infection drugs" by people in folk medicine.

Five *Cistus* species belonging to Cistaceae family grow naturally in Turkey. The genus *Salvia* widely distributed in Turkey is represented by 94 taxa belonging to 89 species, with a 50% ratio of endemism.[3] *Cistus* and *Salvia* species have been used against high fever, rheumatic pain, peptic ulcer, stomachache, urinary inflammations, catarrh, cold, wounds, stomachache, flatulence, constipation, rheumatic pain, wards, sunstroke, and hemorrhage in Turkish folk medicine.[4-10] It has been shown that *Cistus* and *Salvia* species have some activities, including antimicrobial[11-14] antioxidant,[11,14-16] anti-inflammatory,[17-19] analgesic,[18,20,21] and antiviral[14,22-25] activities.

In our previous studies, we demonstrated *in vitro* inhibitory effects of *Cistus laurifolius* leaves on interleukin-1 alpha and beta (IL-1α, and IL-1β) and tumor necrosis factor (TNF) biosynthesis. The extract and fractions obtained from the leaves were found to be ineffective on TNF inhibition, but effective on IL-1 inhibition. The methanolic extract and the hexane (Hx) and chloroform ($CHCl_3$) fractions were found to have remarkable IL-1α inhibitory effects in high concentrations.[17] Th ee active compounds were isolated from the $CHCl_3$ extract of *C. laurifolius*. Of these three compounds, quercetin 3-methyl ether exhibited the highest inhibitory effect on *H. pylori* at the concentration of 3.9 µg/mL.[13] Likewise, analgesic activity of the same extract was also demonstrated. *C. laurifolius* $CHCl_3$ extract and its precipitated fraction exhibited central analgesic effect in mice.[20] These studies support folkloric utilization of the plant. In addition to these confi m activities, we aimed to evaluate bioactivities of these plants.

In this study, antibacterial and antifungal activities of the ethanol (EtOH), Hx, $CHCl_3$, buthanol (BuOH), and remaining-water (*r*-H_2O) extracts of *C. laurifolius* were assessed against *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Acinetobacter*

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baumannii, Staphylococcus aureus, Enterococcus faecalis, and their isolates as well as yeasts namely; *Candida albicans* and *Candida parapsilosis*. Also, we evaluated the efficacy of *C. laurifolius* and *Salvia wiedemannii* extracts (EtOH, Hx, $CHCl_3$, BuOH, and *r*-H_2O) on *Herpes simplex virus*-1 (HSV-1) and *Parainfluenza*-3 (PI-3) viruses. The effects of these extracts were compared to reference drugs (i.e. ampicillin, ofl xacin, levofl xacin, ketoconazole, fluconazole, acyclovir, and oseltamivir).

MATERIALS AND METHODS

Plant material

The leaves of *C. laurifolius* and the aerial parts of *S. wiedemannii* were collected in the vicinity of Kurtboğazı (Ankara-May 2005) and Yunak (Ankara-May 2003) in Turkey, respectively. Voucher specimens were identifi d by a comparison with authentic specimens that had already been identifi d by Prof. Dr. Ekrem Sezik. Authenticated voucher specimens (*C. laurifolius*-GUEF No: 97B002, *S. wiedemannii*-GUEF No: 2379) were stored in the herbarium of Faculty of Pharmacy at Gazi University, Ankara, Turkey.

Preparation of the extract and its fractions

The air-dried and powdered plant (*C. laurifolius*; 510 g, *S. wiedemannii*; 360 g) was macerated in 95% EtOH (2200 mL EtOH) for 6 h in water bath adjusted to 40°C. Then, the macerate was filtrated, and this procedure was repeated three times. The combined extracts were evaporated to dryness *in vacuo* using a rotary evaporator (*C. laurifolius* EtOH extract 32.35%, *S. wiedemannii* EtOH extract 31.38%). The EtOH extract was redissolved in 90% MeOH/H_2O (1000 mL) and re-extracted with portions of *n*-Hx (3 × 300 mL) for 6 h in water bath adjusted to 40°C. The combined Hx fraction was evaporated to dryness to obtain a sticky residue (the Hx fraction for *C. laurifolius* and *S. wiedemannii*; 4.5% and 3.61%, respectively). Then, the MeOH/H_2O extract was concentrated under reduce pressure in a rotary evaporator and dissolved in H_2O (150 mL). The water extract was re-extracted with portions of $CHCl_3$ (3 × 400 mL) for 6 h in water bath adjusted to 40°C. The combined $CHCl_3$ fraction was evaporated in a rotary evaporator ($CHCl_3$ fraction for *C. laurifolius* and *S. wiedemannii*; 10.98%, and 10.27%, respectively). The aqueous extract was then re-extracted with BuOH saturated with distilled H_2O for 6 h in water bath adjusted to 40°C and evaporated to dryness (BuOH fraction for *C. laurifolius* and *S. wiedemannii*; 6.27%, and 7.77%, respectively). The remaining aqueous part (*r*-H_2O) was lyophilized (*r*-H_2O fraction for *C. laurifolius* and *S. wiedemannii*; 9.6% and 8.61%, respectively).

Bioactivities assay

The ethanolic extract, and Hx, $CHCl_3$, BuOH, and *r*-H_2O fractions dissolved in dimethylsulfoxide (DMSO, 80%) and EtOH (20%) at a fi al concentration of 512 µg/mL. The extracts were sterilized by filtration using 0.22 µm millipore used as the stock solutions. Reference pharmaceutical agents were purchased from Sigma Chemical Co., and dissolved in phosphate buffer solution (ampicillin, pH: 8.0; 0.1 mol/mL), DMSO (ketoconazole) or in water (ofl xacin, levofl xacin, and fluconazole).[26,27] As the standards, Gram-negative strains of *E. coli* ATCC 35218, *P. aeruginosa* ATCC 10145, *P. mirabilis* ATCC 7002, *K. pneumoniae* RSKK 574, *A. baumannii* RSKK 02026, and as Gram-positive strains of *S. aureus* ATCC 25923, and *E. faecalis* ATCC 29212, and its isolates were used for the determination of antibacterial activity. *C. albicans* ATCC 10231 and *C. parapsilosis* ATCC 22019 were used for determination of antifungal activity. Mueller Hinton Broth (MHB; Difco) and Mueller Hinton Agar (MHA; Oxoid) were applied for growing and diluting of the bacteria suspensions. The synthetic medium RPMI-1640 with L-glutamine was buffered to pH: 7 with 3-(N-morpholino)-propansulfonic acid and culture suspensions were prepared as described previously.[26,27] The microdilution method was employed for antibacterial and antifungal activity tests as described previous study.[28,29]

Vero cell line and Madin-Darby bovine kidney cell cultures were grown in Eagle's Minimal Essential Medium (Seromed; Biochromin) enriched with 10% fetal calf serum (Biochrom, 100 mg/mL of streptomycin; 100 IU/mL of penicillin) in a humidifi d atmosphere of 5% carbon dioxide at 37°C as described previously. In order to determine the antiviral activity of the extracts, HSV-1, and PI-3 were used. Maximum cytopathogenic effect ($_{max}$CPE) concentrations as the indicator of antiviral activities of the extracts were determined with maximum nontoxic concentrations (MNTCs) as described previously.[28,29]

RESULTS

The results of comparative antibacterial and antifungal activities of the extracts of *C. laurifolius*, which assayed *in vitro* antibacterial and antifungal activity against standard strains of *E. coli P. aeruginosa*, *P. mirabilis*, *K. pneumoniae, A. baumannii*, *S. aureus*, *E. faecalis* and their drug-resistant strains as well as yeasts *C. albicans*, and *C. parapsilosis* are presented in Table 1.

As shown in Table 1, all of the extracts of *C. laurifolius* showed the same activity against standard Gram-negative bacteria (*E. coli, P. mirabilis*, *K. pneumoniae*, and *A. baumannii*) with minimum inhibitory concentrations (MICs) value of 32 µg/mL. The efficacies of the extracts were lower against resistant isolates (ESβLs enzyme positive; *E. coli*, trimethoprim-sulfamethoxazole, tazobactam-resistant *P. aeruginosa*, trimethoprim-sulfamethoxazole, cefriaxon-resistant *P. mirabilis/K. pneumonia*, and trimethoprim-sulfamethoxazole-resistant *A. baumannii*). MICs for all microbes were two times higher for all extracts. The extracts of *C. laurifolius* showed some degree of antibacterial activity against standard and isolated strains of *P. aeruginosa* at MIC values of 64, 128 µg/mL, respectively. Interestingly, the extracts were found to have good activity against isolated Gram-negative strains of *E. coli*, which showed a close effect to those of the references; ampicillin (MIC; 64 µg/mL).

As for Gram-positive bacteria, MIC values of 64 µg/mL and 128 µg/mL were determined for standard (*S. aureus* ATCC25923, and *E. faecalis* ATCC29212) and drug-resistant strains (methicillin-resistant *S. aureus*, and cephalosporin-resistant *E. faecalis*), respectively.

All extracts had similar MICs values (8 µg/mL) on *C. albicans* and *C. parapsilosis* compared to the references drugs employed (ketoconazole and fluconazole).

The results of antiviral activities of the extracts of *C. laurifolius*, and *S. wiedemannii* are presented in Table 2. As shown in Table 2, the H_2O, $CHCl_3$, and EtOH extracts (16–<0.25 µg/mL) of *S. wiedemannii* had signifi ant antiviral activity on HSV-1 with a MNTC of 16 µg/mL, similar to that of acyclovir. The BuOH extract of *S. wiedemannii* had remarkable inhibitory activity on HSV-1 (32–<0.25 µg/mL). On the other hand, the BuOH and Hx extracts of *C. laurifolius* had less antiviral activity (32–16 µg/mL) on HSV-1 with the MNTC of 32 µg/mL. In particular, the Hx extract of *C. laurifolius* (CPE of 32–8 µg/mL) had signifi ant antiviral activity on PI-3 with a MNTC of 32 µg/mL, similar to that of oseltamivir (CPE of 32–<0.25 µg/mL). Regarding PI-3, the EtOH, BuOH extracts of *C. laurifolius*, and the BuOH extract of *S. wiedemannii* showed the same inhibitory activity (CPE of 64–16 µg/mL).

DISCUSSION

In a previous study, *in vitro* bioactivities of the aqueous extracts of *C. ladanifer* and *C. populifolius* leaves were studied.[11] Their cytotoxicity

Table 1: Antimicrobial activity of the *C. laurifolius* extracts and references expressed as minimum inhibitory concentrations (MICs; μg mL^{-1})

Extracts	Gram negative bacteria										Gram positive bacteria				Yeasts	
	E. coli		*P. aeruginosa*		*P. mirabilis*		*K. pneumoniae*		*A. baumannii*		*S. aureus*		*E. faecalis*		*C. albicans*	*C. parapsilosis*
	ATCC	Isol[a]	ATCC	Isol.	ATCC	Isol.	ATCC	Isol.	ATCC	Isol.	ATCC	Isol.	ATCC	Isol.		
EtOH	32	64	64	128	32	64	32	64	32	64	64	128	64	128	8	8
r-H_2O	32	64	64	128	32	64	32	64	32	64	64	128	64	128	8	8
$CHCl_3$	32	64	64	128	32	64	32	64	32	64	64	128	64	128	8	8
BuOH	32	64	64	128	32	64	32	64	32	64	64	128	64	128	8	8
Hexane	32	64	64	128	32	64	32	64	32	64	64	128	64	128	8	8
AMP_b	2	64	-	-	2	4	2	4	2	4	<0.12	8	0.5	1	NT	NT
OFX_c	0.12	1	1	4	<0.12	1	<0.12	1	0.12	2	0.5	4	1	2	NT	NT
LVX_d	<0.12	0.25	1	2	<0.12	1	<0.12	1	0.12	2	0.5	4	0.5	2	NT	NT
KET_e	[g]-	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	1	1
FLU_f	[h]NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	4	4

Isol[a]: Isolated stran; AMP[b]: Ampicilline; OFX[c]: Ofl xasine; LVX[d]: Levofl xacine, KET[e]: Ketoconazole, FLU[f]: Fluconazole, [g]-: No activity observed, NT[h]: Not tested. *E. coli* isolates; (+ESβLs enzyme), *P. aeruginosa* isolates (resist to trimethoprim-sulfamethoxazole, tazobactam), *P.mirabilis/K. pneumonae* isolates (resist to Trimethoprim-sulfamethoxazole, cefriaxon), *A. baumannii* isolates (resist to trimethoprim-sulfamethoxazole), *S. aureus* (resist to methicillin), *E. faecalis* (resist to cephalosporin)

Table 2: Antiviral activity together with CPEs of the extract of the *C. laurifolius* and *S. wiedemannii* and references as MICs (μg mL^{-1}) values

Extracts	MDBK cells (mg mL^{-1})			Vero cells (mg mL^{-1})		
	MNTC[a] (mg mL^{-1})	CPE[b] inhibitory concentration HSV-1		MNTC (mg mL^{-1})	CPE inhibitory concentration PI-3	
		Max.	Min.		Max.	Min.
CL[c]-EtOH	64	64	32	64	64	16
CL-r-H_2O	32	e-	-	64	-	-
CL-$CHCl_3$	32	-	-	128	-	-
CL--BuOH	32	32	16	64	64	16
CL-Hexane	32	32	16	32	32	8
SW[d]-BuOH	32	32	<0.25	32	64	16
SW-H_2O	16	16	<0.25	16	-	-
SW-$CHCl_3$	16	16	<0.25	16	-	-
SW-EtOH	16	16	<0.25	16	-	-
SW-Hexane	64	64	64	64	-	-
Acyclovir	16	16	<0.25	NT	NT	NT
Oseltamivir	NTf	NT	NT	32	32	<0.25

MNTC[a]: Maximum non-toxic concentration, CPE[b]: Cytopathogenic effect, CL[c]: *Cistus laurifolius*, SW[d]: *Salvia wiedemannii*, [e]-: No activity observed, NT[f]: Not tested

on human cancer cells was determined. *C. ladanifer* showed remarkable antibacterial activity on *S. aureus*, whereas *C. populifolius* was effective against *E. coli* with MICs values of 154 and 123 μg/mL, respectively. Also, cytotoxic activity of both extracts was observed against breast cancer cells.

The water, methanol, $CHCl_3$, ethyl acetate, and butanol extracts, obtained from leaves and the fruits of the five *Cistus* species (*C. creticus* L., *C. laurifolius* L., *C. monspeliensis* L., *C. parvifl rus* Lam., and *C. salviifolius* L.), were investigated against the following microorganisms: *S. aureus*, *Streptococcus faecalis*, *Bacillus subtilis*, *B. cereus*, *P. aeruginosa*, *E. coli*, and *C. albicans* with disc diffusion method.[12] All extracts of *C. laurifolius* showed activity against *S. aureus*, whose inhibition zones varied between 8 mm and 14 mm. Additionally, methanol, $CHCl_3$ extract, ethyl acetate extract, n-butanol extract, and water extracts exerted activity against *B. subtilis* (between 8 mm and 11 mm) and *B. cereus* (between 9 mm and13 mm). Only the butanol extract showed activity against *S. faecalis* (8 mm) and *E. coli* (11 mm). Cefazolin (*S. aureus*; 23.21 mm, *B. subtilis*; 23 mm, *B. cereus*; 20 mm) and ciprofl xacin (*S. faecalis*; 22 mm, *E. coli*; 27 mm) used as the references. None of the extracts of *C. laurifolius* were active against *P. aeruginosa* and *C. albicans*. As a matter of fact, no comparison could be made between the results of our and the aforementioned study,[12] depending on difference on the methods used in both studies. In our study, all extracts exhibited antimicrobial activity.

C. incanus extract which was rich in polyphenolics exerted antiviral activity against influenza A virus (H_7N_7) *in vitro* and *in vivo*.[22] Relevantly, antiviral activity of *Salvia* species has been evaluated. Ogutcu *et al.* studied the methanol, Hx, and dichloromethane extracts of *S. limbata* and *S. sclarea*. Anti-influenza drug rimantadine and (E)-5-(2-bromov inyl)-2′-deoxyuridine hydrochloride were used as the references. The dichloromethane extract of *S. sclarea* and the methanol extract of *S. limbata* showed high anti-*infl enza virus* activity while the methanol extract of *S. sclarea* had a limited antiherpetic activity.[14] The inhibitory activity of the aqueous extract of *S. officinalis* on HSV-1 and HSV-2 was tested using RC-37 cells (African green monkey kidney cells). In the study, acyclovir was the positive control. The aqueous extracts of *S. officinalis* had signifi ant antiviral activity on HSV-1 (0.777 ⌇⌇ g/mL) and HSV-2 (1.359 μg/mL).[23] In another study, antiviral activities of seven extracts of *S. miltiorrhiza* were evaluated.[24] The ethyl acetate and water extracts of the plant inhibited viral RNA synthesis; however, the other extracts did not have any protective activity. Fifty percentage inhibitory concentrations of the ethyl acetate and water extracts on *Enterovirus* 71 were 0.742 ± 0.042 mg/mL and 0.585 ± 0.018 mg/mL, respectively. Likewise, another study showed antiviral activity (anti-human deficie cy virus) with no cytoxicity of two compounds (lithospermic acid and lithospermic acid B) isolated from the roots of *S. miltiorrhiza*.[25] Th CC_{100} doses for lithospermic and lithospermic acid B were >297 μM and >223 μM, respectively.

Recently, resistance to conventional antiviral drugs is on rise. Therefore, there is a strong need for novel effective substances synthesized or isolated from plants. Th s study shows for the fi st time that *C. laurifolius* and *S. wiedemannii* are very promising plants regarding their antiviral and cytotoxic effects. However, further studies are necessary on the isolation and mechanism of the active component (s) of *C. laurifolius* and *S. wiedemannii*.

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Conflicts of interest

There are no confli ts of interest.

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