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Cistus incanus: a natural source of antimicrobial metabolites

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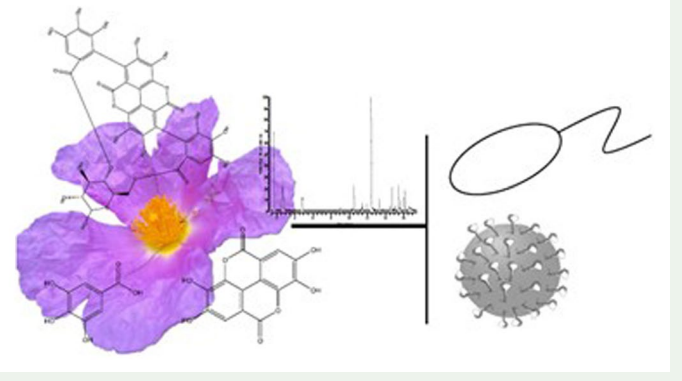
ABSTRACT

The discovery of natural molecules with antimicrobial properties has become an urgent need for the global treatment of bacterium and virus infections. *Cistus incanus*, a Mediterranean shrub species, represents a valuable source of phytochemicals with an interesting wide-spectrum antimicrobial potential. In this study, we analysed the spectrum of molecules composing a commercial hydroalcoholic extract of *C. incanus* finding ellagitannins as the most abundant. The effect of the extract and its main constituents (gallic acid, ellagic acid and punicalin) was assessed as co-treatment during viral (HSV-1, HCoV-229E, SARS-CoV-2) and bacterial infection (*Staphylococcus aureus* and *Escherichia coli*) of cells and as pre-treatment before virus infections. The results indicated a remarkable antiviral activity of punicalin, against SARS-CoV-2, by pre-treating both the viral and the host cells, and a major sensitivity of *S. aureus* to the *C. incanus* extract compared to *E. coli*. The present study highlights broad antimicrobial potential of *C. incanus* extract.



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1. Introduction

In the modern era, the pharmaceutical sector remains significantly inspired by plant-derived compounds and plant extracts continue to be an important source of promising drug candidates. A renewed scientific attention regards the potential use of natural drugs against infectious diseases driven by the rapid spread of microbial epidemics along with drug-resistant infectious diseases. This represent a global threat to human health, even in developing countries (Limmathurotsakul et al. 2019; Rawson et al. 2020). Current research trends in the field of natural drugs emphasise the evaluation of herbals (botanicals) used by diverse human populations in traditional medicine. In particular, there is a growing interest for the herbal Chinese medicine, spurred in part by the in 2015 Nobel Prize in medicine awarded to three scientists who identified artemisinin from *Artemisia annua* as effective treatment against malaria. Numerous papers are emerging that describe the biochemical characterisation and bioactivity of extracts coming from herbs of the Chinese traditional medicine (Feng et al. 2019; Malode et al. 2021; Pantharos et al. 2022). On the contrary, similar studies on Western and Mediterranean herbals are still limited (Dziedziński et al. 2021; Tomasella et al. 2022), although the practice of using wild plants for medicinal reasons continues today in small rural communities of the Mediterranean region (Emre et al. 2021).

Among Mediterranean herbals, Cistus is widely utilised by several communities, and the use of its resin (labdanum) has been documented in southeastern Europe for over 2,500 years (Barrajón-Catalán et al. 2011; Chovanec 2016). Also known as rockroses, *Cistus* is a perennial shrub belonging to the Cistaceae family. The genus *Cistus* includes more than 20 species of which *Cistus x incanus* L., *Cistus ladanifer* L., *Cistus monspeliensis*, and *Cistus salviifolius* are the most widely employed for healing purposes (Kubica et al. 2017). Several *Cistus* species have been used in various forms within the Mediterranean basin, as poultice, infusions, decoctions, and ointments (Tomou et al. 2022). In Sardinia and Turkey local people used *Cistus* extracts as cure for gastrointestinal disorders (Mastino et al. 2017; Fecka et al. 2020). In Morocco, seeds of *Cistus* are consumed as appetiser food and thought to possess aphrodisiac qualities (El Youbi et al. 2016). In addition, *Cistus* species have been also introduced in the perfume industry given their aromatic properties (Nicoletti et al. 2015). In Spain, for example, *Cistus* ointment is a noteworthy product in the cosmetic, employing it for the treatment of skin infections and wound healing (Alarcón et al. 2015). *Cistus incanus* is included in the BELFRIT botanical list of food supplements. The *folium* and *gummi* are recommended as digestive and bronchial fluidifiers, while the *herba* is indicated as an antioxidant and to enhance respiratory functions (Cousyn et al. 2013).

The metabolic repertoire and the related bioactivity of *Cistus* extracts have been recently reviewed by Zalegh et al. and Tomou et al. (Zalegh et al. 2021; Tomou et al. 2022). Catechins, tannins, flavonols, diterpenes and sesquiterpenes are the main groups of specialised metabolites detected within *Cistus* species (Venditti et al. 2015; Gaweł-Bęben et al. 2020; Zalegh et al. 2021). Glycosidic derivatives of kaempferol, quercetin and myricetin, monomeric flavan-3-ols, proanthocyanidins, hydrolysable tannins and simple phenolic acids (e.g. gallic, ellagic, gentisinic) were described as typical for *Cistus* plants. Particularly, monoterpenes, sequiterpenes and labdane-type

diterpenes are the main compounds contained in leaves, stems and brown resin of these plants (Fecka et al. 2020). Early studies described the efficiency of the diterpene (+)-19-acetoxy-cis-clerodan-3-ene-15-oic acid, extracted from *C. monspeliensis* leaves, against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* (Kolocouris et al. 2001). More recently, Carev and co-workers (Carev et al. 2020) tested *C. salviifolius* and *C. creticus* aqueous extracts, enriched in myricetin-3-hexoside and myricetin-rhamnoside, against fungi and bacteria, indicating the major antimicrobial activity against the opportunistic yeast *Candida albicans* and both Gram-positive and Gram-negative bacteria. Similarly, Salomè-Abarca and collaborators (Salomé-Abarca et al. 2020) tested extracts from 10 different ecotypes of *C. monspeliensis* from Sardinia (Italy). They discoved their effectiveness against *Fusarium oxysporum*, and authors hypothesised an additive or even a synergist activity of flavonoids and diterpenes present in these extracts. The potential use of *Cistus* extract has been suggested also against viruses (Zalegh et al. 2021). Already in 2007, Droebner and collaborators (Ehrhardt et al. 2007) found the so-called CYSTUS052, a specific extract from *C. incanus* rich in polymeric polyphenol, to be effective against influenza A virus, without negative impact on systemic immune response suggesting the eligible use of this remedy. About ten years later, *C. incanus* aqueous extracts have been studied to contrast human immunodeficiency virus (HIV) with multidrug resistance profile, and Filoviruses. The study showed an inhibition percentage higher than 80%, proving how the extracts were able to block viral particles' attachment and inhibit viral entry into host cells (Rebensburg et al. 2016). Considering this, *C. incanus* extracts have been largely investigated allowing the identification of the massive presence of polyphenols (Gaweł-Bęben et al. 2020; Dziurka et al. 2021), which could play a role in fighting not only viruses, but also bacteria. For this reason, *C. incanus* extract has been considered also as an alternative means of overcoming antimicrobial resistance.

In this study, we examined the qualitative and quantitative profile of a commercial hydroalcoholic extract of C. incanus and its antimicrobial properties against human pathogens, including *Escherichia coli* and *Staphylococcus aureus*. Moreover, we experimentally tested *C. incanus* extract against viruses belonging to the Coronaviridae and Herpesviridae families. The main metabolites involved in the observed effects were also identified. The goal of our work was to broaden current knowledge regarding the role that *C. incanus* and the specialised metabolites characterising its extracts can play against a wide spectrum of microbes and viruses, even towards the latest SARS-CoV-2 coronavirus.

2. Results and discussion

2.1. Chemical profile and citotoxicity of Cistus incanus extract

The increasing number of multidrug-resistant microorganisms represents a cogent problem for human health worldwide, and this number will continue to increase. There is the necessity of exploring new drugs that can represent alternatives in the management of some serious infectious processes, starting from natural drugs provided by nature. *Cistus* genus is represented by different species of plant shrubs that are part of the Mediterranean flora (Raimundo et al. 2018; Casadesús et al. 2021;

Coello et al. 2021). The taxonomy of the Cistus genus is intricate, presenting challenges in the accurate identification of individual species. This complexity is exacerbated by the polymorphism observed in both vegetative and generative organs, as well as the potential hybridisation among closely related species, leading to the differentiation of numerous subspecies (Guzmán and Vargas, 2005; Barrajón-Catalán et al. 2016; Fecka et al. 2020). The focus of this study is a commercial extract of *Cistus x incanus*, a hybrid derived from *Cistus albidus* and *Cistus crispus*, although the exact subspecies remains unidentified. According to Starzec and collegues, there are currently three subspecies of *C. incanus*, namely *corsicus*, and *creticus*. Consequently, the issue of synonymy is evident in Cistus species, where *Cistus incanus* is also referred to as *Cistus creticus*, *Cistus villosus*, or pink or hairy rockrose (Viapiana et al. 2017). The difference in biochemical composition is also dependent on the subspecies and can vary according to the region (Starzec et al. 2023).

The available data on specialised metabolites of the genus *Cistus* indicates that the main bioactive compounds are polyphenols. Specifically, compounds such as flavonol glycosides, flavan-3-ols, proanthocyanidins, hydrolysable tannins and phenolic acids (gallic and ellagic acids) were identified as typical for *Cistus* (Tomou et al. 2022). In this study, we conducted a comprehensive analysis of the metabolic profile of a *C. incanus* extract, aiming to identify putative molecules contributing to the extract's bioactivity (Figure S1). The identified compounds are reported in Table 1 with their detailed UHPLC-HRMS/MS data. In total, twenty-two major peaks were characterised, and the identified compounds (confirmed by reference standards or tentatively identified by HRMS/MS data) were previously reported in the *Cistus* genus (Barrajón-Catalán et al. 2011; Barros et al. 2013; Tomás-Menor et al. 2013; Gaweł-Bęben et al. 2020), with the exception of the compounds **8**, **13** and **15**. According to the literature, compounds **13** and **15** may be reaction artefacts, generated by the extraction process (Venditti, 2020). Gallic acid (GA, **1**), its ethyl ester (**13**), ellagic acid (EA, **17**) and three ellagitannins (**3**, **8** and **15**) resulted as the most abundant compounds of the investigated *C. incanus* extract (Table 1). Punicalin (**3**), the predominant ellagitannin, is regarded as a distinctive ellagitannin of *Cistus* species, although its abundance strongly depends on the genotype, origin, and environmental conditions (Tomás-Menor et al. 2013; Barrajón-Catalán et al. 2016). Ellagitannins are specialised metabolites commonly found in certain berries (blackberries, strawberries, raspberries) (Van de Velde et al. 2018; Klewicka et al. 2020; Karlińska et al. 2021) and are also abundant in grapes and pomegranate (Vignault et al. 2020; Suručić et al. 2021). The healing role of ellagitannins has been recognised in several other plants used in Asia for medical purposes such as *Camellia japonica*, *Cornus officinalis*, *Rosa rugosa*, and *Agrimonia Pilosa*, all of which enriched in ellagitannins (Lipińska et al. 2014). Other polyphenols present in the extract, albeit in markedly lower content, are flavan-3ols (**5**, **9** and **11**) their related dimers (**2**, **4**, **6** and **10**), the flavonols myricitrin (**16**), quercitrin (**21**) and myricetin (**22**), and apigenin-6,8-di-C-glucoside (**14**). As minor compounds, three neolignans (-) were tentatively identified in the studied extract based on their occurrence in *Cistus* spp (Sadhu et al. 2006; Barrajón-Catalán et al. 2011) and HRMS/MS spectra (Table 1). Before assessing the bioactivity of *C. incanus* extract and its main compounds, gallic acid (GA), ellagic acid (EA) and punicalin (P), we evaluated the potential toxicity on Vero cell monolayers by MTT test. Tested concentration did not affect cell viability,

Table 1. UHPLC-HRMS/MS data of compounds detected in *cistus incanus* extract.

N[a]	Compound	RT (min)	[M - H]- (*m/z*)	Molecular formula	Error (ppm)	Diagnostic product ions (*m/z*)[b]	L.I.[c]	mg/g[d]
1	gallic acid	1.69	169.0131	$C_7H_6O_5$	−0.5	**125.0232**	1	15.3
2	(epi)gallocatechin dimer	2.20	609.1253	$C_{30}H_{26}O_{14}$	2.4	441.0827, 423.0724, 305.0668, 255.0299, 177.0188, **125.0233**	2	
3	punicalin	2.61	781.0527	$C_{34}H_{22}O_{22}$	1.1	600.9892, 392.9886, 300.9988, 298.9833, **270.9882**	1	13.3
	punicalin	2.77	781.0526	$C_{34}H_{22}O_{22}$	0.9			
4	(epi)gallocatechin dimer	3.39	609.1253	$C_{30}H_{26}O_{14}$	2.3	441.0826, 423.0717, 305.0661, 255.0296, 177.0184, **125.0233**	2	
5	(epi)gallocatechin	4.82	305.0663	$C_{15}H_{14}O_7$	2.4	261.0769, 219.0658, 137.0233, **125.0233**, 109.0283	2	
6	(epi)gallocatechin-(epi)catechin	5.53	593.1305	$C_{30}H_{26}O_{13}$	2.6	425.0873, 407.0771, 303.0506, 289.0717, 245.0817, **177.0184**, 125.0232, 109.0283	2	
7	hexoside of gallic acid ethyl ester	8.84	359.0986	$C_{15}H_{20}O_{10}$	3.6	**197.0448**, 182.0212, 153.0546, 138.0311	2	
8	valoneic acid dilactone	9.04	469.0048	$C_{21}H_{10}O_{13}$	2.2	**425.0152**, 407.0042, 379.0088, 335.0192, 299.9912	2	1.3
9	(epi)gallocatechin	10.32	305.0666	$C_{15}H_{14}O_7$	3.2	287.0559, 261.0769, 219.0658, 137.0233, **125.0233**, 109.0283	2	
10	(epi)catechin dimer	10.46	577.1352	$C_{30}H_{26}O_{12}$	2.0	425.0882, 407.0767, 289.0716, 245.0815, 161.0234, **125.0232**	2	
11	catechin	10.55	289.0713	$C_{15}H_{14}O_6$	2.3	245.0814, 203.0706, 151.0390, **123.0440**, 125.0233, 109.0282	1	
12	roseoside	11.96	431.1919	$C_{19}H_{30}O_8$	1.8	**385.1866**, 223.1335, 205.1228, 179.0553, 161.044, 153.0911	2	
13	gallic acid, ethyl ester	12.44	197.0445	$C_9H_{10}O_5$	0.3	169.0132, **125.0233**	2	2.9
14	apigenin-6,8-diC-glucoside (vicenin-2)	12.83	593.1514	$C_{27}H_{30}O_{15}$	2.2	473.1113, 413.0844, **383.0770**, 353.0670, 325.0707	2	
15	valoneic acid bislactone, ethyl ester	14.49	497.0362	$C_{23}H_{14}O_{13}$	2.3	450.9942	2	1.2
16	myricetin-3-deoxyhexose (myricitrin)	14.75	463.0878	$C_{21}H_{20}O_{12}$	1.5	317.0290, **316.0222**, 287.0197, 271.0248, 178.9911, 151.0027	2	
17	ellagic acid	14.77	300.9987	$C_{14}H_6O_8$	2.5	**283.9961**, 229.0137, 185.0234, 163.0391	1	2.4
18	dihydrodehydrodiconiferyl alcohol deoxyhexose	15.12	505.2077	$C_{26}H_{34}O_{10}$	1.8	359.1499,	2	
19	guaiacylglycerol-O-4′-dihydroconiferyl alcohol deoxyhexose	15.46	523.2179	$C_{26}H_{36}O_{11}$	1.0	**475.1972**, 329.1397, 327.1456, 195.0657, 165.0547, 150.0313, 149.0223	3	
20	guaiacylglycerol-O-4′-dihydroconiferyl alcohol deoxyhexose	15.7	523.2181	$C_{26}H_{36}O_{11}$	1.3	475.1974, 329.1385, 327.1447, 195.0656, 165.0548, **150.0312**, 149.0223	3	
21	quercetin-3-deoxyhexose (quercitrin)	16.07	447.0929	$C_{21}H_{20}O_{11}$	1.5	301.0349, **300.0275**, 271.0249, 255.0297, 163.0025, 151.0026	2	
22	myricetin	16.61	317.0302	$C_{15}H_{10}O_8$	3.1	**271.0243**, 178.9977, 151.0027, 137.0233	1	

[a]Compounds are numbered according to their elution order.
[b]In bold the base peak of MS/MS spectrum.
[c]L.I.: level of identification, assigned according to Sumner (Sumner et al. 2007).
[d]Results are expressed as mg/g of dry extract.

except for a slight decrease at the highest tested concentrations (100 and 200 µg/mL) (Figure S2), confirming the safety of the extracts.

2.2. Antiviral activity of Cistus extract

Cistus extracts are considered natural remedies due to their attributed anti-inflammatory, antitumoral, vasodilator, antispasmodic and antimicrobial properties (Barrajón-Catalán et al. 2016). In this study, we analysed effects of *Cistus* extracts against different human pathogenic viruses.

To date, *Cistus* extracts have been tested against a wide range of viruses, including influenza virus (Droebner et al. 2007; Ehrhardt et al. 2007), human immunodeficiency virus (HIV), Ebola virus (Rebensburg et al. 2016), and Dengue virus (Kuchta et al. 2020). These studies have demonstrated potent antiviral activity in the early phases of viral infection. In this study, we tested the activity of *C. incanus* extract against HSV-1 and coronaviruses, including the pandemic SARS-CoV-2. HSV-1 is responsible for the common oral lesions, but it is rarely associated to severe manifestations, e.g. neonatal herpes, keratitis, and encephalitis (de Mello et al. 2016). HSV-1 is an enveloped virus with a linear double-stranded DNA, and usually Latinises in the neuronal ganglia causing recurrent events (de Mello et al. 2016). Some studies, reported this virus as strongly related to the age of the patient, leading those aged ≥40 years to be more affected than the rest of the population. Although it is predominantly transmitted through oral shedding HSV-1 can be transmitted sexually, leading to genital herpes. For instance, in Asia, it was demonstrated that this transmission route is starting to play a significant role in the spread of infection (Kadhr et al. 2018).

Another enveloped virus, highly related to the seasonal cold, is the human coronavirus 229E (CoV-229E) that contains a linear single-stranded RNA (Kesheh et al. 2021). Strictly associated is virus SARS-CoV-2, which shares many features with HCoV-229E, but differently from it, caused and still is causing a huge number of deaths due to serious upper respiratory failures (Kesheh et al. 2021). sCoVs cocirculate endemically with other common respiratory viruses, and coinfections are very often observed; these viruses were found to infect mainly people aged <1, 6-26, >64 years. In particular, the data showed that CoV-229E affects patients according to sex differences, with males being more prone to be infected than females (Nickbakhsh et al. 2023).

We investigated the mechanism of action of *C. incanus* extracts by using three antiviral experimental schemes at non-cytotoxic concentrations against the viral models: 1) co-treatment assay, each of the three viruses was incubated simultaneously with *C. incanus* extract on Vero cell monolayer (Figures 1A and 3B,C); 2) virus pre-treatment assay, *C. incanus* extract was incubated with the virus to test its antiviral activity (Figures 1D and 3E,F) and 3) cell pre-treatment assay, *C. incanus* extract was added on cells before viral infection (Figures 2G and 4H,I). As it can be observed in Figure 1, the minimum concentration of *Cistus* extracts effective against virus varied depending on the method and the virus studied. The best results for HSV-1 were obtained after virus pre-treatments indicating that *C. incanus* extract interfered directly with HSV-1 particles before they could interact with the cell. In fact, 100% of virus inhibition was observed at a concentration of 3.12 µg/mL of extract (Figure 1D), representing a four-fold more efficient effect compared to the same concentration used

in the co-treatment test (Figure 1A). Dell'Annunziata and collaborators explained that HSV-1 is characterised by a lipid envelope containing four glycosylated proteins primarily responsible for the fusion of the virus with the host cell membrane. Considering this, the diverse group of phytochemicals present in *C. incanus* extract may be capable of interfering with this fusion (Dell'Annunziata et al. 2022). A recent study further suggested that ellagitannins can be effective in inhibiting the biding between HSV-1 and cell surface through virus inactivation (El-Aguel et al. 2022).

Positive results were observed for SARS-CoV-2 pre-treatment, and these effects were maintained when cells were simultaneously co-treated with the extract and the virus (Figure 1C). This result confirms what has recently been observed for CYSTUS PANDALIS®, where it has also been suggested that the peculiar polyphenol composition of *Cistus x incanus* prevents virus from entering cells by reducing the binding between the receptor present on the surface of host cells (ACE2) and the S1 subunit

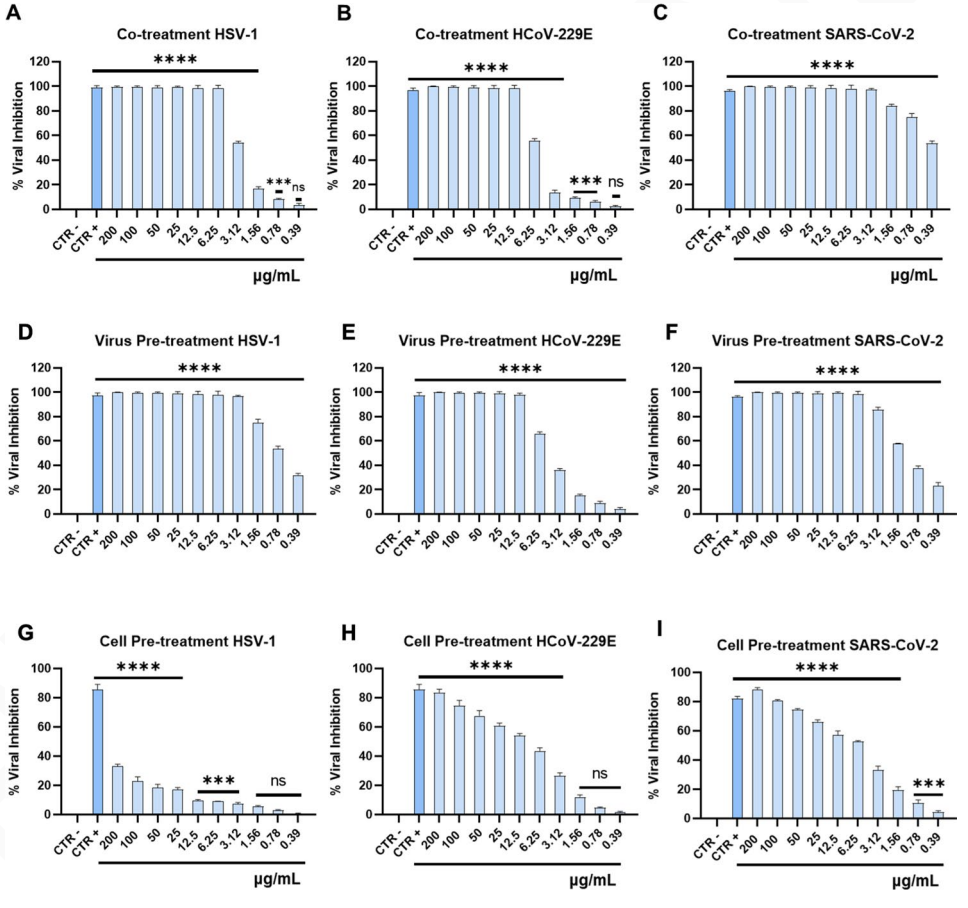

Figure 1. (A–C) Co-treatment assay of *C. incanus* extract. Vero cell monolayers were treated with the extract (0.39-200 µg/mL) and infected with HSV-1 (A), HCoV-229E (B), or SARS-CoV-2 (C). D, E, F in the virus pre-treatment test, HSV-1 (D), HCoV-229E (E) or SARS-CoV-2 (F) were treated with the extract (0.39–200 µg/mL) and then diluted on the cell monolayer. (G), (H), and (I) in the cell pre-treatment test, vero cell monolayers were treated with the extract and then infected with HSV-1 (G), HCoV-229E (H) or SARS-CoV-2 (I). ****$p < 0.0001$; ***$p < 0.001$; ns: nonsignificant.

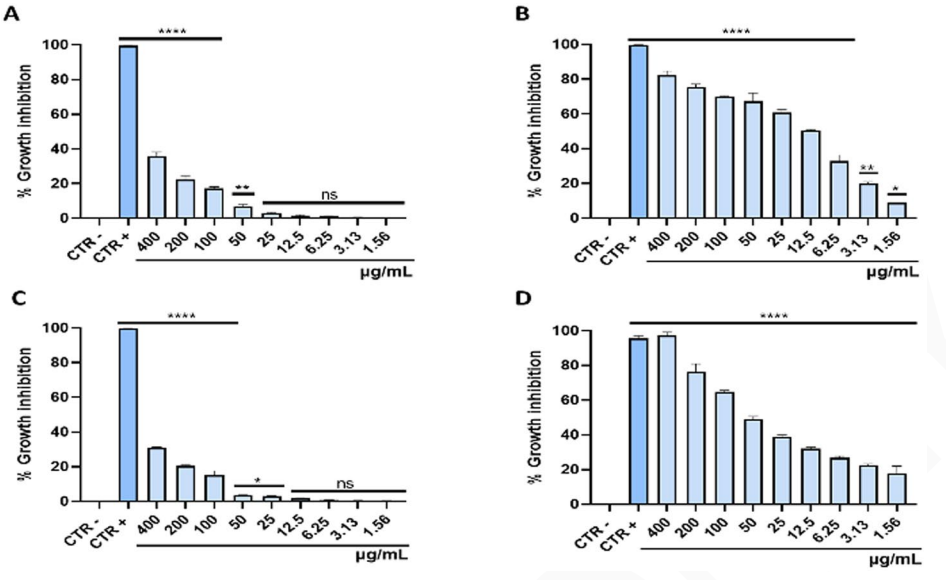

Figure 2. Growth inhibition rate (%) of S. aureus exposure with (A) *C. incanus* extract (****p value < 0.0001, ** p value = 0.002, *p value = 0.0475), (B) gallic acid (****p value < 0.0001, **p value = 0.0086, *p value = 0.0481), (C) ellagic acid (****p value < 0.0001, *p value = 0.0139 and 0.456, ns: not significant), (D) punicalin (****p value < 0.0001).

of the Spike protein or the receptor-binding domain of the virus (Traeder 2021). To attain 100% viral inhibition of HCoV-229E in either co-treatment or virus pre-treatment (Figures 1B and 3E), a higher concentration of the extract (a minimum 12.5 µg/mL) had to be used compared to SARS-CoV-2 and HSV-1 (3.12-6.25 µg/mL). This difference might arise from different binding affinities of phenolic compounds with protein components of the virus. The predominant effect of the Cistus compounds directly on the virus, rather than protecting cells, was further supported by the reduced inhibition of infectivity observed when cells were pre-treated with the extract (Figures 1G and 3H,I), and no inhibition ability was detected in post-treatment (i.e. *C. incanus* extract added to the cells already infected with the virus, data not shown). In particular, for HSV-1 a 100% viral inhibition was never achieved (Figure 1G), while for coronavirus this was attained only at the highest concentration tested (200 µg/mL) (Figures 1H and 3I).

To pinpoint the specific component of *C. incanus* extract with the highest antiviral potential, we conducted the same experimental schemes described for the total extract (except for the post-treatment test, because no viral inhibition was observed) using the three main constituents of investigated extract: gallic acid (GA), ellagic acid (EA) and punicalin (P) (Figure S3). In co-treatment and virus pre-treatments, the individual molecules exhibited a less potent effect, in terms of minimum concentration used, compared to the total extract (Figure S3A and Figure 4B– F). Among molecules, punicalin emerged as the most active compound against viruses. In some cases, i.e. virus pre-treatment against HSV-1 and SARS-CoV-2, its antiviral action was comparable to that of the total extract (Figure 1 and Figure S3). Molecular docking results obtained by Suručić et al. (Suručić et al. 2021) established the high affinity that punicalin,

identified in pomegranate (*Punica granatum* L.), has for the viral Spike protein and the host cell proteins ACE2, TMPRSS2 and furin required by the SARS-CoV-2 for the cell entry. In fact, both *in vitro* and *in silico* studies have identified in pomegranate juice a potential beneficial food for alleviating COVID-19 side effects. This has been attributed to its main components punicalin and punicalagin (Tito et al. 2021; Banihani 2022). In other studies, punicalin was found to cause a dramatic alteration of DNA replication of hepatitis B virus, as it inhibits HBeAg (viral protein) and cccDNA in a dose-dependent manner (Liu et al. 2016). These results may explain why *C. incanus* antiviral potential is very effective during the virus pre-treatment assay against HSV-1, a virus with a linear double-stranded DNA. Although punicalin was the most effective molecule tested in most of the cases, its inhibitory activity did not completely overlap with that reached when the full extract was used. This may be due to a limited combined action of punicalin and other phytochemicals contained within *Cistus* extract.

2.3. Antibacterial activity of Cistus extract

To assess *C. incanus* extract antibacterial potential, we employed two different bacteria strains, *E. coli* and *S. aureus*, representing Gram-negative and Gram-positive bacteria, respectively. It has been reported that fruit extracts rich in ellagitannins are effective against both Gram-positive and Gram-negative bacteria. For example, these have been proven to inhibit *S. aureus*, *Pseudomonas aeruginosa*, *E. coli* growth, as well as on certain Clostridia species (Li et al. 2015; Gullon et al. 2016; Baradaran Rahimi et al. 2020). In this study, *E. coli and S. aureus* were exposed to the extract at concentrations ranging from 400 to 1.56 µg/mL for 20 h, and the antibacterial effect was expressed as percentage of growth inhibition compared to bacteria treated with the extract solvent only (negative control, CTR-). A more potent antibacterial activity was observed against *S. aureus* in all tested conditions compared to *E. coli*. A growth inhibition rate of 35.9% was found after exposing of *S. aureus* to a concentration of 400 µg/mL of total extract, which significantly decreased at concentration below 100 µg/mL (Figure 2A). The analyses of the single molecules again confirmed the inefficacy of *Cistus* aqueous extract against *E. coli*, as the use of single molecules did not alter bacterial growth (Figure S4B and Figure 6C,D). Concerning *S. aureus*, the highest antibacterial effect was observed with gallic acid and punicalin, with MIC50 values of 40.2 and 75.4 µg/mL, respectively (Figures 2B and 5D). Consequently, the two single molecules were even more efficient than the total extract (an average of 3-folds more efficient for both molecules). In line with this, Liu et al. (Liu et al. 2017) demonstrated that the expression of *ica* genes involved in the formation of poly-*N*-acetylglucosamine polymer, part of Gram-positive peptidoglycan layer, was significantly downregulated in a dose-dependent manner with increasing concentrations of gallic acid, establishing the bactericidal potential of this compound against *S. aureus*. Punicalin also showed an interesting antibacterial effect, and this is in line with what recently observed (Ravi et al. 2019, Álvarez-Martínez et al. 2021) in *S. aureus*.

The proposed general molecular mechanism explaining how ellagitannins limit bacterial growth lies in the reaction of these molecules with sulfhydryl groups of proteins present in bacterial cell walls, resulting in toxicity (Singh et al. 2019). Hence, we hypothesise that the variation in the effects on the two types of bacteria can be

simply explained by the structural difference in the cell membrane of Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria. Gram-negative bacteria possess an outer peptidoglycan layer that acts more efficiently as barrier for antimicrobial agents.

3. Experimental

All details are provided in the supplementary material.

4. Conclusion

In the current report, we provide new insights into the antiviral and antimicrobial bioactivities of a commercial hydroalcoholic extract of *C. incanus* (*Cistus x incanus*). Our findings demonstrate that gallic acid, ellagic acid and punicalin are significant compounds exerting inhibitory effect against different pathogenic bacteria and virus strains, including Gram-positive and Gram-negative bacteria, as well as coronavirus and herpesviruses. To comprehensively understand the mechanisms underpinning the bioactivity of Cistus extract, future investigations will expand the tests to include pathogenic eukaryotic organisms, such as *C. albicans*. Additionally, these future analyses will delve into more detailed aspects by integrating cellular and biochemical analyses. The current results no only reinforce the traditional use of Mediterranean medicinal plants for antiviral and antimicrobial purposes, but also highlight *Cistus* as a potential neglected crop for bioactive compound production.

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Disclosure statement

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