Evaluation of the anti-SARS-CoV-2 properties of essential oils and aromatic extracts.

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Research Article

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Abstract

Essential oils and aromatic extracts (oleoresins, absolutes, concretes, resinoids) are often used as food flavorings and constituents of fragrance compositions. The flavor and fragrance industry observes significant growth in sales of some natural materials during the COVID-19 outbreak. Some companies worldwide are making false claims regarding their essential oils or blends to be effective (or indirectly points towards this conclusion) against coronaviruses even though the available data on the activity of plant materials against highly pathogenic human coronaviruses is very scarce. Our exploratory study aimed to develop pioneering knowledge and provide the first experimental results on the inhibitory properties of hundreds of flavor & fragrance materials against SARS-CoV-2 main- and papain-like proteases, and antiviral potential of the most active protease inhibitors. As essential oils are volatile products, they could provide an interesting subsidiary inhalation therapeutic strategy in the long term.

Introduction

Essential oils (EOs) are plant-derived products and often consist of dozens to hundreds of volatile compounds. They are produced only by physical methods like mechanical pressing or distillation (hydrodistillation, steam distillation) ¹. Aromatic extracts (AEs) on the other hand are obtained by extraction with organic solvents by a classical solid-liquid extraction, solid-solid extraction (enfleurage), or with the aid of modern equipment (supercritical fluid and microwave extractors). Essential oils and aromatic extracts are often used as food flavorings, perfuming agents, pharmaceuticals, and the former for the means of aromatherapy too ². The complexity of EOs and AEs matrix is an undesirable trait in drug discovery, but on the other hand, they are useful in some areas of medical therapy, and they can be a promising source of new drugs ³. Essential oils and aromatic extracts are not considered as highly effective antiviral agents, and in the flavor and fragrance scientific community, the interest is shifted more towards the assessment of antimicrobial properties of these plant-derived products. Studies regarding antiviral properties of volatile natural materials mostly concern *in-vitro* evaluations ^{4,5}, *in-vivo* testing of these materials is very rare ^{6,7}, and *in-vivo* human data is virtually non-existent.

The flavor and fragrance industry observes significant growth in sales of some natural materials during the coronavirus disease-2019 (COVID-19) outbreak. It is attributed in part to the aromatherapeutic properties of the selected products, but also in part to fraudulent marketing. Some companies worldwide are making false claims regarding their essential oils or blends to be effective (or indirectly points towards this conclusion) against coronaviruses, and (in the same marketing message) to boost the immune system during respiratory illnesses.

Coronaviruses (fam. *Coronaviridae*) are crown-like enveloped viruses comprising single-stranded, positive-sense RNA. Host targets for coronaviruses are animals and humans. Before the current outbreak HCoV-229E, HCoV-NL63, HCoV-OC43, HCoV-HKU1, SARS-CoV, and MERS-CoV were known to be able to infect humans ⁸. The first four are the cause of (in most cases) mild illnesses (common cold), whereas infections with SARS-CoV and MERS-CoV can develop into a severe respiratory disease that could be

fatal⁹. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is responsible for the current outbreak of the disease (COVID-19), is the seventh coronavirus known to infect humans^{10,11}. Due to the international travel and human-human interactions, the virus spread rapidly, which has resulted in more than 200 million infections and over 4 000 000 deaths (according to worldometers.info, accessed August 17th 2021). The world scientific community and pharmaceutical industry are devoted to finding an effective therapy for the COVID-19. In addition to the existing protective vaccinations against COVID-19^{12,13}, targeted drug therapy plays a very important role, however despite many efforts it has not been sufficiently developed so far. A preliminary study shows that drugs from many groups such as antibacterials, antiprotozoals, immunomodulators, angiotensin II receptor blockers, bradykinin B2 receptor antagonists, corticosteroids, anthelmintic, H2 blockers, and anticoagulants are considered as potential therapeutics in COVID-19 treatment¹⁴.

Two of the very well characterized and promising drug targets are the main protease (M^{pro}, 3CL^{pro}) and the papain-like protease (PL^{pro}), which have a key role in viral replication and transcription¹⁵⁻¹⁷.

Coronaviral M^{pro} and PL^{pro} inhibitors have been designed and assessed over the years, and Michael acceptor inhibitors¹⁸, ebiselen, and its derivatives can be promising drug leads^{16,19,20}. In the present study, we wanted to address the question of whether common flavor and fragrance materials are inhibitors or have the potential to be a source for effective inhibitors of the SARS-CoV-2 main and papain-like proteases, and whether these inhibitors exhibit *in vitro* antiviral properties. As essential oils are volatile products, they could provide an interesting subsidiary inhalation therapeutic strategy in the long term. To the best of our knowledge, this is the first experimental study regarding the evaluation of the potential of flavors and fragrances as a part of the search for the recent anti-coronaviral strategies.

Results And Discussion

Natural flavorants and fragrances exhibit different inhibitory activities on both SARS-CoV-2 cysteine proteases. 538 samples of essential oils, aromatic extracts, and F&F raw materials of various origins were screened for their inhibitory properties on SARS-CoV-2 M^{pro} and PL^{pro}. Screening (spectrofluorimetric enzymatic assay) was carried out with the employment of fluorogenic substrates designed by our group in previous works on SARS-CoV-2 proteases^{21,22}. Detailed results for each material are presented in the supplementary data (Table S1). No or the lowest inhibitory activities were observed for all materials with high-monoterpene and monoterpenoid content. The most active natural flavorants and fragrances (inhibitory activity >70%, Table 1) constitute mostly materials with both volatile, and non-volatile fractions like resinoids (storax, benzoin Sumatra and Siam, galbanum, and tolu), gums (elemi, storax, and labdanum), and absolutes (rocket, spinach, and oakmoss). The inhibitory potential of complex mixtures like natural F&F materials is directly related to their composition, which depends on such factors as source plant species, its country of origin, part of the plants used, and method of isolation. Clear differences can be observed for example between lovage root and leaf essential oils, where the former

exhibits higher inhibitory activity on both SARS-CoV-2 proteases (77% and 39% for M^{pro} and PL^{pro} respectively, table 1) than the latter (16% and 0% for M^{pro} and PL^{pro} respectively, table S1, entry 52).

Flavorants and fragrances derived from *Cistus ladanifer* are very good examples showing the distribution of activity within materials derived from one plant species. *Cistus ladanifer* is a pyrophoric plant and a source of highly prized labdanum gum. When the plant's flowers begin to fade, the shrub develops leafy twigs. Its branches are covered with secretory hairs, which release abundant quantities of gum with an amber-like fragrance. The gum was historically harvested by combing goats, whose coats were covered with the exudate as they crisscrossed the hillsides²³. Nowadays cistus branches are gathered and processed industrially. Hydrodistillation or steam diffusion of cistus branches results in cistus traditional essential oil, which has a lemony, fresh, and amber-like olfactory profile. The addition of an extract from distillation water to the traditional oil gives ciste (full) essential oil with predominant amber-like, warm, and gourmand notes. Extraction of cistus twigs and branches with hydrocarbon-based solvent leads to a cistus concrete, which can be processed by ethanolic extraction that gives traditional cistus absolute with dry wood and sweet notes or by molecular distillation resulting in cistus SEV absolute with balsamic, smoky, and hot notes. On the other hand, young cistus branches can be dipped in the hot solution of sodium carbonate. This solution is acidified with sulphuric acid and raw labdanum gum is obtained. Its hydrodistillation results in labdanum oil with intensive amber-like, woody notes. Extraction of gum with a hydrocarbon solvent leads to labdanum concrete, which after ethanolic extraction gives labdanum absolute that possesses alcoholic, balsamic, and mild notes. Ethanolic extraction of raw gum leads to labdanum resinoid with balsamic, sweet, and amber-like olfactory profile. The acid fraction of a mixture of labdanum gum and resinoid processed by molecular distillation leads to labdasur – aroma material with animalic and cheese-like notes. It should be noted that despite the same plant origin, cistus isolates present different olfactory properties. This means that some constituents are absent in one isolate, while present in another, which may result in different biological activities. And in this case, the inhibitory activities of various cistus isolates on SARS-CoV-2 M^{pro} are different, *e.g.* labdanum gum – 74%, labdanum resinoid – 33%, ciste EO – 34%, cistus absolute – 0%, cistus SEV absolute – 42%.

In some materials, the non-volatile part is mostly responsible for inhibitory activity. It is evident in the case of elemi (*Canarium luzonicum*) gum (97% M^{pro} inhibition vs 33% in the case of elemi essential oil), and galbanum (*Ferula galbaniflua*) resinoid (78% vs 18% (EO) M^{pro} inhibition). Very illustrative examples of the influence of the method of isolation are turmeric-derived flavorants and fragrances where most probably polar volatile part is responsible for the M^{pro} inhibitory activity (essential oil - 64%; isolation method: water/steam distillation vs CO₂ extract – 33% inhibition), and polar non-volatile part for PL^{pro} inhibitory activity (essential oil – 6% and CO₂ extract 5% vs oleoresin 64% inhibition). In the case of turmeric oleoresin, its polar non-volatile part also enhances the M^{pro} inhibitory activity (89%).

The *Cupressaceae* family constitutes a more abundant example of active essential oils. Cade crude *ex-sabine* EO was the most active (94%) against M^{pro}, together with blue cypress and Siam wood essential

oils. These activities were also retained for Chinese cedarwood (*Cupressus funebris*) and Virginian cedarwood (*Juniperus virginiana*) EOs (Table S1, 57%, and 60% inhibition respectively).

Most of the activity of the tested samples was attributed to the inhibition of the SARS-CoV-2 main protease. M^{pro} is the primary protease of the virus, the papain-like protease has a regulatory role, and both of them are essential for the processing of the polyprotein. In addition, the PL^{pro} has an important role to play in counteracting the innate immunity of the host cell. Ultimately, the best inhibitor would be the one that has high activities against one or both enzymes. There were only two examples (out of 400 tested) of such high activities – turmeric oleoresin (89% M^{pro} and 64% PL^{pro} inhibition) and petitgrain mandarin essential oil (85% M^{pro} and 100% PL^{pro} inhibition).

Table 1. Inhibition activity of selected F&F materials against SARS-CoV-2 cysteine proteases.

English common name	Source plant botanical name	Main compounds of the natural product	Country of origin	M ^{pro} [%] ^a	PL ^{pro} [%] ^a
Altingiaceae					
Storax gum	Liquidambar styraciflua	Cinnamyl cinnamate 3-Phenylpropyl cinnamate	Honduras	90.5±3.6	8
Storax resinoid	Liquidambar orientalis		Honduras	93.1±0.7	13
Amaranthaceae					
Spinach absolute	Spinacia oleracea	Flavonoids	Egypt	76.6±5.9	15
Apiaceae					
Galbanum resinoid	Ferula gummosa Boiss. syn. Ferula galbaniflua Boiss. et Buhse	(+)-Nopinone (+)-Eremorphilene β-Amyrin	Iran	78.2±0.8	11
Lovage root EO	Levisticum officinale Koch	(Z)-Ligustilide	Hungary	76.5±1.9	39
Brassicaceae					
Rocket absolute	Eruca vesicaria	Erucic acid	Egypt	100	26
Burseraceae					
Elemi gum	Canarium luzonicum (Blume) A. Gray	β-Amyrin, brein, elemadienoic acid β-phellandrene Elemol Elemicin	Philippines	97.1±1.9	17
Cistaceae					
Labdanum gum refined	Cistus ladanifer	Labdanoic acid	Spain	73.7±2.2	0
Cupressaceae					
Blue cypress EO	Callitris intratropica	(+)-Bulnesol (-)-Guaiol	Australia	73.1±2.7	0
Cade crude ex-sabine EO	Juniperus phoenicea	α-Pinene δ–3-Carene β-Phellandrene	Spain	94.0±0.6	31
Siam wood EO	Fokienia hodginsii	Fokienol (E)-Nerolidol	Vietnam	83.8±1.5	3
Fabaceae					
Cabreuva red EO	Myrocarpus fastigiatus Allemao	(E)-Nerolidol	Paraguay	78.6±0.9	19
Tolu resinoid	Myroxylon	Benzyl benzoate	Venezuela	77.3±4.6	15

	<i>balsamum</i> (L.) Harms	Benzyl cinnamate			
<i>Parmeliaceae</i>					
Oakmoss absolute	<i>Evernia Prunastri</i>	Evernin, Atranorin β -Orcinolcarboxylate	North Macedonia	71.4 $\pm$ 3.1	16
<i>Poaceae</i>					
Vetiver EO	<i>Chrysopogon zizanioides</i> (L.) Roberty syn. <i>Vetiveria zizanioides</i> (L.) Nash	Khusimol Vetiselinol	Haiti	74.2 $\pm$ 4.3	0
<i>Rutaceae</i>					
Petitgrain mandarin (PM) EO	<i>Citrus reticulata</i> Blanco syn. <i>Citrus nobilis</i> Andrews	Dimethyl anthranilate	Egypt, Spain	85.3 $\pm$ 6.2	100
<i>Santalaceae</i>					
Sandalwood EO	<i>Santalum austro-caledonicum</i>	α -Santalol	New Caledonia	70.9 $\pm$ 3.3	4
<i>Styracaceae</i>					
Benzoin Siam resinoid	<i>Styrax tonkinensis</i>	Benzoic acid Benzoates	Laos	99.7 $\pm$ 0.4	17
Benzoin Sumatra resinoid	<i>Styrax benzoin</i>	Cinnamic acid Cinnamates	Indonesia	72.9 $\pm$ 2.5	9
<i>Zingiberaceae</i>					
Turmeric oleoresin	<i>Curcuma longa</i>	Curcuminoids β -Phellandrene <i>ar</i> -Turmerone	India	89.0 $\pm$ 0.7	64.3 $\pm$ 1.6
<i>Zygophyllaceae</i>					
Guaiacwood EO	<i>Bulnesia sarmientoi</i>	(+)-Bulnesol (-)-Guaiol	Paraguay	72.0 $\pm$ 5.1	15

^a Inhibitory activity for the concentration of 50 μ g/mL of each F&F material. Numbers represent the mean value from two experiments (inhibition < 50%) or five experiments (inhibition > 50%).

All the main compounds of oils, gums, and resinoids listed in Table 1 have been studied whether they can be potentially considered as potent inhibitors of the SARS-CoV-2 M^{pro} and SARS-CoV-2 PL^{pro}. They were docked in the active center of the enzymes, in the S1 cavity, but with large binding freedom (20 Å) due to

their large shape. Mostly they bind at the S1-S1' cavity but are also shifted towards further cavities on the substrates and the products on both sides (Figure 1A and 1B).

Structural similarity of main compounds outside families can be noticed, and they could be divided into six main groups: benzoates and cinnamates, aliphatic acids, aromatic oxygenated compounds, low-molecular cyclic oxygenated compounds, terpene hydrocarbons, and terpenoid alcohols. The first group of compounds includes derivatives of organic acids based on the skeleton of benzoic or cinnamic acid. The extension of the ester or skeleton length could have a positive effect, increasing the inhibitory activity. Cinnamic acid esters could be more active than their shorter benzoate counterparts. Complex matrices of the remaining materials do not allow a direct comparison of the structure-inhibition relationship. The additional oxygen, hydroxy, or methoxy moieties or heteroatoms in other main groups of major constituents of the most active natural materials could not directly increase the inhibition of the enzyme. It is difficult to say whether it is certain, especially when a given natural product has an equal amount of individual ingredients.

However, the main constituents can be important for the inhibitory activity, for example (+)-bulnesol and (-)-guaiol prevail in blue cypress and guaiac wood essential oils. The results of inhibition for the M^{pro} are almost the same, but for the PL^{pro} the additional ingredients of guaiac wood oil increase its activity against this enzyme. The variation of the inhibition could be related to the different ratios of the two major compounds in blue cypress and guaiac wood essential oils.

One compound contributes to the overall inhibitory activity of the petitgrain mandarin essential oil. The essential oil isolated from mandarin leaves was the most potent in the preliminary screening studies. It comprises 6 main constituents (Table 2): α -pinene, β -pinene, *p*-cymene, (+)-limonene, γ -terpinene, dimethyl anthranilate (DMA), with the last one being the most abundant.

Table 2. The main constituents of the petitgrain mandarin (*Citrus reticulata* blanco var. Mandarin) oils

		RI ^a		Area ^d [%]			Identification method ^e
		DB-5	lit. DB-5	<i>EO</i>	<i>LFr</i>	<i>HFr</i>	
1	α -pinene	931	932 ²⁴	2.19	3.65	2.42	RI, MS, ref.
2	β -pinene	973	972 ²⁵	2.18	3.51	1.92	RI, MS, ref.
3	<i>p</i> -cymene	1023	1025 ²⁶	14.7	9.09	2.19	RI, MS, ref.
4	(+)-limonene	1027	1024 ²⁷	8.26	11.3	8.34	RI, MS, ref.
5	γ -terpinene	1057	1056 ^{b 28}	17.9	30.2	20.2	RI, MS, ref.
6	dimethyl anthranilate (DMA)	1409	1407 ^{c 29}	43.5	35.1	60.6	RI, MS, ref.

^a Experimental and literature RIs relative to *n*-alkanes on DB-5 capillary column unless otherwise stated. ^b RI for DB-5MS column. ^c RI for SLB-5MS column. ^d Area percentage of the main components; *EO* – essential oil, *LFr* – a light fraction of the oil, *HFr* – a heavy fraction of the oil. ^e Methods: RI – identification based on RI comparison with literature data; MS – identification based on mass spectra comparison with those of Wiley Registry of Mass Spectral Data library (8th edition), and Adams library of essential oil components (edition 4.1); ref. – co-injection with an authentic sample.

Petitgrain mandarin essential oil was fractionated on kugelrohr, three fractions and distillation residue were analyzed chromatographically, and their activity on both SARS-CoV-2 proteases was assessed (Table 3, the activity of fractions determined at the same concentration as the raw material during screening phase). Before fractionations, the hypothesis was set that the anthranilate part of the oil is responsible for the inhibitory activity (other materials with high monoterpene content exhibited low or no activity). The hypothesis was correct as the inhibition rate increases with the increase of dimethyl anthranilate content. The same can be observed for two fractions of PM essential oil (during the production of the oil two fractions emerge – lighter than water, and heavier one - the combination of both gives the commercial PM oil). It was further confirmed with the evaluation of pure natural dimethyl anthranilate, which fully inhibits both SARS-CoV-2 proteases in the same experimental conditions.

Table 3. Discovery of the most active constituents of the petitgrain mandarin (*Citrus reticulata* blanco var. Mandarin) oil against SARS-CoV-2 M^{pro} and PL^{pro}.

		Area ^a [%]							
		<i>EO</i>	<i>Fr. 1</i>	<i>Fr. 2</i>	<i>Fr. 3</i>	<i>Res.</i>	<i>LFr</i>	<i>HFr</i>	DMA
1	α -pinene	2.19	4.07	0.7	0.03	0.0	3.65	2.42	-
2	β -pinene	2.18	4.36	1.2	0.03	0.0	3.51	1.92	-
3	<i>p</i> -cymene	14.7	27.5	20.2	0.6	0.06	9.09	2.19	-
4	limonene	8.26	16.4	9.9	0.2	0.03	11.3	8.34	-
5	γ -terpinene	17.9	33.5	27.1	0.8	0.05	30.2	20.2	-
6	dimethyl anthranilate (DMA)	43.5	7.0	33.8	93.0	92.7	35.1	60.6	100
	M ^{pro} inh. [%]	85	3	62	100	100	70	87	100
	PL ^{pro} inh. [%]	100	79	100	100	100	100	100	100

^a Area percentage of the main components of the petitgrain mandarin oil and its fractions: *Fr.* – distillation fraction number; *Res.* – distillation residue; *EO* – essential oil, *LFr* – a light fraction of the oil, *HFr* – a heavy fraction of the oil.

The detailed inhibition properties of the petitgrain mandarin essential oil, natural dimethyl anthranilate, and other most active essential oils and aromatic extracts were assessed (Table 4). As could be suspected from the fractionation studies, PM oil and DMA are significantly stronger inhibitors of SARS-CoV-2 papain-like protease. The best inhibitors of M^{pro} are benzoin Siam and Sumatra resinoids, and rocket extracts (absolute and concrete) with the IC₅₀ in range of 3.41 to 5.24 µg/mL.

Table 4. Inhibition parameters of essential oils and aromatic extracts and SARS-CoV-2 M^{pro} and PL^{pro}.

Natural material	SARS-CoV-2 M ^{pro} IC ₅₀ [µg/mL] ([µM])	SARS-CoV-2 PL ^{pro} IC ₅₀ [µg/mL] ([µM])
Essential oils		
Petitgrain mandarin EO	>100	22.9±4.6
DMA	>100	5.20±1.22 (31.5±7.4)
Guaiacwood EO	48.01 ± 4.51	-
Blue cypress EO	48.22 ± 3.02	-
Lovage root EO	70.86 ± 4.65	-
Siam wood EO	76.10 ± 1.23	-
Extracts		
Benzoin Siam resinoid	5.24 ± 0.22	-
Benzoin Sumatra resinoid	4.20 ± 0.15	-
Galbanum resinoid	34.52 ± 2.27	-
Storax resinoid	46.31 ± 3.52	-
Turmeric oleoresin	12.01 ± 1.31	96.15 ± 2.09
Labdanum gum refined	47.44 ± 1.60	-
Nasturtium absolute	72.79 ± 1.12	-
Rocket absolute	3.41 ± 0.32	-
Rocket concrete	4.79 ± 0.04	-
<i>Tasmania lanceolata</i> extract	49.98 ± 1.99	-

Our exploratory study aimed to develop pioneering knowledge, and provide the first experimental results on the inhibitory properties of hundreds of flavor & fragrance materials against SARS-CoV-2 main- and papain-like proteases. The majority of available information is related to SARS-CoV. Only several plant extracts have been evaluated on activity against the key proteases, and they exhibited moderate to low inhibitory potential (Table 5, entries 3-5) with comparison to two natural plant constituents 3-isothaflavin-3-gallate and tannic acid, which had relatively good inhibitory activities on the SARS-CoV main protease (Table 5, entries 1 and 2).

Table 5. Inhibitory activity of plant extracts and their constituents on the key SARS-CoV proteases.

No	Material	SARS-CoV M ^{pro} IC ₅₀	SARS-CoV PL ^{pro} IC ₅₀	Ref
1	3-Isothaflavin-3-gallate	7 µM	-	30
2	Tannic acid	3 µM	-	
3	<i>Houttuynia cordata</i> aq extract	~1000 µg/mL	-	31
4	<i>Psoralea corylifolia</i> seeds EtOH extract	-	15 µg/mL	32
5	<i>Rheum palmatum</i> EtOH extract	38.09 µg/mL	-	33

Molecular docking studies reveal the detailed binding method of the DMA in the active sites of both enzymes. Dimethyl anthranilate was docked in the active centers of the enzymes to investigate ligand-enzyme interactions. Two types of binding were considered. The ligand was allowed to bind freely in 20 Å from the catalytic cysteine (Cys145 for M^{Pro} and Cys111 for PL^{Pro}) or to be forced to interact with the mentioned amino acid.

In the first step of docking, DMA interacted with the catalytical groups of the SARS-CoV-2 M^{Pro} in both ways of docking. The S1-S1' pocket is not filled tightly by the ligand (Figure 2A), which is directed with the methyl group of the ester to the Cys145. The placement is stabilized by the hydrophobic interactions of Cys145—Me (3.28 Å) and His163—Me (3.27 Å). The carbonyl oxygen atom interacts with the histidine as well (3.33 Å). Its position is determined by a strong hydrogen bond with Ser144 (2.47 Å). Another strong hydrogen bond is noticeable for the amino group of the inhibitor with His164 (2.57 Å). The position of the phenyl ring and the *N*-methyl group seems to be rather constrained and the interactions of the amino acids with them are weaker compared to the ester of the carboxyl group. Besides, their positions might be influenced by a strong intermolecular interaction of neighboring COOMe and NHMe groups (1.92 Å).

The optimization of the ligand-enzyme complex moved the dimethyl anthranilate towards the S2-S1 pocket (Figure 2A). The phenyl ring finds itself in the middle of a hydrophobic area of a pocket built by the His41 (4.05 Å), the Cys44 (5.48 Å), and the Met49 (3.98 Å). Intermolecular interaction is not formed because the side chain of the Gln189 creates a strong hydrogen bond (1.90 Å) with the carbonyl oxygen atom, while the methyl ester group interacts with His41 (3.60 Å) and Met165 (4.03 Å). Such positioning of the ligand seems more natural and comparable to the enzyme-substrate complex. This is also confirmed by the large difference in the binding energy $\Delta G_{S1-S1'} = -16.70$ kcal/mol vs $\Delta G_{S2-S1} = -37.08$ kcal/mol.

The amino acids building the S1 and S1' active pockets SARS-CoV-2 PL^{Pro} are closer and catalytical Cys111 is involved in the construction of both. Dimethyl anthranilate is placed very well in the S1 area (Figure 2B). It creates a strong hydrogen bonds between its carbonyl oxygen atom and catalytical amino acids Trp106 (1.90 Å) and Cys111 (3.25 Å). The methyl ester group plays a role of a dovetail interlocking the compound in its position with hydrophobic interactions with Asn109 (3.25 Å), Tyr112 (5.13 Å), Leu162 (3.97 Å). The leucine interacts hydrophobically with the *N*-methyl group (4.17 Å) likewise. Small role in the bindings of the inhibitor plays Cys270, which arranges the aromatic part of the DMA (5.45 Å).

Intramolecular interaction of COOMe and NHMe (1.96 Å) also occurs.

In the S2-S1 pocket, placement is different than in the M^{Pro} (Figure 2B). The phenyl ring interacts with Cys111 (4.22 Å), while Asn110 (3.16 Å) and the hydrophobic amino acids Ile104 (4.40 Å, 3.28 Å for C=O—Me), Ala114 (3.42 Å). The alanine (3.80 Å), Leu118 (5.13 Å) and Ile285 (3.61 Å) position the methyl carboxylate. Carbonyl oxygen atom interacts with the carbonyl oxygen atom of Asp286 (3.34 Å) and by the carbon-hydrogen bond with Lys274 (3.34 Å), but those bonds come from recurring intermolecular interaction (1.89 Å). The binding energy determines that the S2-S1 pocket is also preferred: $\Delta G_{S1-S1'} =$

-17.02 kcal/mol vs $\Delta G_{S2-S1} = -23.59$ kcal/mol. The compound bind well in both selected areas of the both tested enzymes, however, the binding energy confirms they do prefer the S2-S1.

Benzoin resinoids (Siam and Sumatra) demonstrated very promising inhibitory activity of M^{pro}. They are comprised of derivatives of benzoic and cinnamic acids. Four main constituents were evaluated *in-silico* on SARS-CoV-2 M^{pro} (Figure 3): benzyl benzoate (Fig. 3A), benzyl cinnamate (Fig. 3B), cinnamyl cinnamate (Fig. 3C), and cinnamic acid (Fig. 3D). For the general nature of the inhibition, we assumed that the unsaturated parts of the compounds are *trans*-isomers.

Benzyl derivatives (Fig 3A and B) are directed by the phenyl ring of the acid part towards the deep part of the S2 pocket of the active center. Arenes are stabilized by the multiple hydrophobic interactions with the lipophilic amino acid such as His41, Met49. However, none of the amino acids create the hydrogen bond between ligand and protein. Hydrogen interaction occurs when the backbone is elongated. Carbonyl oxygen interacts with the amino group's hydrogen atom from the Glu166. The attracted ligands are too distant to form a weak phenyl-Cys145 interaction. Nevertheless, it may expose the double bond from the ester part and create the possibility of forming a covalent bond between the aforementioned cysteine and the backbone of the compound.

Benzoin Sumatra resinoid has the most prominent antiviral activity. The most active protease inhibitors were also tested for their antiviral activity using a fully replication-competent SARS-CoV-2 strain in VeroE6-GFP cell culture (Table S2). The benzoin Sumatra resinoid showed selective inhibition (half-maximal effective concentration (EC_{50}) = 31.5 ± 2.4 μ g/mL) with limited toxicity (50% cytotoxic concentration (CC_{50}) = 85.5 ± 1.9 μ g/mL). The best PL^{pro} inhibitor petitgrain mandarin essential oil showed lower antiviral activity (>100 μ g/mL).

Available data on the activity of plant materials against highly pathogenic human coronaviruses is scarce. A few studies have been conducted on anti-SARS-CoV activities of plant extracts, and essential oils (Table 6). Evaluation of cinnamon bark and clove buds aqueous extracts (Table 6, entries 1-5) have shown that hydroalcoholic extract of cinnamon bark and its butanol fraction possess moderate inhibitory activity against SARS-CoV infection. Screening of 121 Chinese herbs resulted in the identification of two compounds (Table 6, entries 6 and 7), tetra-*O*-galloyl- β -D-glucose from *Galla chinensis*, and luteolin from *Veronica linariifolia*, which could inhibit SARS-CoV infection in a dose-dependent fashion. Only one reference addresses the evaluation of anti-SARS-CoV activities of essential oils³⁴ (Table 6, entries 10-16) with laurel EO being the most active (modestly).

Table 6. The anti-SARS-CoV activity of plant extracts and their constituents.

No	Material	SARS-CoV IC ₅₀	Ref
1	<i>Cinnamomi cortex</i> (<i>Cinnamomum verum</i> , cinnamon bark) aq extract (CCE)	43.1 µg/mL	35
2	<i>Cinnamomi cortex</i> (<i>Cinnamomum verum</i> , cinnamon bark) hydroalcoholic extract (CC)	10.7 µg/mL	
3	Butanol fraction of CC	7.8 µg/mL	
4	Aqueous fraction of CC	39.7 µg/mL	
5	<i>Caryophylli flos</i> (<i>Syzygium aromaticum</i> , clove buds) aq extract	50.1 µg/mL	
6	tetra- <i>O</i> -galloyl-β-D-glucose	4.5 µM	36
7	Luteolin	10.6 µM	
8	Glycyrrhizin	>607.6 µM	
9	Glycyrrhizin	300 µg/mL	37
10	<i>Laurus nobilis</i> EO	120 µg/mL	34
11	<i>Juniperus oxycedrus</i> ssp. <i>oxycedrus</i> EO	270 µg/mL	
12	<i>Thuja orientalis</i> EO	130 µg/mL	
13	<i>Cupressus sempervirens</i> ssp. <i>pyramidalis</i> EO	700 µg/mL	
14	<i>Pistacia palaestina</i> EO	>1000 µg/mL	
15	<i>Salvia officinalis</i> EO	870 µg/mL	
16	<i>Satureja thymbra</i> EO	No activity	

In addition to the *in-vitro* evaluations, several randomized controlled trials showed some promise of Chinese herbal medicine combined with conventional medicine for the treatment of SARS but the evidence was insufficient due to the low methodological quality of the trials ³⁸.

Relevant data on the activity of plant materials against MERS-CoV and SARS-CoV-2 is even more scarce, as most of the references are focused on *in-silico* evaluations of natural products (including essential oils constituents) ³⁹⁻⁴¹ or perspectives in this field ⁴². Two of the few meaningful references describe the antiviral value of the hydroalcoholic extract of *Echinacea purpurea* herb and roots (Echinaforce®) against MERS-CoV and SARS-CoV-2 ^{43,44}. 50 µg/ml of the Echinaforce® (final concentration of ethanol was 0.2% and it was proven that tested coronaviruses were not sensitive to this residual concentration) fully blocked infectivity of MERS-CoV and SARS-CoV-2.

Despite the lack of experimental *in-vitro* data on the potential of flavor and fragrance materials against SARS-CoV-2, two clinical trials evaluate the potential of the commercial product containing essential oils constituents (Listerine® - it was originally EO-based, now it comprises a mixture of eucalyptol, (-)-menthol, thymol, and methyl salicylate) to eliminate SARS-CoV-2 in the throat and nasopharynx of COVID-19 patients. The first trial (⁴⁵, NCT04410159) was completed and has shown high viral clearance [two negative RT-PCR (of swabs from oropharynx and nasopharynx) results at least 24 hours apart] rate for 1% iodine-povidone (Betadine®) and „essential oils” (Listerine®) gargles (100% and 80% respectively) 4 days after intervention (median time without intervention – 14 days). These results shall be treated with caution though – testing groups were very small (5 patients in each gargle group). The second „gargle”

clinical trial (NCT04584684) „Antiviral Efficacy and Acceptability of Therapeutic Antiseptic Mouth Rinses for Inactivation of COVID SARS-2 Virus” is ongoing. Its goal is to test the efficacy of antiseptic mouth rinses (e.g. Listerine®) to inactivate SARS-CoV-2 in the saliva of COVID-19 positive patients (480 patients). Listerine® is a hydroalcoholic mixture of essential oils constituents. The ethanol concentration is at the level of 21%, which is significant but it has been proven that the concentration of ethyl alcohol for the complete inactivation of SARS-CoV-2 must be $\geq 30\%$ (⁴⁶, 20% EtOH concentration resulted in a reduction factor of only 1.1).

F&F materials are important for society as they are commonly used in various areas of human interest, have established toxicological profiles, and are relatively safe. Until recently, the tools to assess the inhibitory potential of chemicals against the key SARS-CoV-2 proteases were limited. Our recent developments ^{21,22} provided a framework for the discovery of new materials/molecules with a potential therapeutic value. Results of this study give the scientific community the selection criteria for further *in vitro* and *in vivo* testing of the most promising EOs, AEs, and isolated natural compounds. They can be also used for the rational design of SARS-CoV-2 protease inhibitors. Even though important countermeasures for the spread of SARS-CoV-2 are in motion (introduction of vaccines), these results will be important to the field because coronaviruses are changing but coronaviral proteases are conserved (broad application of results in potential future outbreaks).

Materials And Methods

The molecular modeling studies were prepared with the protocol of our earlier studies ⁴⁷. Crystal structures of the SARS-CoV-2 M^{pro} (PDB ID: 6XBH - ⁴⁸) and SARS-CoV-2 PL^{pro} (PDB ID: 6WX4²²) were obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB-PDB). Waters, ligands, and ions were deleted, and the proteins were protonated at experimental pH and optimized ⁴⁹. Before docking, the structures of the inhibitors were protonated in experimental pH typical for each enzyme and optimized by LigPrep ⁵⁰. Induced Fit Docking protocol was used for molecular docking studies. The site of the enclosing box was set at 20 Å on the centroid of the catalytical cysteines ⁵¹. The VSGB (variable-dielectric generalized Born) model, which incorporates residue-dependent effects, was used. The solvent was water. The active center amino acids were optimized within 5.0 Å of ligand poses, and Glide redocking was carried out with the XP (extra precision) algorithm. The top three poses for ligand were saved. The last steps, rePrime refinement, and MM-GBSA (molecular mechanics-generalized Born surface area) calculations were performed to calculate the Gibbs free energies with protein flexibility, and the distance from the ligand was also set to 5.0 Å. The one with the lowest free binding energy was considered for publication. The protocol and all the parameters were the same for the second docking, but the ligands were forced to interact with the catalytical cysteine (Cys145 or Cys111) during the IFD.

Reagents

Essential oils and related products

All samples of investigated plant volatile products were generously donated by members of the industry – manufacturers of the products at the best possible quality. Nomenclature of the products according to specifications.

They were:

A. Fakhry & Co.

Essential oils of bitter orange (fruit) distilled, petitgrain bigarade *sur fleurs*, cabbage rose, carrot seed, celery seed, celery leaf, chamomile blue, cinnamon basil, tropical basil, sweet basil, lemon basil, clary sage, coriander seed, coriander leaf, dill leaf, jasmine, anise seed, cumin, lantana, leek, fennel bitter, fennel sweet, neroli, neroli BdN, parsley seed, parsley leaf, petitgrain bigarade, petitgrain key lime, petitgrain mandarin, winter savory, summer savory, tagetes, geranium, petitgrain mandarin, bitter orange distilled, rosemary, caraway, aniseed, spearmint, sweet marjoram, mature bitter orange (“red”) expressed, immature bitter orange (“green”) expressed, onion, and garlic **absolutes of** artichoke, cabbage rose, cabbage rose leaves, calendula, carnation, carrot leaf, cassie, *Rosa damascena*, bitter orange blossom hydrolate, honeysuckle, jasmine, mango leaf, nasturtium, nettle, olive leaf, rocket, spinach, strawberry leaf, tomato leaf, tropical basil, sweet basil, lemon basil, tropical basil hydrolate, petitgrain bigarade, tagetes, geranium, carrot seed, coriander leaves, fenugreek, geranium hydrolate, rosemary, caraway, sweet marjoram, blue chamomile, cumin, orange flower water, violet leaves, clary sage and **concretes** of cabbage rose, jasmine, lemon basil, sweet basil, tropical basil, geranium, thyme, blue chamomile, cumin, violet leaves, nettle, calendula, artichoke, nasturtium, mango leaves, cassie, strawberry leaves, olive leaves, clary sage, carnation, rocket, and carrot leaves, and hydroalcoholic **extract** of licorice.

Albert Vieille

Essential oils of traditional and complete cistus, **absolutes** of cistus, cistus SEV, and tonka bean.

Amigo & Arditi

Essential oils of cabreuva red, guaiac wood, citronella organic, and petitgrain bitter orange.

Ashna Samai

Essential oils of Irish lace, spiked pepper, copal, ishpingo, and palo santo.

Berje Inc.

Essential oils of angelica root, angelica seed, anise (*Pimpinella anisum*), *Artemisia afra*, rosewood, buchu leaf betulina, buchu leaf crenulata, calamus, cascarilla bark, catnip, coffee, cognac white, cubeb, dill seed, Eucalyptus smithii, fir needle China, lavandin abrialis, lovage leaf, lovage root, mandarin red, milfoil, *Ocotea cymbarum*, parsley leaf, *Rosa damascena* Bulgaria, *Rosa damascena* Turkey, peppermint Yakima, *Pinus pumilio*, perilla, sassafras, savory, siam wood, tagetes, valerian root, and wormwood European, fir balsam, **absolutes** of spruce, *Rosa damascena* Morocco and Bulgaria, orris root **concrete**, **gums** of labdanum and storax.

Bordas S.A.

Essential oils of labdacistus, rue, bitter fennel, clementine, cypriol, laurel, onion, tarragon, tangerine, petitgrain lemon, petitgrain mandarin, vetiver Java, ylang ylang 2nd, citronella Java type, orange CP Valencia, and thyme red, **absolutes** of labdanum, jasmine, spike lavender, thyme red, and thyme grey, **oleoresins** of cumin and turmeric.

BoreA Canada

Essential oils of black spruce twigs and leaves, bark, and wood, jack pine twigs and leaves, and wood, balsam poplar, balsam fir bark, hops, larch tamarack, white spruce, Labrador tea.

Brüder Unterweger.

Essential oils of black pine, maritime pine, silver fir cones, hops, hyssop, yarrow blue 3% and 15% chamazulene, and Venice turpentine I.

Christodoulou Bros S.A.

Essential oils of bitter orange, lemon, mandarin, and white grapefruit.

Citrus and Allied Essences Ltd.

Essential oils of fir needle Siberian, ginger China, lemon California type CP Extra, lemon Argentina Tucuman CP, orange Valencia CP, orange Brazil CP, orange Brazil 5-fold, corn mint India (partially dementholized, *Mentha arvensis*), tangerine Dancy, geranium China.

D.V. Deo Industries

Essential oils of palmarosa, cardamom, gingergrass vetiver, cypriol, angelica root, spikenard, ginger, betel leaf, lemongrass, galangal root, chaulmoogra, and cedarwood.

Dutjahn Sandalwood Oils.

Santalum spicatum **essential oil**.

Essential Oils and Herbs

Melissa and yarrow **essential oils**.

Essential Oils of Tasmania

Essential oils of bitter fennel, coastal tea tree, fennel (70% fenchone), kunzea, lavender, parsley herb, parsley seed, peppermint, rosemary, big badja gum, smoky tea tree, and southern Rosalina, **absolutes** of Boronia, Boronia leaves, Tasmanian pepperberry, and Tasmanian pepperberry **extract**.

Eucaforest

Essential oils of rose geranium, tea tree, lemon-scented tea tree, *Eucalyptus smithii*, *Eucalyptus radiata*, tagete.

Hierbas Patagónicas S.R.L

Essential oils of fabiana, douglas fir, paramela, and pinus ponderosa.

Jacarandas International

Essential oils of psiadia, ylang-ylang I, II, III, and complete, clove bud and leaf, ginger fresh, black pepper, *Helichrysum gymnocephalum*, *Helichrysum bracteiferum*, *Eucalyptus citriodora*, geranium, niaouli, and ravintsara.

Kallin Ltd.

Essential oils of citronella China, garlic China, geranium China, geranium Egypt, ginger China, neroli Morocco, peppermint arvensis China, petitgrain bigarade, sandalwood India, shiu (Ho wood) China, vetivert Bourbon, and ylang-ylang, **absolutes** of coffee, fenugreek, jasmine India, orris, and orange flower, ginger China and pink pepper **CO₂ extracts**.

Mane Kancor Ingredients Private Limited

Essential oils of carrot seed, ajowan, black pepper CO₂, capsicum CO₂, ginger CO₂, cumin seed, black cumin, celery seed, fennel, mace, tuberose **absolute** and **concrete**.

Lebermuth

Essential oils of allspice, blood orange, catnip, cedarwood Himalayan, *Eucalyptus polybractea*, goldenrod, grapefruit red, *Lavandula stoechas*, mandarin red, Peru balsam, *Rosa damascena*, black spruce, tea tree, ylang-ylang I, ylang-ylang II, and neem **oil**.

Les Arômes du Maroc

Mastic and neroli **essential oils**, orange flower water **absolute**, orange flower, and bran **concretes**, and orris germanica butter.

Lluch Essence

Essential oils of cedarleaf, cedarwood Texas, citronella Java type, geranium Egypt, ginger China, ginger Nigeria, gurjun balsam, lavender fresh Bulgarian, lemon Spain, sweet orange CP Valencia, sweet orange CP Brazil, pennyroyal, peppermint Arvensis, thuja, thyme red, star aniseed, cassie, citrate, cognac green, coriander seed, cumin “ex-distilled”, dill leaf, *Eucalyptus radiata*, lemongrass, key lime distilled type Mexico, key lime expressed type Mexico, mandarin green, myrtle, nutmeg, parsley seed, patchouli molecular distillation, patchouli super dark, pimento berry, pimento leaf, pine needle, scotch pine, ravintsara, sweet basil, tropical basil, verbenas, ylang-ylang I, ylang ylang III, **oleoresins** of basil, capsicum 1000000 SHU, paprika 40000 SHU, paprika 60000 SHU, paprika 80000 SHU, pepper black 40/20, and thyme red, copaiba balsam, and gurjun balsam rectified (copaene).

Metainy

Cotula and neroli **essential oils**.

Nelixia

Cardamom **essential oil**, storax gum and raw gum, peru balsam and raw peru balsam.

New Zealand Mānuka Group

Essential oils of mānuka (MBTK 5+), mānuka (MBTK 20+), mānuka (MBTK 25+), and kanuka.

Payan Bertrand S.A.

Essential oils of galbanum, myrrh, olibanum, opoponax, storax, oakmoss **absolute, resinoids** of galbanum, myrrh, olibanum, opoponax, storax, benzoin Siam, and benzoin Sumatra.

Plant's Power

Essential oils of magnolia flower, goldenrod, catnip, rhododendron, fir balsam needle, Labrador tea, sweet fern, black spruce, and yuzu.

Robertet S.A.

Bran, blackcurrant buds, and broom **absolutes**.

Ultra International

Essential oils of angelica root, artemisia, *Artemisia taurica*, blood orange, blue cypress, buchu leaf, buddha wood, coriander herb, *Eucalyptus kochii*, hinoki, kumquat, kunzea, lemon myrtle, Rosalina, **CO₂ extracts** of juniper berry, star anise, turmeric, vetiver, black seed, cardamom green, coffee arabica, coffee robusta, and ginger.

Van Aroma

Essential oils of clove stem 85% MD, clove stem 85% dark, cajeput, and **CO₂ extracts** of cocoa butter, black pepper, clove bud, turmeric, cubeb, cassia bark, and ginger, and benzoin crystals.

Vessel Essential Oils

Essential oils of lavender organic, oregano liquid fire, oregano organic, lavandin organic, peppermint organic, sweet orange organic, lemon organic, mandarin organic, pine organic, sea fennel organic, melissa organic, helichrysum organic, thyme organic, laurel organic, sage organic, rosemary organic, white fir organic, blue chamomile organic, mastic organic, rose organic, wild carrot seed organic, sweet marjoram organic, cypress organic, geranium organic, clary sage organic, yarrow organic, sweet fennel organic, juniper berry organic, anise organic, dill organic, cistus organic, Roman chamomile, spearmint, grapefruit organic oil, coriander seed oil, petitgrain organic, basil organic ct linalool, inula, frankincense Somalia, Emerald frankincense, frankincense Ethiopia, frankincense India, frankincense Oman.

The remaining samples of essential oils and other aromatic materials were bought from **PerfumersWorld Ltd**. Essential oils were named according to the ISO 4720:2018 norm - *Essential oils – Nomenclature* where possible.

Enzymes

The expression of recombinant enzymes - SARS-CoV-2 M^{pro} and PL^{pro} is described in our previous works ^{15,22}.

ACC-labeled substrates

The synthesis of ACC-labeled substrate for M^{pro} (Ac-Abu-Tle-Leu-Gln-ACC) and PL^{pro} (Ac-Leu-Arg-Gly-Gly-ACC) is described in our previous works ^{21,22}.

Inhibitor screening

SARS-CoV-2 M^{pro} (75 nM) was preincubated in assay buffer 20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.3 for 10 min at 37°C. Then, the enzyme was added to wells containing inhibitors (50 µg/mL essential oils or aromatic extracts) and the mixture was incubated for 30 min at 37°C. After the incubation period, fluorogenic substrate (Ac-Abu-Tle-Leu-Gln-ACC) was added to the wells (final concentration 50 µM). ACC liberation was monitored for 30 min at 37°C ($\lambda_{\text{ex}} = 355 \text{ nm}$, $\lambda_{\text{em}} = 460 \text{ nm}$) using Molecular Devices Spectramax Gemini XPS spectrofluorometer. Negative control experiments were carried out in the absence of the enzyme. Each experiment was repeated twice (inhibition $\leq 50\%$) or five times (inhibition $> 50\%$). The same experiments were performed for SARS-CoV-2 PL^{pro}. SARS-CoV-2 PL^{pro} (150 nM) was preincubated in assay buffer 50 mM Tris, 5 mM NaCl, 0.075% BSA, 5 mM DTT, pH 7.5 for

10 min at 37°C. The assay conditions were the same as described above (Ac-Leu-Arg-Gly-Gly-ACC was used as the substrate to measure SARS-CoV-2 PL^{Pro} residual activity).

IC₅₀ determination

For selected inhibitors, the IC₅₀ value was determined. Serial dilutions of inhibitors in assay buffer were prepared on 96-well plates (20 µL of each dilution in wells). SARS-CoV-2 M^{Pro} (75 nM) or SARS-CoV-2 PL^{Pro} (100 nM) was preincubated in assay buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.3; 50 mM Tris, 5 mM NaCl, 0.075% BSA, 5 mM DTT, pH 7.5 respectively) for 10 min at 37°C. Then, 60 µL of the enzyme was added to the wells containing serial dilutions of inhibitors (ranging from 1 µg/mL to 80 µg/mL) and the mixture was incubated for 30 min at 37°C. After that time, 20 µL of the substrate (Ac-Abu-Tle-Leu-Gln-ACC for SARS-CoV-2 M^{Pro} or Ac-Leu-Arg-Gly-Gly-ACC for SARS-CoV-2 PL^{Pro}) was added to each well. Measurements were carried out at 37°C for 40 min (λ_{ex} = 355 nm, λ_{em} = 460 nm). The experiments were repeated three times. IC₅₀ values were determined in GraphPad Prism software using non-linear regression (dose-response – Inhibition equation) and presented as relative enzyme activity vs. inhibitor concentration.

Antiviral activity of essential oils and aromatic extracts toward a SARS-CoV-2 strain in VeroE6-GFP cell culture

VeroE6-GFP cells were seeded at a density of 25000 cells/well in 96-well plates (Greiner Bio One, catalogue no. 655090) and pre-treated with three-fold serial dilutions of the compounds overnight. On the next day (day 0), cells were infected with the SARS-CoV-2 inoculum at a multiplicity of infection (MOI) of 0.001 tissue culture infectious dose (TCID₅₀) per cell. The number of fluorescent pixels of GFP signal determined by High-Content Imaging (HCI) on day 4 post-infection (p. i.) was used as a read-out. Percentage of inhibition was calculated by subtracting background (untreated-infected control wells) and normalizing to the untreated-uninfected control wells (also background subtracted). Experiments were carried out twice. The 50% effective concentration (EC₅₀) was determined using logarithmic interpolation. Potential toxicity of compounds was assessed in a similar set-up in treated-uninfected cultures where metabolic activity was quantified at day 5 using the MTS assay as described earlier⁵². The 50% cytotoxic concentration (CC₅₀) was calculated by logarithmic interpolation.

Gas chromatography (FID)

GC analyses were performed using a Shimadzu GC-2010 Plus gas chromatograph equipped with an FID detector and DB-5 (0.25 mm i.d. × 30 m, 0.25 µm film thickness, Agilent, Santa Clara, USA) capillary column. The injection port was maintained at 250 °C. The split ratio was set as 25:1, and 1 µL of the

sample was injected. The oven temperature was set at 40 °C and increased to 300 °C at a rate of 2 °C/min, with a constant nitrogen carrier gas flow of 1.5 mL/min. The linear retention indices (RIs) of the compounds were calculated using the retention times of *n*-alkanes from C₈ to C₂₆.

Gas chromatography-mass spectrometry (GC-MS)

GC-MS analyses were performed using an Agilent 7890A gas chromatograph equipped with an HP-5-MS capillary column (0.25 mm i.d. × 30 m, 0.25 µm film thickness, Agilent, Santa Clara, USA) combined with a WATERS GCT high-resolution mass spectrometer (TOF, EI+). The injection port was maintained at 250 °C. 1 µL of the sample was injected in the splitless mode. The oven temperature was held at 40 °C and raised to 300 °C at a rate of 2 °C/min, with a constant helium carrier gas flow of 1.5 mL/min. Mass spectra in electron impact (EI) mode were recorded at 70 eV ionization energy.

Essential oil fractionation

Distillation of the petitgrain mandarin essential oil was carried out using Buchi B-585 oven equipped with the kugelrohr accessory. 11 g of the essential oil was placed in a 40 mL round-bottom flask and was fractionated under controlled distillation set-up (2 mbar, 40-260°C). Three fractions (3.22 g, 2.09 g, 3.91 g) and a residue (1.78 g) were analyzed using GC-FID and GC-MS.

Declarations

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Abbreviations used

Abu, 2-aminobutanoic acid; ACC, 7-amino-4-carbamoylmethylcoumarin; RFU, relative fluorescence unit; Tle, *tert*-leucine; EO, essential oil, AE, aromatic extract; MBTK, manuka oil beta triketones; DMA, dimethyl anthranilate

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Author contributions

Conceptualization: D.J.S.; project administration: D.J.S., M.S.; investigation: D.J.S., W.R., M.Z., M.S., D.J., L.V.; methodology: D.J.S., W.R., M.Z., D.J.; molecular modeling studies: M.T.; obtainment of natural samples: D.J.S., W.B.; enzyme expression: L.Z., X.S., Z.L., D.N., R.H., S.O.; statistical analysis: D.J.S., M.Z.; writing - original draft preparation: D.J.S, M.T., W.R., M.S.; writing - review and editing: D.J.S, M.T., W.R., M.Z., M.S., W.B., R.H., S.O., D.J., M.D.; funding acquisition: D.J.S., M.D. All authors have read and agreed to the published version of the manuscript.

Competing interest

Authors declare no competing interests.

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Figures

Figure 1

Modeling of the main compounds of the natural products onto crystal structures of of SARS-CoV-2-Mpro (A, PDB: 6XBH) and SARS-CoV-2-PLpro (B, PDB: 6WX4) with approximate scaling of the S4-S1' active cavities. Ligands surface is colored green and represents the total volume of all of the studied compounds in the active centers. The surface of the enzymes is colored light blue (Mpro) or light pink (PLpro).

Figure 2

The interactions of the dimethyl anthranilate in the active centers of SARS-CoV-2 Mpro (A) and SARS-CoV-2 PLpro (B). The binding for each enzyme is shown for the S1- S1' and S2-S1 pockets. The surface of the enzymes is colored light blue (Mpro) or light pink (PLpro). The surface of the methyl N-methylanthranilate is colored dark brown for the S1-S1' pockets and green for the S2-S1 pockets. The ligands and the side chains of the amino acids are shown as sticks, the bond order is not shown.

Figure 3

The interactions of benzyl benzoate (A), benzyl cinnamate (B), cinnamyl cinnamate (C), and cinnamic acid (D) in the active center of SARS-CoV-2 Mpro. The ligands and the side chains of the amino acids are shown as sticks, the bond order is not shown.

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