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Discovery of Bioactive Natural Products for the Treatment of Acute Respiratory Infections – An Integrated Approach*

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ABSTRACT

In this work, an integrated approach for the identification of new antiviral agents from natural sources for the treatment of acute respiratory infections is presented. The approach comprises (i) the selection of starting material based on traditional knowledge, (ii) phenotypic screening of extracts for antiviral activity, and (iii) the implementation of *in silico* predictions to identify antiviral compounds and derive the molecular mechanism underlying their biological activity. A variety of starting materials from plants and fungi was selected for the production of 162 extracts. These extracts were tested in cytopathic effect inhibition assays against influenza virus A/Hong Kong/68 (HK/68), rhinovirus A2 (RV-A2), and coxsackie virus B3 (CV-B3). All extracts were also evaluated regarding their cytotoxicity. At an IC_{50} threshold of 50 µg/mL, 20, 11, and 14% of all tested extracts showed antiviral activity against HK/68, CV-B3, and RV-A2, respectively. Among all active extracts (n = 47), 68% showed antiviral activity against one of the investigated viruses, whereas 31% inhibited at least two viruses. Herein, we present a comprehensive dataset of probed extracts along with their antiviral activities and cytotoxicity. Application examples presented in this work illustrate the phytochemical workflow for the identification of antiviral natural compounds. We also discuss the challenges, pitfalls, and advantages of the integrated approach.

Introduction

Acute respiratory infections (ARIs) affect the lives of millions of people each year. They are the leading cause of morbidity and mortality related to infectious diseases worldwide [1]. ARIs are typically caused by enteroviruses (EVs), e.g., coxsackie viruses (CVs) and the closely related rhinoviruses (RVs), or influenza A viruses (IAVs) and influenza B viruses (IBVs). Virus infections might also occur in combination with bacterial infections caused by *Streptococcus pneumoniae, Staphylococcus aureus, Haemophilus influenzae*, or *Pseudomonas aeruginosa* [2–4].

Neither vaccines nor antiviral drugs are available for the prevention or treatment of EV infections [5, 6]. For IAV and IBV infections, the gold standard for prevention is vaccination [7, 8]. Treatment options are limited to ion channel blockers (M2 inhibitors) and NAIs. Most circulating IAVs are resistant to approved M2 inhibitors (amantadine and rimantadine in particular) [9]. Also, the portfolio of NAIs is small, with oseltamivir and zanamivir being the only two NAIs approved in most countries. Further NAIs include laninamivir, approved in Japan, and peramivir, approved in Japan, China, South Korea, and the USA [10]. Japan was the first country to approve the stockpiling of favipiravir, an RNA polymerase inhibitor, for use during influenza pandemics in 2016 [11].

The risk of emerging NAI-resistant IAVs was demonstrated by the recent local [12, 13] and global [14] outbreaks of seasonal

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ABBREVIATIONS

HK/68	influenza virus A/Hong Kong/68 (H3N2)
ARI	acute respiratory infection
CC_{50}	50% cytotoxic concentration
CPE	cytopathic effect
CV	coxsackie virus
CV-B3	coxsackie virus B3
EV	enterovirus
RV	rhinovirus
RV-A2	rhinovirus type A2
IAV	influenza A viruses
IBV	influenza B viruses
IV	influenza viruses
LLE	lead-like enhanced extract
MDCK	Madin-Darby canine kidney
NA	neuraminidase
NAI	neuraminidase inhibitor
RNA	ribonucleic acid
SI	selectivity index

H1N1 IAV that acquired mutations compensating the fitness loss caused by the H275Y mutation [12].

The lack of anti-EV drugs, the limited efficacy of NAIs against IVs, resistance issues, and the limited availability of favipiravir demand for the identification of novel highly active anti-EV and anti-IV agents as leads for drug development. Natural products are a primary resource for the discovery and development of new antivirals [15]. For the identification of bioactive secondary metabolites from plant, fungal, microbial, or marine sources, a variety of approaches such as (i) the exploitation of chemotaxonomic or ethnopharmacological knowledge, (ii) extract screening followed by bioassay-guided isolation, and (iii) computational approaches have been developed and applied [16, 17]. Probing multicomponent mixtures and their constituents is generally approached by two different strategies: (1) phenotypic screening, where the identification of promising starting material is based on the constituents' ability to trigger a desired biological response without knowledge of the underlying mode of action, and (2) target-based screening, where a specific working hypothesis serves as a starting point for the identification of bioactive compounds effective on a specific molecular target [18].

In this study, an integrated approach combining, in particular, knowledge from traditional medicine and phenotypic screening of extracts is presented. The objective was to identify antiviral natural products against three pathogens involved in ARIs: HK/68, RV-A2, and CV-B3. The application, scope, and limitations of this integrated approach are discussed and supported by a number of examples. Screening data of 162 extracts are presented to guide the selection of further promising candidates for the discovery of natural products targeting viral proteins.

Results and Discussion

Ethnopharmacological sources comprising the books "*De materia medica*" by Pedanios Dioscurides [19], "*Naturalis historiae libri*" by Plini the Elder [20], "*Das große Buch der Heilpflanzen*" and sources cited therein [21], and the published final report of an EU-Interreg-II project, "*Volksmedizin in Tirol*" [22] served as a starting point. The key words "cough", "cold", "catarrh", "sore throat", "fever", "lung disease", "pneumonia", "flu", "bronchitis", and "pleuritis" were used for searching in these four sources to select plant and fungal species with a traditional background for the treatment of symptoms related to ARIs.

The final selection comprised 141 diverse plant and fungal species belonging to 66 different families, with Asteraceae (10%), Lamiaceae (10%), Apiaceae (6%), and Fabaceae (4%) being the most prominent ones (▶ **Table 1**). For some of the selected species, individual extracts were produced from different organs, resulting in a total of 162 extracts.

A recently reported protocol for the preparation of LLEs [23, 24] was adapted for the production of small-scale extracts of the acquired natural materials. Additionally, 15 plant extracts (produced either by extraction with dichloromethane, methanol, ethanol, or water) were included in the extract screening (▶ **Table 1**).

The ability of extracts to inhibit the virus-induced CPE was evaluated with a phenotypic assay described previously [25, 26]. In this work, multicomponent mixtures were screened in a cellular model to test whether the contained compounds were able to protect the cells from the respective virus. The observed effects served as a basis for further experiments to determine the bioactive compounds and their biological targets. All 162 extracts were tested for their anti-influenza activity against HK/68 in MDCK cells. The majority of the extracts (88%) were also tested against CV-B3 and RV-A2 in HeLa cells (▶ **Table 1** and **Fig. 1 a**). Forty-seven extracts (29%) were active (i.e., having IC_{50} values no higher than 50 µg/mL) against at least one of the three viruses. Of these extracts, 33 (20%), 16 (11%), and 20 (14%) extracts were active against HK/68, CV-B3, and RV-A2, respectively. IC_{50} values were below 30 µg/mL for 21 (13%), 8 (6%), and 14 (10%) extracts, respectively. Sixty-eight percent showed antiviral activity (defined as IC_{50} values no higher than 50 µg/mL) against one of the investigated viruses, whereas 15 extracts (31%) inhibited at least 2 viruses. Cytotoxicity was determined for all samples as a prerequisite for the estimation of selectivity of antiviral activity. The SI of each antiviral active extract was calculated to evaluate the specificity of antiviral activity (▶ **Table 1**). Raw data for the determination of IC_{50} and CC_{50} values (and respective CI) of the most active extracts are given in **Table 2S**, Supporting Information.

Two-dimensional plots of activity and cytotoxicity data allowed for the prioritization and targeted selection of starting materials for further phytochemical and pharmacological investigations (▶ **Fig. 1**). In particular, the extracts were classified into four different categories based on their measured antiviral activity and cytotoxicity (▶ **Fig. 1 a**).

▶ **Table 1** Antiviral activity and cytotoxicity of extracts. Their mean IC_{50} values against IV A/Hong Kong/68 (HK/68), coxsackie virus (CV-B3), and rhinovirus (RV-A2) in the CPE inhibition assay (MDCK cells for HK/68; HeLa cells for CV-B3 and RV-A2) as well as their mean CC_{50} values are presented (n = 3).

Code	Species	Family	Organ	Extract type	HK/68		CV-B3		RV-A2		CC_{50} [µg/mL] in	
					IC_{50} [µg/mL] of CPE	Selectivity index [CC_{50}/IC_{50}]	IC_{50} [µg/mL] of CPE	Selectivity index [CC_{50}/IC_{50}]	IC_{50} [µg/mL] of CPE	Selectivity index [CC_{50}/IC_{50}]	MDCK cells	HeLa cells
1	*Abutilon theophrasti* Medik.	Malvaceae	seeds	LLE	n. a.	–	n. a.	–	n. a.	–	> 100	> 100
2	*Aegle marmelos* (L.) Corrêa	Rutaceae	fruit	LLE	n. a.	–	n. d.	–	n. d.	–	> 100	n. d.
3	*Allium sativum var. sativum* L.	Amaryllidaceae	bulb	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
4	*Anchieta pyrifolia* (Mart.) G. Don	Violaceae	herb	LLE	n. a.	–	n. a.	–	n. a.	–	> 100	> 100
5	*Andographis paniculata* (Burm. F.) Nees	Acanthaceae	herb	LLE	79	> 1.3	n. a.	–	n. a.	–	> 100	64
6	*Angelica sinensis* (Oliv.) Diels	Apiaceae	root	LLE	n. a.	–	n. a.	–	n. a.	–	> 100	> 100
7	*Annona squamosa* L.	Annonaceae	seeds	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	6.3
8	*Aquilegia vulgaris* L.	Ranunculaceae	herb	LLE	n. a.	–	n. d.	–	n. d.	–	> 100	n. d.
9	*Arctostaphylos uva-ursi* (L.) Spreng.	Ericaceae	leaves	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	82
10	*Arisaema sp.*	Araceae	rhizome	LLE	68	> 1.5	n. a.	–	32*	–	> 100	68
11	*Artemisia absinthum* L.	Asteraceae	herb	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	129
12	*Artemisia anomala* S. Moore	Asteraceae	herb	LLE	n. a.	–	n. a.	–	n. a.	–	> 100	> 100
13	*Artemisia argyi* Levl. & Vant.	Asteraceae	leaves	LLE	24	2.5	n. a.	–	32*	–	60	67
14	*Artemisia vulgaris* L.	Asteraceae	herb	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
15	*Aster tataricus* L. f.	Asteraceae	root	LLE	n. a.	–	n. a.	–	n. a.	–	> 100	> 100
16	*Azadirachta indica* A. Juss.	Meliaceae	fruit	LLE	n. a.	–	n. d.	–	n. d.	–	84	n. d.
17	*Boswellia serrata* Roxb. ex Colebr.	Burseraceae	resin	D	9.0	2.4	n. d.	–	n. a.	–	21	16
18	*Buddleja officinalis* Maxim.	Loganiaceae	flowers	LLE	n. a.	–	n. a.	–	n. a.	–	> 100	81
19a	*Burkea africana* Hook.	Fabaceae	bark	D	24	3	32*	–	29	2.1	71	60
19b	*Burkea africana* Hook.	Fabaceae	bark	M	5.6	11	n. a.	–	n. a.	–	63	16
20a	*Burkea africana* Hook.	Fabaceae	heartwood	D	22	> 4.5	n. a.	–	n. a.	–	> 100	> 100
20b	*Burkea africana* Hook.	Fabaceae	heartwood	M	48	> 2.1	32*	–	n. a.	–	> 100	74
21	*Calamintha menthifolia* L.	Lamiaceae	herb	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
22	*Calendula officinalis* L.	Asteraceae	flowers	LLE	n. a.	–	n. d.	–	n. d.	–	> 100	n. d.
23	*Capsella bursa-pastoris* (L.) Medik.	Brassicaceae	herb	LLE	44	2.9	n. a.	–	n. a.	–	126	> 200
24	*Carlina acaulis* L.	Asteraceae	herb	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	145
25	*Carlina acaulis* L.	Asteraceae	root	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
26	*Carum carvi* L.	Apiaceae	fruit	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
27	*Castanea sativa* Mill.	Fagaceae	leaves	LLE	n. a.	–	46	1.7	n. a.	–	n. d.	80

continued

▶ **Table 1** *Continued*

Code	Species	Family	Organ	Extract type	HK/68		CV-B3		RV-A2		CC_{50} [µg/mL] in	
					IC_{50} [µg/mL] of CPE	Selectivity index [CC_{50}/IC_{50}]	IC_{50} [µg/mL] of CPE	Selectivity index [CC_{50}/IC_{50}]	IC_{50} [µg/mL] of CPE	Selectivity index [CC_{50}/IC_{50}]	MDCK cells	HeLa cells
28a	*Centaurea ragusina* L.	Asteraceae	leaves	LLE	2.7	2.4	n. a.	–	n. a.	–	6.4	9.0
28b	*Centaurea ragusina* L.	Asteraceae	leaves	H_2O	n. a.	–	n. d.	–	n. d.	–	62	n. d.
28c	*Centaurea ragusina* L.	Asteraceae	leaves	E	n. a.	–	n. d.	–	n. d.	–	2.3	n. d.
29	*Cetraria islandica* (L.) Ach.	Parmeliaceae	thallus	LLE	44	3.3	50*	–	50*	–	148	91
30	*Chenopodium ambrosioides* (L.) Mosyakin & Clemants	Amaranthaceae	leaves	LLE	75	> 1.3	n. a.	–	n. a.	–	> 100	> 100
31	*Chrysanthemum indicum* L.	Asteraceae	flowers	LLE	n. a.	–	n. a.	–	n. a.	–	> 100	> 100
32	*Cinnamomum mairei* H. Lév.	Lauraceae	bark	LLE	n. a.	–	n. a.	–	n. a.	–	> 100	> 100
33	*Cordia curassavica* (Jacq.) Roem. & Schult.	Boraginaceae	leaves	LLE	30	2.0	n. a.	–	n. a.	–	60	31
34	*Cynanchum paniculatum* (Bunge) Kitag. ex H.Hara	Apocynaceae	root	LLE	n. a.	–	n. a.	–	n. a.	–	4.7	> 100
35	*Cynanchum stauntonii* (Decne.) Schltr. ex H.Lév.	Apocynaceae	root	LLE	9.1	2.1	n. a.	–	n. a.	–	19	> 100
36	*Cynomorium songaricum* Rupr.	Cynomoriaceae	herb	LLE	n. a.	–	n. a.	–	n. a.	–	> 100	> 100
37	*Daucus carota* L.	Apiaceae	herb	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
38	*Drynaria fortunei* (Kunze) J. Sm.	Polypodiaceae	rhizome	LLE	n. a.	–	n. a.	–	n. a.	–	> 100	> 100
39	*Elettaria cardamomum* (L.) Maton	Zingiberaceae	fruit	LLE	n. a.	–	n. d.	–	n. d.	–	> 100	n. d.
40	*Epimedium sagittatum* (Sieb. & Zucc.) Maxim.	Berberidaceae	herb	LLE	86	> 1.2	n. a.	–	n. a.	–	> 100	> 100
41	*Equisetum arvense* L.	Equisetaceae	herb	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
42	*Equisetum hiemale* L.	Equisetaceae	herb	LLE	n. a.	–	n. a.	–	n. a.	–	> 100	> 100
43	*Euphrasia officinalis ssp. rostkoviana* Hayne (L.)	Orobanchaceae	herb	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
44	*Evodia rutaecarpa* (Juss.) Benth.	Rutaceae	fruit	LLE	n. a.	–	1.0*	–	n. a.	–	48	0.8
45	*Fagopyrum esculentum* Moench.	Polygonaceae	seeds	LLE	n. a.	–	n. a.	–	n. a.	–	> 200	> 100
46	*Foeniculum vulgare* L.	Apiaceae	fruit	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	152
47	*Fomes fomentarius* J. J. Kickx. (strain 19)	Polyporaceae	fruit body	E	n. a.	–	n. a.	–	n. a.	–	> 100	> 100
48	*Fomitopsis pinicola* Karst. (strain 10)	Fomitopsidaceae	fruit body	E	n. a.	–	n. a.	–	n. a.	–	18	16
49	*Forsythia suspensa (Thunb.) Vahl*	Oleaceae	fruit	LLE	22	3.5	32*	–	n. a.	–	76	40
50	*Fraxinus sp.*	Oleaceae	bark	LLE	91	> 1.1	n. a.	–	91	> 1.1	> 100	> 100
51	*Galeopsis tetrahit* L.	Lamiaceae	herb	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200

continued

▶ **Table 1** *Continued*

Code	Species	Family	Organ	Extract type	HK/68		CV-B3		RV-A2		CC_{50} [µg/mL] in	
					IC_{50} [µg/mL] of CPE	Selectivity index [CC_{50}/IC_{50}]	IC_{50} [µg/mL] of CPE	Selectivity index [CC_{50}/IC_{50}]	IC_{50} [µg/mL] of CPE	Selectivity index [CC_{50}/IC_{50}]	MDCK cells	HeLa cells
52	*Ganoderma applanatum* (Pers.) Pat. (strain 12)	Ganodermataceae	fruit body	E	n.a.	–	n.a.	–	10*	–	18	16
53	*Ganoderma lucidum* Karst.	Ganodermataceae	fruit body	LLE	37	2.5	n.a.	–	n.a.	–	94	54
54	*Ganoderma sinense* Zhao, Xu & Zang	Ganodermataceae	fruit body	LLE	n.a.	–	n.a.	–	n.a.	–	> 100	> 100
55	*Ganoderma tsugae* Murill.	Ganodermataceae	fruit body	E	n.a.	–	n.a.	–	28	2.0	48	55
56	*Gardenia jasminoides* Ellis.	Rubiaceae	fruit	LLE	n.a.	–	n.a.	–	n.a.	–	> 100	> 100
57	*Glechoma hederacea* L.	Lamiaceae	herb	LLE	n.a.	–	n.a.	–	> 50	–	n. d.	109
58	*Gleditsia sinensis* Lam.	Fabaceae	fruit	LLE	n.a.	–	n.a.	–	n.a.	–	66	29
59	*Gloeophyllum odoratum* Imazeki (strain 23)	Gloeophyllaceae	fruit body	E	13	> 7.7	36	2.2	19	4.1	> 100	77
60	*Gloeophyllum odoratum* Imazeki (strain 28)	Gloeophyllaceae	fruit body	E	9.4	> 11	16	2.5	27	1.4	> 100	39
61	*Gloeophyllum odoratum* Imazeki (strain 54)	Gloeophyllaceae	fruit body	E	15	> 6.8	31	> 3.3	16	> 6.3	> 100	> 100
62	*Glycyrrhiza glabra* L.	Fabaceae	root	LLE	n.a.	–	n.a.	–	47	> 4.3	n. d.	> 200
63	*Hedera helix* L.	Araliaceae	leaves	LLE	n.a.	–	n.a.	–	n.a.	–	n. d.	> 200
64	*Helianthus annuus* L.	Asteraceae	flowers	LLE	n.a.	–	n.a.	–	n.a.	–	n. d.	> 200
65	*Hepatica nobilis* Schreb.	Ranunculaceae	leaves	LLE	n.a.	–	n.a.	–	n.a.	–	n. d.	> 200
66	*Hericium erinaceus* (Bull.) Pers.	Hericiaceae	fruit body	LLE	n.a.	–	n.a.	–	n.a.	–	> 100	> 100
67	*Imperata cylindrica var. major* (Nees) C. E. Hubb	Poaceae	root	LLE	n.a.	–	n.a.	–	n.a.	–	> 100	> 100
68	*Inonotus obliquus* (Ach. ex Pers.) Pilát	Hymenochaetaceae	fruit body	LLE	31	4.7	n.a.	–	n.a.	–	147	52
69	*Ischnoderma benzoinum* Karst. (strain 38)	Fomitopsidaceae	fruit body	E	n.a.	–	n.a.	–	32*	–	68	71
70	*Kaempferia galanga* L.	Zingiberaceae	rhizome	LLE	n.a.	–	n.d.	–	n.d.	–	> 100	n. d.
71	*Lactuca sativa* L.	Asteraceae	herb	LLE	n.a.	–	n.d.	–	n.d.	–	> 100	n. d.
72	*Laetiporus sulphureus* (Bull.) Murrill (strain 43)	Fomitopsidaceae	fruit body	E	n.a.	–	n.a.	–	n.a.	–	> 100	62
73	*Lantana camara* L.	Verbenaceae	leaves	LLE	n.a.	–	32*	–	n.a.	–	66	25

continued

▶ **Table 1** *Continued*

Code	Species	Family	Organ	Extract type	HK/68		CV-B3		RV-A2		CC_{50} [µg/mL] in	
					IC_{50} [µg/mL] of CPE	Selectivity index [CC_{50}/IC_{50}]	IC_{50} [µg/mL] of CPE	Selectivity index [CC_{50}/IC_{50}]	IC_{50} [µg/mL] of CPE	Selectivity index [CC_{50}/IC_{50}]	MDCK cells	HeLa cells
74	*Lepidium apetalum* Willd.	Apiaceae	seeds	LLE	n. a.	–	32*	–	n. a.	–	> 100	88
75	*Liquidambar orientalis* Mill.	Hamamelidaceae	resin	LLE	47	2.0	n. a.	–	13*	–	93	31
76	*Lonicera japonica* Thunb.	Caprifoliaceae	twigs	LLE	n. a.	–	n. a.	–	n. a.	–	> 100	> 100
77	*Lophaterum gracile* Brongn.	Poaceae	herb	LLE	n. a.	–	n. a.	–	n. a.	–	> 100	> 100
78	*Lycopodium clavatum* L.	Lycopodiaceae	herb	LLE	n. a.	–	n. a.	–	50*	–	n. d.	78
79	*Lycopodium clavatum* L.	Lycopodiaceae	spores	LLE	n. a.	–	n. a.	–	n. a.	–	> 200	122
80	*Lycopus lucidus var. hirtus* Regel.	Lamiaceae	herb	LLE	76	> 1.3	32*	–	n. a.	–	> 100	96
81	*Lygodium japonicum* (Thunb.) Sw.	Lygodiaceae	spores	LLE	41	> 2.4	n. a.	–	n. a.	–	> 100	> 100
82	*Magnolia sp.*	Magnoliaceae	flowers	LLE	n. a.	–	n. a.	–	n. a.	–	> 100	40
83	*Matricaria chamomilla* L.	Asteraceae	flowers	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
84	*Melissa officinalis* L.	Lamiaceae	leaves	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	84
85	*Morus alba* L.	Moraceae	root bark	M	30	> 3.4	n. d.	–	n. d.	–	> 100	n. d.
86	*Nelumbo nucifera* Gaertn.	Nelumbonaceae	leaves	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	75
87	*Nelumbo nucifera* Gaertn.	Nelumbonaceae	root	LLE	48	1.5	22	1.9	13*	–	74	42
88	*Nelumbo nucifera* Gaertn.	Nelumbonaceae	seeds	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
89	*Origanum vulgare* L.	Lamiaceae	herb	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
90	*Papaver rhoeas* L.	Papaveraceae	flowers	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
91	*Papaver somniferum* L.	Papaveraceae	seeds	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
92	*Peucedanum ostruthium* (L.) Koch	Apiaceae	herb	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
93	*Peucedanum ostruthium* (L.) Koch	Apiaceae	root	LLE	48	1.2	n. a.	–	n. a.	–	66	45
94	*Pharbitis sp.*	Convolvulaceae	seeds	LLE	32	1.2	n. a.	–	n. a.	–	39	20
95	*Phellinus robustus* (L.) Quel. (strain 25)	Hymenochaetaceae	fruit body	LLE	92	> 1.2	n. a.	–	n. a.	–	> 100	88
96	*Pimenta dioica* (L.) Merr.	Myrtaceae	fruit	LLE	n. a.	–	n. d.	–	n. d.	–	> 100	n. d.
97	*Pimpinella anisum* L.	Apiaceae	fruit	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
98	*Pimpinella major* (L.) Huds.	Apiaceae	root	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	155
99	*Pinguicula vulgaris* L.	Lentibulariaceae	herb	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
100	*Piper nigrum* L.	Piperaceae	fruit	LLE	n. a.	–	11	1.5	n. a.	–	n. d.	17
101	*Piptoporus betulinus* Karst. (strain 29)	Fomitopsidaceae	fruit body	E	10*	–	10*	–	8.3	2.3	54	22
102	*Piptoporus betulinus* Karst. (strain 39)	Fomitopsidaceae	fruit body	E	10*	–	10*	–	9.9	3.8	40	38

continued

▶ **Table 1** *Continued*

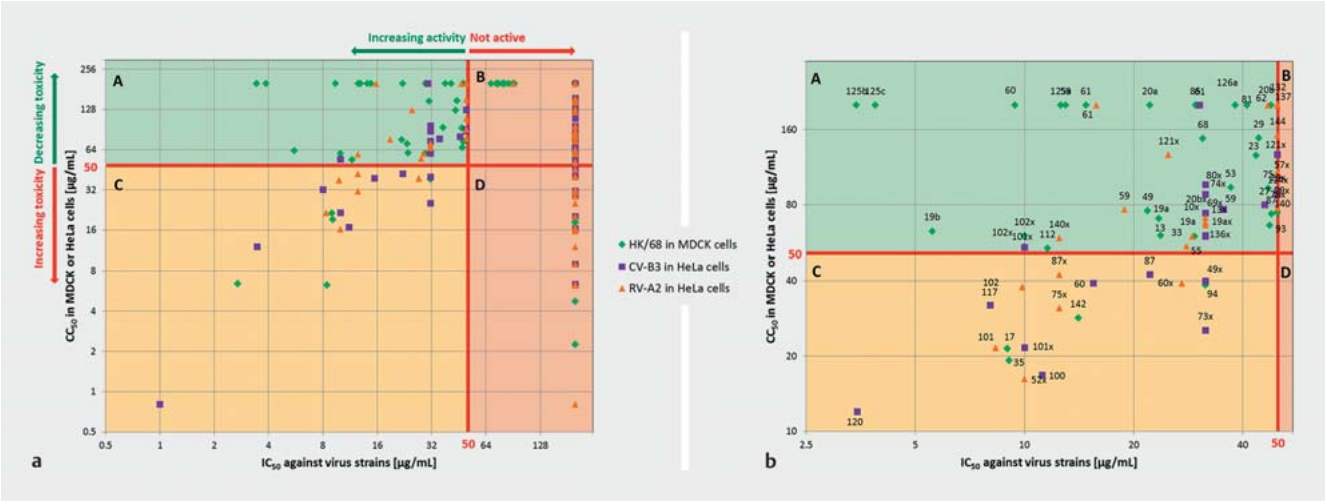
Code	Species	Family	Organ	Extract type	HK/68		CV-B3		RV-A2		CC_{50} [µg/mL] in	
					IC_{50} [µg/mL] of CPE	Selectivity index [CC_{50}/IC_{50}]	IC_{50} [µg/mL] of CPE	Selectivity index [CC_{50}/IC_{50}]	IC_{50} [µg/mL] of CPE	Selectivity index [CC_{50}/IC_{50}]	MDCK cells	HeLa cells
103	*Plantago lanceolata* L.	Plantaginaceae	leaves	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
104	*Polygala senega* L.	Polygalaceae	root	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	147
105	*Polygala sp.*	Polygalaceae	root	LLE	n. a.	–	n. a.	–	n. a.	–	> 100	> 100
106	*Polygala vulgaris* L.	Polygalaceae	herb	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
107	*Polygonum aviculare* L.	Polygonaceae	herb	LLE	n. a.	–	n. d.	–	n. d.	–	> 100	n. d.
108	*Polypodium vulgare* L.	Polypodiaceae	root	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
109	*Potentilla anserinae* L.	Rosaceae	herb	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
110	*Potentilla aurea* L.	Rosaceae	herb	LLE	n. a.	–	n. d.	–	n. d.	–	> 100	n. d.
111	*Prunella grandiflora* D. Torre & Sarnth.	Lamiaceae	herb	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
112	*Pterocarpus santalinus* L. f.	Fabaceae	wood	LLE	12	4.6	n. d.	–	n. d.	–	54	n. d.
113	*Pyrrosia sp.*	Polypodiaceae	leaves	LLE	n. a.	–	n. a.	–	n. a.	–	> 100	> 100
114	*Ribes nigrum* L.	Grossulariaceae	leaves	LLE	n. a.	–	> 50	–	n. a.	–	n. d.	89
115	*Ribes nigrum* L.	Grossulariaceae	fruit	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
116	*Rosa canina* L.	Rosaceae	fruit	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
117	*Rosmarinus officinalis* L.	Lamiaceae	leaves	LLE	n. a.	–	8.0	4.0	n. a.	–	n. d.	32
118	*Rubus fruticosus* L.	Rosaceae	leaves	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
119	*Rubus fruticosus* L.	Rosaceae	root	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
120	*Ruta graveolens* L.	Rutaceae	herb	LLE	8.5	< 0.7	3.5	3.5	n. a.	–	< 6.3	12
121	*Salvia glutinosa* L.	Lamiaceae	herb	LLE	n. a.	–	50*	–	25*	–	n. d.	127
122	*Sambucus nigra* L.	Adoxaceae	flowers	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
123	*Sambucus nigra* L.	Adoxaceae	fruit	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
124	*Saussurea costus* (Falc.) Lipsch.	Asteraceae	root	LLE	n. a.	–	n. d.	–	n. d.	–	56	n. d.
125a	*Sclerocarya birrea* (A. Rich.) Hochst.	Anacardiaceae	bark	D	13	> 7.9	n. a.	–	n. a.	–	> 100	46
125b	*Sclerocarya birrea* (A. Rich.) Hochst.	Anacardiaceae	bark	M	3.4	> 29	n. a.	–	n. a.	–	> 100	> 100
125c	*Sclerocarya birrea* (A. Rich.) Hochst.	Anacardiaceae	bark	E	3.9	> 26	n. d.	–	n. d.	–	> 100	n. d.
126a	*Sclerocarya birrea* (A. Rich.) Hochst.	Anacardiaceae	heartwood	D	38	> 2.6	n. a.	–	n. a.	–	> 100	96
126b	*Sclerocarya birrea* (A. Rich.) Hochst.	Anacardiaceae	heartwood	M	n. a.	–	n. a.	–	n. a.	–	> 100	> 100
127	*Scrophularia nodosa* L.	Scrophulariaceae	herb	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
128	*Scrophularia nodosa* L.	Scrophulariaceae	root	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
129	*Scutellaria barbata* D. Don	Lamiaceae	herb	LLE	n. a.	–	n. a.	–	n. a.	–	> 100	> 100

continued

▶ **Table 1** *Continued*

Code	Species	Family	Organ	Extract type	HK/68		CV-B3		RV-A2		CC_{50} [µg/mL] in	
					IC_{50} [µg/mL] of CPE	Selectivity index [CC_{50}/IC_{50}]	IC_{50} [µg/mL] of CPE	Selectivity index [CC_{50}/IC_{50}]	IC_{50} [µg/mL] of CPE	Selectivity index [CC_{50}/IC_{50}]	MDCK cells	HeLa cells
130	*Sida cordifolia* L.	Malvaceae	herb	LLE	n. a.	–	n. d.	–	n. d.	–	> 100	n. d.
131	*Sinomenium acutum* (Thunb.) Rehd. & Wils.	Menispermaceae	twigs	LLE	n. a.	–	n. a.	–	n. a.	–	> 100	> 100
132	*Solanum dulcamara* L.	Solanaceae	twigs	LLE	n. a.	–	n. a.	–	50	> 4	n. d.	> 200
133	*Solanum paniculatum* L.	Solanaceae	leaves	LLE	n. a.	–	n. a.	–	n. a.	–	77	51
134	*Solanum pseudoquina* A. St.-Hil.	Solanaceae	leaves	LLE	81	> 1.2	n. a.	–	n. a.	–	> 100	87
135	*Solanum torvum* Sw.	Solanaceae	leaves	LLE	n. a.	–	n. a.	–	n. a.	–	> 100	> 100
136	*Sophora flavescens* Ait.	Fabaceae	root	LLE	n. a.	–	32*	–	n. a.	–	> 100	60
137	*Stachys officinalis* L.	Lamiaceae	herb	LLE	n. a.	–	n. a.	–	50	> 4	n. d.	> 200
138	*Stachys sylvatica* L.	Lamiaceae	herb	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
139	*Stemona sp.*	Stemonaceae	root	LLE	n. a.	–	n. a.	–	n. a.	–	> 100	> 100
140	*Styrax calamitus* L.	Styracaceae	resin	LLE	50	1.5	n. a.	–	13*	–	74	59
141	*Syzygium aromaticum* (L.) Merr. & L. M.Perry	Myrtaceae	flowers	LLE	73	> 1.4	n. d.	–	n. d.	–	> 100	n. d.
142	*Terminalia chebula* Retz.	Combretaceae	fruit	LLE	14	2.0	n. a.	–	n. a.	–	28	33
143	*Teucrium chamaedrys* L.	Lamiaceae	herb	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
144	*Thymus pulegioides* L.	Lamiaceae	herb	LLE	n. a.	–	n. a.	–	50	3.0	n. d.	152
145	*Tilia cordata* Mill.	Malvaceae	flowers	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
146	*Trametes gibbosa* (Pers.) Fr. (strain 52)	Polyporaceae	fruit body	E	n. a.	–	n. a.	–	n. a.	–	> 100	> 100
147	*Tussilago farfara* L.	Asteraceae	leaves	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
148	*Vaccinium vitis-ideae* L.	Ericaceae	leaves	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
149	*Valeriana officinalis* L.	Valerianaceae	root	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
150	*Verbascum densiflorum* Bertol.	Scrophulariaceae	flowers	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
151	*Verbena officinalis* L.	Verbenaceae	herb	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
152	*Veronica officinalis* L.	Plantaginaceae	herb	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
153	*Viola odorata* L.	Violaceae	herb	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
154	*Viola tricolor* L.	Violaceae	herb	LLE	n. a.	–	n. a.	–	n. a.	–	n. d.	> 200
155	*Viscum coloratum* (Komar.) Nakai	Loranthaceae	herb	LLE	n. a.	–	n. a.	–	n. a.	–	> 100	> 100

*No IC_{50} could be determined due to toxicity. The last noncytotoxic test concentration with an antiviral effect is reported instead. Abbreviations: n. a. = not active, n. d. = not determined, D = dichloromethane, M = methanol, LLE = lead-like enhanced extracts [23, 24], H_2O = water, E = ethanol



▶ **Fig. 1** **a** Activity of extracts against HK/68, CV-B3, and RV-A2 versus their cytotoxicity (CV-B3 and RV-A2 in HeLa cells; HK/68 in MDCK cells). Candidates with high antiviral activity (IC_{50} ≤ 50 µg/mL) and low cytotoxicity (CC_{50} ≥ 50 µg/mL) are top left (green quadrangle). General activity (IC_{50} ≤ 50 µg/mL) and cytotoxicity frontiers (CC_{50} ≥ 50 µg/mL) are indicated by bold, red lines. All inactive extracts were set to an IC_{50} = 200 µg/mL in order to be able to display them in the graphic. Accordingly, CC_{50} values above 100 or 200 µg/mL were set to 200 µg/mL. **b** Enlarged section of **a** revealing the identity of respective extracts (see ▶ **Table 1**). Extracts where it was not possible to determine an IC_{50} value due to interference with cytotoxicity are marked with "x".

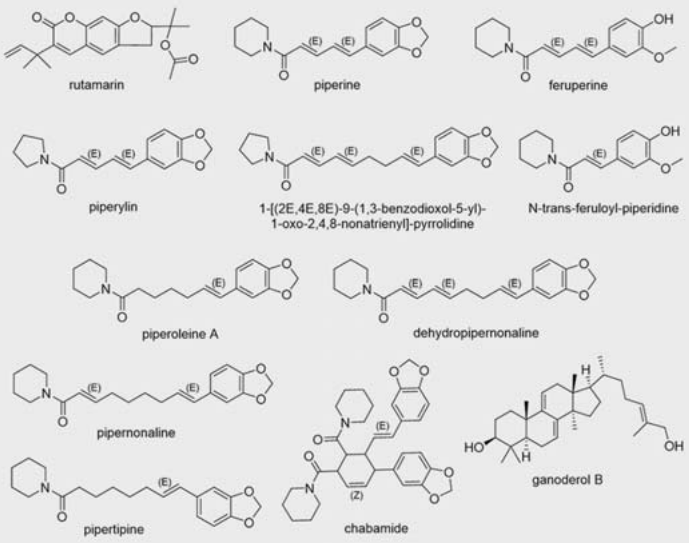
Category A extracts (i.e., extracts located in quadrant A of ▶ **Fig. 1 a**) had distinct antiviral activity (IC_{50} ≤ 50 µg/mL) and no or low cytotoxicity (CC_{50} ≥ 50 µg/mL), giving an SI > 5. These extracts were considered to be most promising for further processing. Category B and D extracts (located in the respective quadrants of ▶ **Fig. 1 a**) showed weak or no antiviral activity against the tested viruses (IC_{50} ≥ 50 µg/mL) and were therefore not investigated further. Category C extracts (located in quadrant C of ▶ **Fig. 1 a**) were active (IC_{50} ≤ 50 µg/mL), but also cytotoxic (CC_{50} ≤ 50 µg/mL). These extracts are potentially worthwhile investigating because the observed cytotoxicity may be mediated by components other than those responsible for the extract's antiviral activity, and those components could potentially be separated. However, cytotoxic compounds may be able to mimic biological activity and cause false-positive assay readouts (measurements potentially affected by this type of assay interference are indicated with an asterisk in ▶ **Table 1**). The probability of false-positive outcomes resulting from cytotoxicity is a function of the ratio of CC_{50} and IC_{50}, denoted as the SI. The higher the SI, the lower is the risk of false-positive results. Accordingly, extracts with SI values greater than 2 are candidates worthwhile investigating further. An enlarged depiction of this area of interest (quadrant A and the upper part of quadrant C) is shown in ▶ **Fig. 1 b**.

In the following paragraphs, we report on the most relevant findings obtained from the extract screening and show how the integrated approach can help to overcome some of the pitfalls in the discovery of antiviral natural compounds.

The majority of extracts was prepared according to the protocol for the preparation of LLE [23, 24]. In addition, some of the already available extracts were produced without the application of defatting or tannin depletion procedures. These include the extracts from the bark of *Burkea africana* (no. 19a and 19b) and *Sclerocarya birrea* (no. 125a, 125b, and 125c), which showed potent anti-influenza activity without a significant level of cytotoxicity (**Table 2S**, Supporting Information). These plant materials as well as the fruits of *Terminalia chebula* (no. 142) are known to contain high amounts of tannins. Tannins tend to build nonselective protein complexes [27–29] and were previously shown to prevent virus adsorption to the host cells [30–32]. The focus of our project was to identify novel antiviral compounds active against viral targets other than those involved in adsorption. Accordingly, following the specific antiviral activity in category A, extracts from the bark of *B. africana* and *S. birrea*, and the fruits of *T. chebula* were generated on a larger scale for phytochemical investigations. For further evaluation of the antiviral activity of these extracts, a polyamide column was employed to separate the extracts into fractions free of polyphenols, fractions with low molecular weight polyphenols, and fractions with polymerized polyphenols (e.g., tannins).

In the case of *S. birrea* and *T. chebula*, the *in vitro* results indicated a strong influence of tannins on the antiviral activity. While the tannin-depleted fractions were not or only weakly active in the CPE assay, an antiviral effect was observed for the fractions with polyphenols present. Therefore, the phytochemical investigation of these two plant materials was discontinued.

As recently published, the situation was different in the case of *B. africana*, where tannin-free fractions showed distinct activities against influenza HK/68, with IC_{50} values of about 3 µg/mL [33]. Accordingly, polyphenol-rich fractions were neglected, whereas the tannin-free fractions were investigated further (**Fig. 2S**, Supporting Information). Eight novel triterpene saponins from lupane and oleanane types were identified as the bioactive principles (**Fig. 2S**, Supporting Information). In the CPE assay, the most active compounds showed IC_{50} values between 0.05 µM and



▶ **Fig. 2** Chemical structures of the tested pure compounds.

0.27 µM against HK/68 and the 2009 pandemic H1N1 strain A/Jena/8178/09 [33].

To explore the limitations of this integrated approach, the extracts of *Piper nigrum* fruits (no. 100) and *Ruta graveolens* herbs (no. 120) were selected for further analysis. These extracts out of category C exerted significant antiviral activities, but also problematic levels of cytotoxicity (SI between 1 and 2). The aim here was to determine whether the antiviral activity and cytotoxicity are mediated by different components and whether those could be separated.

The extract of *R. graveolens* (no. 120; **Figs. 4S** and **5S**, Supporting Information) obtained IC_{50} values of 8.5 and 3.5 µg/mL in the phenotypic assay with HK/68 and CV-B3, respectively. Due to cytotoxicity (CC_{50} = 12 µg/mL in HeLa cells and below 6.3 µg/mL in MDCK cells), no exact IC_{50} for RV-A2 could be determined but was estimated by visual evaluation to be in the range of 12 to 25 µg/mL. Sixteen metabolites isolated from aerial parts of *R. graveolens* were evaluated in a previous study [34]. These are the alkaloids S-ribalinine, arborinine, isoplatydesmine, (−)-edulinine, and norgraveoline, the coumarins 7-methoxycoumarin, 6,7,8-trimethoxycoumarin, daphnoretin methyl ether, rutamarin, isoimperatorine, psoralen, bergapten, and 8-methoxy psoralene, and the phenyl propionic acid derivatives methyl 3-hydroxy-3-(4-hydroxy-3,5-dimethoxyphenyl) propanoate, methyl 3-(6-hydroxy-7-methoxy-benzofuran-5-yl) propanoate, and methyl 3-(4-hydroxy-3,5-dimethoxyphenyl)oxirane-2-carboxylate). In that study, the potential targets of these metabolites were predicted with a pharmacophore-based *in silico* approach. For one out of five metabolites predicted as inhibitors of the RV coat protein (i.e., arborine), an inhibitory activity was detected by an experiment against the capsid protein of RV-A2 (IC_{50} = 3.2 µM) [34]. Also 6,7,8-trimethoxycoumarin (not picked up by the *in silico* approach) showed activity against the capsid protein of RV-A2, with an IC_{50} of 12 µM. The CC_{50} values of both of these compounds were greater than 50 µM. This case demonstrates the successful isolation of noncytotoxic, antiviral constituents from a category C extract. In the present study, the 16 metabolites previously isolated from *R. graveolens* were further assayed for their CPE inhibition on HK/68 and CV-B3. The furanocoumarin rutamarin protected cells from a HK/68- and CV-B3-induced CPE with IC_{50} values of 2.7 µM and 5.1 µM, respectively (▶ **Table 2**, **Fig. 2**). All other constituents were inactive (data not shown). Since rutamarin is one of the main constituents identified for this extract [34], its cytotoxic and antiviral activity might reflect that of the whole extract. However, an effect of the high level of cytotoxicity of rutamarin (CC_{50} in MDCK cells: 4.7 µM; CC_{50} in HeLa cells: 4.6 µM) on the observed anti-influenza and anti-CV-B3 activity cannot be excluded.

The extract of *P. nigrum* fruits (no. 100; **Figs. 6S** and **7S**, Supporting Information) revealed an antiviral activity against CV-B3 (IC_{50} = 11 µg/mL). However, due to significant cytotoxicity observed with HeLa cells (CC_{50} = 16.8 µg/mL), the obtained antiviral activity data are questionable. For a more detailed analysis, ten piperamides that have been extracted as part of a previous study [35] [i.e., piperine, feruperine, piperylin, 9-(1,3-benzodioxol-5-yl)-1-(1-pyrrolidinyl) 2E,4E,8E-nonatrien-1-one, N-trans-feruloyl-piperidine, piperoleine A, dehydropipernonaline, pipernonaline, chabamide, and pipertipine] were scrutinized for their CV-B3 inhibiting CPE (▶ **Fig. 2**). The most active compounds were chabamide (IC_{50} = 9.1 µM; CC_{50} = 11 µM), piperoleine A (IC_{50} = 22 µM; CC_{50} = 25 µM), and dehydropipernonaline (IC_{50} = 24 µM; CC_{50} = 34 µM) (see ▶ **Table 2**). However, in this case, attempts to separate the antiviral and cytotoxic constituents were not successful.

Phenotypic assays can provide valuable information for the prioritization of extracts for phytochemical processing and pharmacological analysis. There is a clear added value in integrating computational methods into this screening setup. *In silico* methods can, e.g., guide the identification of the mode of action or mechanism of toxicity. They can also identify the most promising

▶ **Table 2** Antiviral activity of selected pure compounds. Their mean IC_{50} values against IV A/Hong Kong/68 (HK/68), coxsackie virus (CV-B3), and rhinovirus (RV-A2) in the CPE inhibition assay (MDCK cells for HK/68; HeLa cells for CV-B3 and RV-A2) as well as their mean CC_{50} values are presented (n = 3).

Name	Inhibition of virus-induced cytopathic effect						Cytotoxicity	
	HK/68		CV-B3		RV-A2		CC_{50} (CI95) [µM] in	
	IC_{50} (CI95) [µM] in MDCK cells	Selectivity index [CC_{50}/IC_{50}]	IC_{50} (CI95) [µM] in HeLa cells	Selectivity index [CC_{50}/IC_{50}]	IC_{50} (CI95) [µM] of CPE in HeLa cells	Selectivity index [CC_{50}/IC_{50}]	MDCK cells	HeLa cells
Rutamarin	2.7 (2.4–3.2)**	1.7	5.1 (3.6–7.1)**	0.9	n. d.		4.7*	4.6*
Piperine	n. d.		n. a.		41 (15–79)	2.2	n. d.	88 (77–87)
Feruperine	n. d.		n. a.		n. a.		n. d.	> 100
Piperylin	n. d.		51 (32–75)	> 1.9	79 (36–139)	> 1.3	n. d.	> 100
1-[(2E,4E,8E)-9-(1,3-Benzodioxol-5-yl)-1-oxo-2,4,8-nonatrienyl]-pyrrolidine	n. d.		61 (44–79)	> 1.6	n. a.		n. d.	> 100
N-trans-feruloyl-piperidine	n. d.		n. a.		n. a.		n. d.	> 100
Piperoleine A	n. d.		22 (18–25)	1.2	n. a.		n. d.	25 (18–34)
Dehydropipernonaline	n. d.		24 (14–35)	1.4	n. a.		n. d.	34 (24–44)
Pipernonaline	n. d.		~ 32	0.7	n. a.		n. d.	21 (13–31)
Chabamide	n. d.		9.1 (7.1–11)	1.2	n. a.		n. d.	11 (8.6–14)
Pipertipine	n. d.		n. a.		n. a.		n. d.	21 (16–27)
Ganoderol B	17 (13–31)	> 5.9	n. a.		65 (39–93)	> 1.5	> 100	> 100

*Tested only once; **tested only twice; n. a. = not active; n. d. = not determined

constituents of extracts for isolation and testing on a target of interest, e.g., for influenza NA [36–38]. In a previously performed computational study, we identified secondary metabolites present in the fruit body extract of *Ganoderma lucidum* Karst. (no. 53) that likely exhibit activity on anti-influenza and anti-RV targets [39]. A database of 279 known constituents of the fungus (mostly lanostane-type triterpenes) was compiled and screened with a pharmacophore-based approach for activity against a selection of viral targets. As one outcome of this study, ganoderol B (▶ **Fig. 2**) was identified as a potential inhibitor of the RV coat protein [39], and was therefore selected here for experimental testing on HK/68 and RV-A2. In the phenotypic CPE assay, previously isolated ganoderol B [40] showed moderate activity against HK/68 and RV-A2 (IC_{50} = 17 µM and 65 µM, respectively; ▶ **Table 2**), even though the crude extract (no. 53; **Figs. 8S** and **9S**, Supporting Information) did not show any activity against RV-A2 (▶ **Table 1**).

With the integrated strategy for the identification of bioactive compounds from extracts that we present in this work, several of the shortcomings of extract screening (e.g., false-positive assay readouts caused by interference or cytotoxicity provoked by multicomponent mixtures) can be leveraged. In particular, the combination of ethnopharmacological knowledge with effective, phenotypic screening technologies can accelerate and improve the prioritization of promising extracts as starting materials. Furthermore, the integration of computational methods can contribute valuable insights on the mode of action and mechanism of toxicity of individual constituents and provide guidance to *in vitro* analyses.

The data on extracts and their biological and toxicological profiles reported in this work shall serve as a starting point for future investigations. Moreover, the generated extract library can serve as a valuable platform for the scientific community. This collection of well-defined LLEs is now available for further studies including biological tests, analytical studies, or a combination of both.

Besides promising extracts with no or low cytotoxicity (i.e., category A extracts), extracts from the upper part of quadrant C in ▶ **Fig. 1 b** (i.e., category C extracts that exhibited antiviral activity but also some degree of cytotoxicity) may also be worthwhile probing for bioactive ingredients, while bearing in mind that the observed cytotoxicity may interfere with the phenotypic assay.

Materials and Methods

Natural materials

Some of the plant and mushroom materials were collected in Tyrol/Austria and identified by J. M. Rollinger or U. Peintner (Institute of Microbiology, University of Innsbruck, Austria). Further plant material was purchased from commercial providers, and some materials/extracts were obtained from collaboration partners (**Table 1S**, Supporting Information). Voucher specimens are

deposited in the Herbarium of the Department of Pharmacognosy, University of Vienna, Austria, unless otherwise stated in **Table 1S**, Supporting Information.

Generation of small-scale extracts

Combined dichloromethane and methanol extracts of plant and fungal species were prepared as described recently [23, 24]. Briefly, ground-dried material was defatted with *n*-hexane. The dried, defatted material was then extracted successively with dichloromethane and methanol, whereby the two resulting extracts were combined. Finally, tannin depletion via polyamide gel was carried out in order to remove compounds likely to cause assay interference.

In a few cases, the materials were not defatted and the dichloromethane (D) and methanol (M) extract were kept separately. Moreover, ethanol (E) or aqueous (H_2O) extracts were also generated for a small number of natural materials.

Cell culture and viruses

H3N2 IV strain HK/68 (strain collection of the Department of Virology and Antiviral Therapy, Jena, Germany), CV-B3 (CV-B3 Nancy; Institute of Poliomyelitis and Virus Encephalitides, Moscow, Russia), and RV-A2 (Medical University of Vienna, Vienna, Austria) were used in antiviral studies. IVs were propagated in MDCK cells (Friedrich-Loeffler Institute, Riems, Germany) in serum-free Eagle's minimum essential medium, 2 µg/mL trypsin, and 1.2 mM bicarbonate [41]. CV-B3 and RV-A2 were grown and tested in Eagle's minimal essential medium supplemented with 2% neonatal calf serum (PAA, Cölbe, Germany). Cells were proved to be free of mycoplasma contamination before using. Titers of virus stocks were determined according to Reed and Muench [42] in MDCK cells and HeLa cells, respectively.

Determination of cytotoxicity and cytopathic effect inhibition

The CC_{50} is defined as the concentration of a sample (in our case extract or pure compound) where the viability of treated cells in comparison to untreated control cells (mean viability of six controls is set to 100%) is reduced by half [25]. The IC_{50} is the concentration of a virus inhibitor (in our case extract or pure compound) where the response (grade of cell destruction, i.e., CPE caused by the virus) is reduced by half [25]. The IC_{50} was determined on 2-day-old confluent MDCK cell monolayers (for HK/68) and on 1-day-old and 2-day-old confluent HeLa Ohio cells for RV-A2 and CV-B3, respectively [43]. The cells were grown in 96-well plates as described previously (maximum tested concentration: 100 or 200 µg/mL for extracts and 100 µM for compounds) [25]. The maximum applied solvent concentration was 0.5% (v/v). Cytotoxicity was analyzed 72 h after adding the extracts. CPE inhibition was measured 48 h after infection for HK/68 and CV-B3, and 72 h after infection for RV-A2. A multiplicity of infection of 0.003, 0.001–0.002, and 0.02 $TCID_{50}$/cell of HK/68, CV-B3, and RV-A2, respectively, resulted in a complete CPE at this time point. CC_{50} and IC_{50} values were calculated from mean dose-response curves of at least three independent experiments. Linear regression using Microsoft Excel was therefore used in the linear scaled dose-dependent sample concentrations (in µg/mL) (▶ **Tables 1** and **2S, Fig. 1S**, Supporting Information).

These CC_{50} and IC_{50} values were used (i) to easily evaluate the specificity of antiviral activity after calculating the SI (CC_{50}/IC_{50}) and (ii) for categorization of activity. Then, the mean CC_{50} and IC_{50} values and confidence intervals of most active extracts (SI > 5) as well as some antiviral but also cytotoxic examples (SI < 5) were calculated. Oseltamivir, guanidine hydrochloride, and pleconaril served as positive controls for HK/68, CV-B3, and RV-A2, respectively.

Supporting information

References to the origin and voucher specimens of the natural materials (deposited in the herbarium) including their registration numbers (**Table 1S**), data leading to the determination of the IC_{50}s/CC_{50}s (individual and means) as well as their confidence intervals (**Table 2S**) and graphs depicting dose-dependencies (**Fig. 1S**) for the most active extracts and further investigated extracts, and chromatograms of HPLC analyses and structures of main constituents for the most relevant extracts (**Figs. 2S–9S**) are available as Supporting Information.

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Conflict of Interest

The authors declare no conflict of interest.

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