Neuroactivity screening of botanical extracts using microelectrode array (MEA) recordings

Regina G.D.M. van Kleef [a], Michelle R. Embry [b], Constance A. Mitchell [b], Remco H. S. Westerink [a,*]

[a] *Neurotoxicology Research Group, Division of Toxicology, Institute for Risk Assessment Sciences (IRAS), Faculty of Veterinary Medicine, Utrecht University, P.O. Box 80.177, NL-3508 TD Utrecht, the Netherlands*
[b] *Health and Environmental Sciences Institute, Washington, DC, USA*

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ABSTRACT

Toxicity testing of botanicals is challenging because of their chemical complexity and variability. Since botanicals may affect many different modes of action involved in neuronal function, we used microelectrode array (MEA) recordings of primary rat cortical cultures to screen 16 different botanical extracts for their effects on cell viability and neuronal network function in vitro. Our results demonstrate that extract materials (50 μg/mL) derived from goldenseal, milk thistle, tripterygium, and yohimbe decrease mitochondrial activity following 7 days exposure, indicative of cytotoxicity. Importantly, most botanical extracts alter neuronal network function following acute exposure. Extract materials (50 μg/mL) derived from aristolochia, ephedra, green tea, milk thistle, tripterygium, and usnea inhibit neuronal activity. Extracts of kava, kratom and yohimbe are particularly potent and induce a profound inhibition of neuronal activity at the low dose of 5 μg/mL. Extracts of blue cohosh, goldenseal and oleander cause intensification of the bursts. Aconite extract (5 μg/mL) evokes a clear hyper-excitation with a marked increase in the number of spikes and (network) bursts. The distinct activity patterns suggest that botanical extracts have diverse modes of action. Our combined data also highlight the applicability of MEA recordings for hazard identification and potency ranking of botanicals.

1. Introduction

Humans have relied on natural products for ages for a variety of purposes, including medicinal, cosmetic, and dietary applications. Botanicals, which are primarily plant-derived products but also can include algae and fungi, are used globally in modern times, with a growing interest in natural remedies and holistic approaches to health (Smith et al., 2022). Botanical extracts have a long-standing use in for example Ayurveda, Traditional Chinese Medicine (TCM), and Indigenous healing practices, but their use is also resurging in Western societies, driven by a desire for natural alternatives to conventional pharmaceuticals and as dietary supplements (Clarke et al., 2015). This has led to the widespread availability and consumption of botanical-based products, including herbal supplements, essential oils, and botanical-infused cosmetics.

While botanicals may offer potential therapeutic benefits, it is crucial to address safety concerns associated with their use. For example, the chemical composition of botanicals can vary significantly, making it

challenging to standardize their potency. Variations in growing conditions, extraction methods, and plant species can influence the chemical content and concentration of bioactive compounds, which in some cases can lead to inconsistent benefits and unwanted side effects (Mitchell et al., 2022; Huie, 2002). In extreme cases, permanent organ damage and even death can occur. While not common, adverse effects of botanicals have been reported, including botanical-drug interactions (e.g., St. John's wort [*Hypericum perforatum*]; Gurley et al., 2008), liver toxicity (e.g., comfrey [*Symphytum officinale*] containing pyrrolizidine alkaloids; Brown et al., 2016), nephrotoxicity (e.g., *Aristolochia fangchi* containing aristolochic acids; Debelle et al., 2008), cardiotoxicity (e.g., ephedra [*Ephedra sinica*]; National Toxicology Program, 1986; Zell-Kanter et al., 2015), and neurotoxicity (e.g., Aconite *[Aconitum napellus]* containing aconitine; Moritz et al., 2005).

Because of their chemical complexity and variability, botanicals are more difficult to assess for toxicity or efficacy compared to single chemicals. However, given the high, intentional exposure from

* Corresponding author.
E-mail addresses: g.vankleef@uu.nl (R.G.D.M. van Kleef), membry@hesiglobal.org (M.R. Embry), cmitchell@hesiglobal.org (C.A. Mitchell), R.Westerink@uu.nl (R.H.S. Westerink).

Table 1

List of source botanicals for extracts used in this study, including their standardized common and scientific names, Distributed Structure-Searchable Toxicity (DSSTox) substance identifier (DTXSID), and the part(s) of the plant used to derive the botanical extract. Botanicals with suspected neuroactive potential are in bold.

Standardized Common Name	Scientific Name	DTXSID	Plant part(s)	Details
Aconite	***Aconitum napellus* L.**	**DTXSID701061676**	**Mixed parts**	95% ethanol extract
Aristolochia fangchi	*Aristolochia fangchi* Y.C. Wu ex L.D. Chou & S.M. Hwang	DTXSID201349132	Root	95% ethanol extract
Ashwagandha	*Withania somnifera* (L.) Dunal	DTXSID201042372	Root	LWS7EP 2005K31; Ethanol: Water (~15:1) extract; 1.56% total withanolides
Asian ginseng	*Panax ginseng* C.A. Mey.	DTXSID1023780	Root	Ginseng Dry Extract 4% (Quintozene free); 4.6% total ginsenosides
Blue cohosh	*Caulophyllum thalictroides* (L.) Michx.	DTXSID401042859	Root & Rhizome	95% ethanol extract
Comfrey	*Symphytum officinale* L.	DTXSID20274226	Root	95% ethanol extract
Ephedra	*Ephedra sinica* Stapf	DTXSID801018482	Aerial Parts	95% ethanol extract
Tea	*Camellia sinensis* (L.) Kuntz	DTXSID0031398	Leaf	Green tea dry decaffeinated extract; 78.5% total catechins (54.6% (−)-epigallocatechin-3-O-gallate)
Goldenseal	*Hydrastis canadensis* L.	DTXSID40274228	Root & Rhizome	95% ethanol extract
Kava	***Piper methysticum* G. Forst.**	**DTXSID901018742**	Root & Rhizome	95% ethanol extract
Kratom	***Mitragyna speciosa* (Korth.) Havil.**	**DTXSID001334842**	**Leaf**	95% ethanol extract
Milk thistle	*Silybum marianum* (L.) Gaertn.	DTXSID8031657	Seed	Milk thistle dry extract; 90.6% of silymarin isomers calc. as silibinin
Oleander	***Nerium oleander* L.**	**DTXSID201042091**	**Leaf**	95% ethanol extract
Usnea	*Usnea* spp.	DTXSID701349537	Whole Lichen	95% ethanol extract
Tripterygium[a]	***Tripterygium wilfordii* Hook. f.**	**DTXSID301349830**	**Root**	95% ethanol extract
Yohimbe	***Pausinystalia johimbe* (K. Schum.) Pierre ex Beille**	**DTXSID4032291**	**Bark**	95% ethanol extract

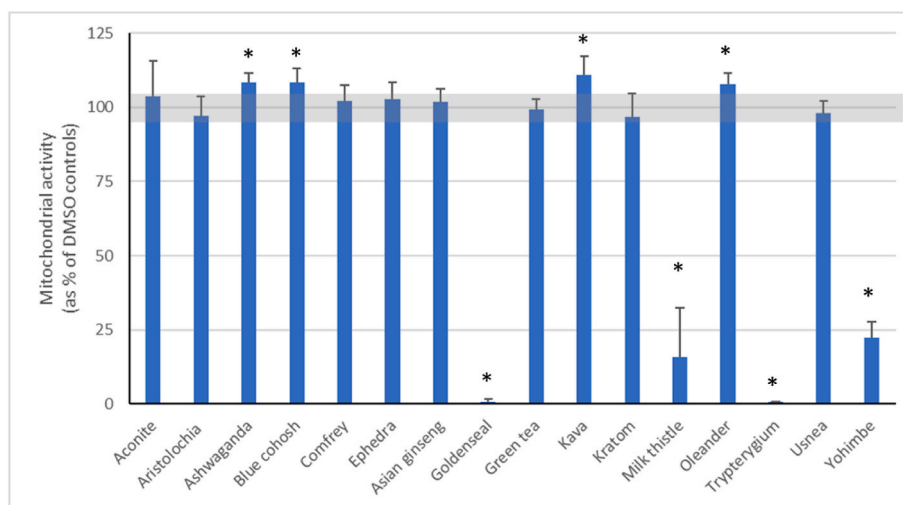[a] Also commonly known as thunder god vine.

Fig. 1. Overview of the effects of botanical extracts on mitochondrial activity. Mitochondrial activity was assessed, as a measure of cell viability, following 7 days of exposure to the botanical extracts at 50 μg/mL. Effects are expressed as mean + SD (from n = 7–39 wells) normalized to DMSO control. Values that do not exceed the BMR of 5%, indicated by the light grey area, are considered to be of limited toxicological relevance. Asterisk indicate values that deviate significantly from DMSO control (p < 0.05).

consumers taking these products and the potential risks associated with the use of botanicals, it is imperative to establish robust safety screening methods, including neurotoxicity evaluations. Since botanicals may affect many different modes of action involved in neuronal function, an extensive battery of assays is needed to assess the neuroactive potential of botanicals on underlying process, like ion channel and receptor function. Alternatively, a screening assay that functionally integrates the underlying processes could be used, like multi-well microelectrode array (MEA) recordings. MEA recordings allow for non-invasive, medium-throughput assessment of changes in neuronal network function in vitro, thereby taking into account effects on multiple neurotoxicity endpoints (Gerber et al., 2021; Hogberg et al., 2011; Johnstone et al., 2010;

McConnell et al., 2012).

Therefore, the aim of the present study is to evaluate the applicability of in vitro MEA recordings for screening and prioritization of botanical extracts for their neuroactive potential. To this aim, we tested 16 different botanical extract materials (Table 1) for their effects on mitochondrial activity and neuronal activity upon acute exposure. This work was conducted as a part of the HESI Botanical Safety Consortium, which is made up of experts from government, industry, and academia working to develop screening strategies that can efficiently screen for botanical-induced toxicity, including neurotoxicity.

Table 2

Effects 24h exposure to botanical extracts on mitochondrial activity. Mitochondrial activity was assessed, as a measure of cell viability, following 24 h of exposure to the botanical extracts at 5 µg/mL and 50 µg/mL. Effects are expressed as mean ± SD (from n = 8–23 wells) normalized to DMSO control. Values that deviate significantly from DMSO control and exceed the BMR of 5% are in bold.

	Average ± SD	p-value
	(% of DMSO control)	
Goldenseal 5 µg/mL	98,6 ± 8,3	n.s.
Goldenseal 50 µg/mL	**91,4 ± 6,3**	<0.001
Milk thistle 5 µg/mL	95,1 ± 9,0	n.s.
Milk thistle 50 µg/mL	**91,0 ± 10,7**	0.007
Triptergium 5 µg/mL	**94,6 ± 6,8**	0.016
Triptergium 50 µg/mL	**74,8 ± 11,0**	<0.0001
Yohimbe 5 µg/mL	98,4 ± 7,6	n.s.
Yohimbe 50 µg/mL	**60,6 ± 9,2**	<0.0001

2. Methods

2.1. Chemicals and botanicals

All botanical test samples (Table 1) were provided as dry extracts by the Botanical Safety Consortium (https://botanicalsafetyconsortium.org/; Mitchell et al., 2022), except for aconite, oleander, and triptergium, which were provided as a stock solution of the extract (100, 88, and 55 mg/mL, respectively) in dimethyl sulfoxide (DMSO).

Details on the sourcing, chemical analysis, and botanical details can be found at NIEHS's CEBS website (https://cebs-ext.niehs.nih.gov/cebs/paper/15717) and are described in Waidyanatha et al., 2023 (submitted). Botanicals were selected based on existing literature with respect to toxicity or safety, from human (adverse event reporting and clinical trials), animal, or mechanistic studies. Ashwagandha extract, Asian ginseng extract (standardized to 4.6% ginsenosides), green tea extract (standardized to 78.5% catechins), and milk thistle extract (standardized to 90.6% silymarin) were obtained from commercial

	SPIKES	BURSTS			NETWORK BURST				
	Number of Spikes	Number of Bursts	Burst Duration	Number of Spikes per Burst	Number of Network Bursts	Network Burst Duration	Number of Spikes per Network Burst	Mean ISI within Network Burst	Area Under Cross-Correlation
Ashwagandha 5 µg/mL	88	92	114	106	89	113	108	101	90
Ashwagandha 50 µg/mL	97	87	*128*	*114*	91	*137*	*118*	107	93
Comfrey 5 µg/mL	102	109	94	98	102	84	96	93	101
Comfrey 50 µg/mL	*77*	*78*	97	101	*67*	128	*117*	118	87
Asian ginseng 5 µg/mL	110	94	105	108	85	102	111	81	97
Asian ginseng 50 µg/mL	101	98	139	93	131	133	*78*	*155*	86

Legend: > 400 | 200 - 400 | 125 - 200 | 75 - 125 | 50 - 75 | 25 - 50 | 0 - 25

Fig. 2. Overview of the effects of ashwagandha, comfrey, and Asian ginseng extracts on nine neuronal activity parameters. Effects are expressed as a percentage of DMSO control and the degree of effect is indicated using a color scheme. Decreases are depicted in blue, increases are depicted in red. Values that do not exceed the BMR of 25% are depicted in light grey and are considered to be of limited toxicological relevance. Values in bold/italic deviate significantly from DMSO control (p < 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Asian ginseng extract, 50 µg/mL

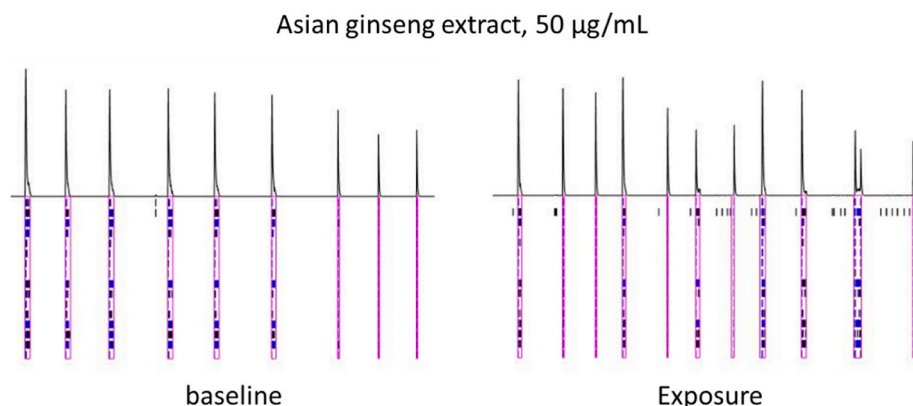baseline   Exposure

Fig. 3. Raster plot showing the activity of the neuronal network in a single, representative well before (baseline) and after exposure to Asian ginseng extract at 50 µg/mL. Activity is recorded at 16 different electrodes and is depicted in black (spikes), blue (bursts) and purple (network burst). The trace on top of the recording shows the cumulative activity recorded at the 16 electrodes. Plots show 90s fragment before (baseline, left) and after (exposure, right) exposure to the botanical extract. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

	SPIKES	BURSTS			NETWORK BURST				Area Under Cross-Correlation
	Number of Spikes	Number of Bursts	Burst Duration	Number of Spikes per Burst	Number of Network Bursts	Network Burst Duration	Number of Spikes per Network Burst	Mean ISI within Network Burst	Area Under Cross-Correlation
Aristolochia 5 µg/mL	93	82	127	115	80	152	119	121	91
Aristolochia 50 µg/mL	47	52	132	100	45	106	104	84	48
Ephedra 5 µg/mL	91	93	120	97	82	96	101	95	92
Ephedra 50 µg/mL	48	44	135	88	43	93	106	98	39
Green tea 5 µg/mL	82	78	95	109	71	109	112	98	82
Green tea 50 µg/mL	28	21	94	58	18	97	115	85	30
Milk thistle 5 µg/mL	99	100	102	100	112	110	89	130	92
Milk thistle 50 µg/mL	55	56	94	80	75	75	72	129	42
Usnea 5 µg/mL	92	85	105	105	80	105	105	102	95
Usnea 50 µg/mL	37	34	110	112	36	115	122	102	37

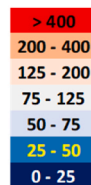Legend: > 400 | 200 – 400 | 125 – 200 | 75 – 125 | 50 – 75 | 25 – 50 | 0 – 25

Fig. 4. Overview of the effects of aristolochia, ephedra, green tea, milk thistle, and usnea extracts on nine neuronal activity parameters. Effects are expressed as a percentage of DMSO control and the degree of effect is indicated using a color scheme. Decreases are depicted in blue, increases are depicted in red. Values that do not exceed the BMR of 25% are depicted in light grey and are considered to be of limited toxicological relevance. Values in bold deviate significantly from DMSO control (p < 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Aristolochia extract, 50 µg/mL

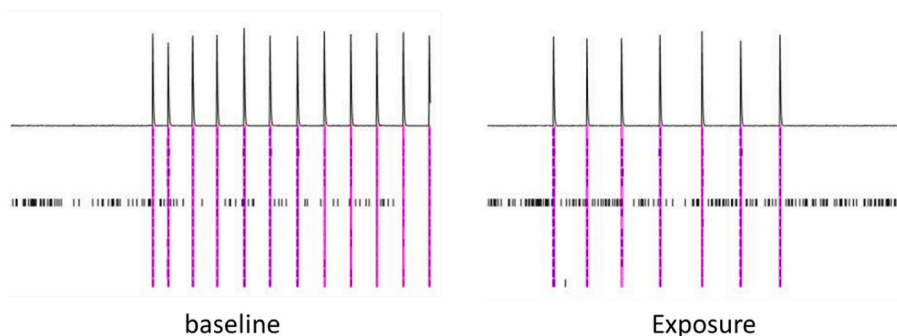baseline   Exposure

Fig. 5. Raster plot showing the activity of the neuronal network in a single, representative well before (baseline) and after exposure to aristolochia extract at 50 µg/mL. Activity is recorded at 16 different electrodes and is depicted in black (spikes), blue (bursts) and purple (network burst). The trace on top of the recording shows the cumulative activity recorded at the 16 electrodes. Plots show 90s fragment before (baseline, left) and after (exposure, right) exposure to the botanical extract. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

extract suppliers. All other botanicals were obtained as crude plant materials and were extracted in-house using 95% ethanol. Some botanicals, including Asian ginseng and milk thistle were selected based on long history of use and animal data that points to safety. Those with suspected neuroactive potential are in bold (Table 1).

Phenol-red free neurobasal-A (NB-A) medium, L-glutamine (200 mM), penicillin/streptomycin (5000 U/mL/5000 mg/mL) and B-27 plus supplement were purchased from Life Technologies (Bleiswijk, the Netherlands). Unless otherwise noted, all other chemicals were obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands. All solutions used in experiments, including control experiments, contained 0.1% DMSO.

2.2. Cell culture

All animal experiments were performed in agreement with Dutch law, the European Community directives regulating animal research (2010/63/EU) and approved by the Ethical Committee for Animal Experiments of Utrecht University. All efforts were made to minimize the number of animals used and their suffering.

Primary cultures of rat cortical neurons were prepared from pups born of timed-pregnant Wistar rats (Envigo, Horst, the Netherlands) on postnatal day 0 or 1 as described previously (Gerber et al., 2021). Briefly, rat pups were decapitated, and the cortex was isolated and placed in ice-cold dissection medium (450 mL NBA medium, 14 g

	SPIKES	BURSTS			NETWORK BURST				Area Under Cross-Correlation
	Number of Spikes	Number of Bursts	Burst Duration	Number of Spikes per Burst	Number of Network Bursts	Network Burst Duration	Number of Spikes per Network Burst	Mean ISI within Network Burst	
Kava 5 µg/mL	15	6	90	35	2	45	56	70	4
Kava 50 µg/mL	0	0			0				0
Kratom 5 µg/mL	38	40	87	101	39	94	128	67	37
Kratom 50 µg/mL	0	0			0				0
Tripterygium 5 µg/mL	73	84	102	96	85	91	93	109	76
Tripterygium 50 µg/mL	19	18	154	107	18	143	106	130	17
Yohimbe 5 µg/mL	59	63	113	103	53	88	107	71	64
Yohimbe 50 µg/mL	7	9	74	73	7	67	70	71	7

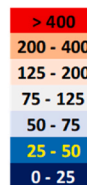Legend: > 400 | 200 – 400 | 125 – 200 | 75 – 125 | 50 – 75 | 25 – 50 | 0 – 25

Fig. 6. Overview of the effects of kava, kratom, tripterygium, and yohimbe extracts on nine neuronal activity parameters. Effects are expressed as a percentage of DMSO control, and the degree of effect is indicated using a color scheme. Decreases are depicted in blue, increases are depicted in red. Values that do not exceed the BMR of 25% are depicted in light grey and are considered to be of limited toxicological relevance. Values in bold deviate significantly from DMSO control (p < 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Yohimbe extract, 50 µg/mL
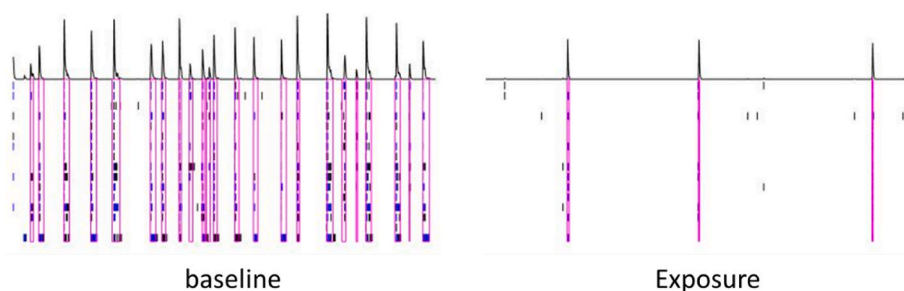

baseline   Exposure

Fig. 7. Raster plot showing the activity of the neuronal network in a single, representative well before (baseline) and after exposure to yohimbe extract at 50 µg/mL. Activity is recorded at 16 different electrodes and is depicted in black (spikes), blue (bursts) and purple (network burst). The trace on top of the recording shows the cumulative activity recorded at the 16 electrodes. Plots show 90s fragment before (baseline, left) and after (exposure, right) exposure to the botanical extract. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

sucrose, 1.25 mL l-glutamine (200 mM), 5 mL glutamate (3.5 mM), 5 mL penicillin/streptomycin and 10 mL B-27, pH 7.4). Cortices were minced and triturated to a homogenous suspension and filtered through an easy strainer (100 µm, Greiner Bio One, Alphen aan den Rijn, The Netherlands). Subsequently, cells were centrifuged for 5 min at 800 rpm. The supernatant was removed, and the pellet was resuspended using 1 mL of dissection medium per rat brain and diluted to a cell suspension containing 2×10^6 cells/mL. Next, drops (50 µL/well) of cell-suspension were seeded on PEI (0.1% PEI solution in borate buffer (24 mM sodium borate/50 mM boric acid in Milli-Q adjusted to pH 8.4)) coated 48-well microelectrode array (MEA) plates (Axion Biosystems Inc., Atlanta, GA, USA) at a density of 1×10^5 cells/well. Cells were allowed to attach in a humidified 5% CO_2/95% air atmosphere for 2 h at 37 °C, before 450 µL dissection medium was added to each well. At four days in vitro (DIV 4), 450 µL dissection medium was replaced by 450 µL glutamate-free medium (450 mL NBA medium, 14 g sucrose, 1.25 mL l-glutamine (200 mM), 5 mL penicillin/streptomycin and 10 mL B-27 plus, pH 7.4). Cells were cultured in 5% CO_2/95% air atmosphere at 37 °C until use at DIV 15.

2.3. Cell viability

To ensure that effects on neuronal activity do not simply reflect changes in cell viability, rat primary cortical cultures were exposed for 7 days to the extracts at 50 µg/mL, after which an Alamar Blue assay was performed to measure mitochondrial activity as measure for cell viability. Those extracts that evoked a decrease in mitochondrial activity were also tested at the low dose of 5 µg/mL. Additionally, these samples were tested at 5 µg/mL and 50 µg/mL following 24 h of exposure.

Following exposure, the exposure medium was replaced by 300 µL

	SPIKES	BURSTS			NETWORK BURST				Area Under Cross-Correlation
	Number of Spikes	Number of Bursts	Burst Duration	Number of Spikes per Burst	Number of Network Bursts	Network Burst Duration	Number of Spikes per Network Burst	Mean ISI within Network Burst	
Aconite 5 µg/mL	378	192	234	113	790	100	108	151	139
Aconite 50 µg/mL	146	176	161	23	542	32	16	203	55
Blue cohosh 5 µg/mL	100	80	116	110	72	122	116	103	92
Blue cohosh 50 µg/mL	92	59	520	181	49	161	182	103	94
Goldenseal 5 µg/mL	112	65	371	186	52	356	223	135	102
Goldenseal 50 µg/mL	0	0	56	3	0				0
Oleander 5 µg/mL	62	25	800	168	42	611	179	525	30
Oleander 50 µg/mL	0	0	50	27	0	156	113	128	0

Legend: > 400 | 200 – 400 | 125 – 200 | 75 – 125 | 50 – 75 | 25 – 50 | 0 – 25

Fig. 8. Overview of the effects of aconite, blue cohosh, goldenseal, and oleander extracts on nine neuronal activity parameters. Effects are expressed as a percentage of DMSO control and the degree of effect is indicated using a color scheme. Decreases are depicted in blue, increases are depicted in red. Values that do not exceed the BMR of 25% are depicted in light grey and are considered to be of limited toxicological relevance. Values in bold deviate significantly from DMSO control (p < 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

pre-warmed Alamar Blue solution (25 µM resorufin in Hanks' Balanced Salt solution) and cells were incubated for 1.5 h at 37 °C, 5% CO_2 and 95% air atmosphere as previously described (Gerber et al., 2021). After the incubation, 200 µL of the Alamar Blue solution was transferred from each well to a transparent 96-well and the conversion of resazurin to resorufin was measured spectrophotometrically at 540/590 nm (excitation/emission). Per experiment, values for exposed wells were normalized to values obtained for DMSO controls (set to 100%). Next, data were combined and averages of metabolic activity per condition were calculated. For each experimental condition, data are presented as average values ± SD (% of control) of primary cultures derived from 7 to 24 wells (n) and 1–3 plates (N), obtained from 1 to 2 independent preparations. Experimental values that exceeded mean ± 2× SD (of their respective treatment) were considered to be outliers (1.7% outliers) and therefore excluded from further analysis.

Benchmark response (BMR) cut-offs were set at 5%, which is based on the average variation in all pooled DMSO control experiments (n = 103, N = 13). Effects that are smaller than the BMR are considered to be of limited toxicological relevance, even if significantly different from control. Statistical analyses were performed using SPSS (IBM SPSS Statistics, version29.0.0.0). Data were tested for significant effects using an unpaired *t*-test, where a p-value ≤0.05 was considered statistically significant.

2.4. MEA recordings

Multi-well MEA plates were used to record spontaneous neuronal activity. MEA plates contain 48 wells per plate, with per well an electrode array of 4 × 4 individual embedded microelectrodes (40–50 µm diameter; 350 µm center-to-center spacing), yielding a total of 768 electrodes, which can be used to record neuronal activity. Recordings were made as previously described (Gerber et al., 2021). All botanical extracts were tested at a dose of 5 and 50 µg/mL. Each well was exposed to only one condition (i.e., one dose of a botanical extract) to prevent potential effects of cumulative dosing.

On DIV 15, a 48-well MEA plate was placed in a Maestro 768-channel

amplifier with integrated heating system, temperature and CO_2 controller, and data acquisition interface (Axion Biosystems Inc., Atlanta, GA, USA). Prior to each recording, MEA plates were allowed to equilibrate for around 5 min, after which a 30-min baseline recording of spontaneous activity was started. Wells with at least three bursting electrodes and with a minimum of one network burst per minute at baseline recording were included for experiments. After the baseline recording, all wells were exposed individually by manually pipetting 55 µL of different doses of the extracts or vehicle (DMSO control) to each active well. Immediately after exposure, acute effects of botanical extracts on spontaneous neuronal activity (spiking and bursting behavior) were measured during a 30-min recording at 37 °C.

2.5. Data analysis and statistics

MEA data analysis was done as described in detail previously (Gerber et al., 2021). Briefly, MEA data acquisition was managed with Axion's Integrated Studio (AxIS version 3.9.1.1). Raw data files were obtained by sampling channels simultaneously with a gain of 1000× and a sampling frequency of 12.5 kHz/channel using a band-pass filter (200–3000 Hz).

These raw data were pre-processed to obtain. spk files. Spikes were detected using the AxIS spike detector (Adaptive threshold crossing, Ada BandFlt v2) with a post/pre-spike duration of 3.6/2.4 ms and a spike threshold of 7 × SD of the internal noise level (rms) of each individual electrode. Spike information was then further analysed using Neural Metrics Tool (v 3.1.7, Axion BioSystems) and custom-made macros in Excel. Bursts were defined using the Poisson surprise method (Legendy and Salcman, 1985) with a minimum of 10 surprises. Network bursts were defined using an adaptive threshold with a minimum of 40 spikes, each separated by a maximum interval set automatically on a well-by-well basis based on the mean spike rate of each well, for a minimum of 15% of the electrodes/well. Data from the last 20 min of the 30-min exposure recording were used for analysis, since this is the most stable timeframe for stable exposure effects (see Hondebrink et al., 2016).

Aconite extract, 5 μg/mL

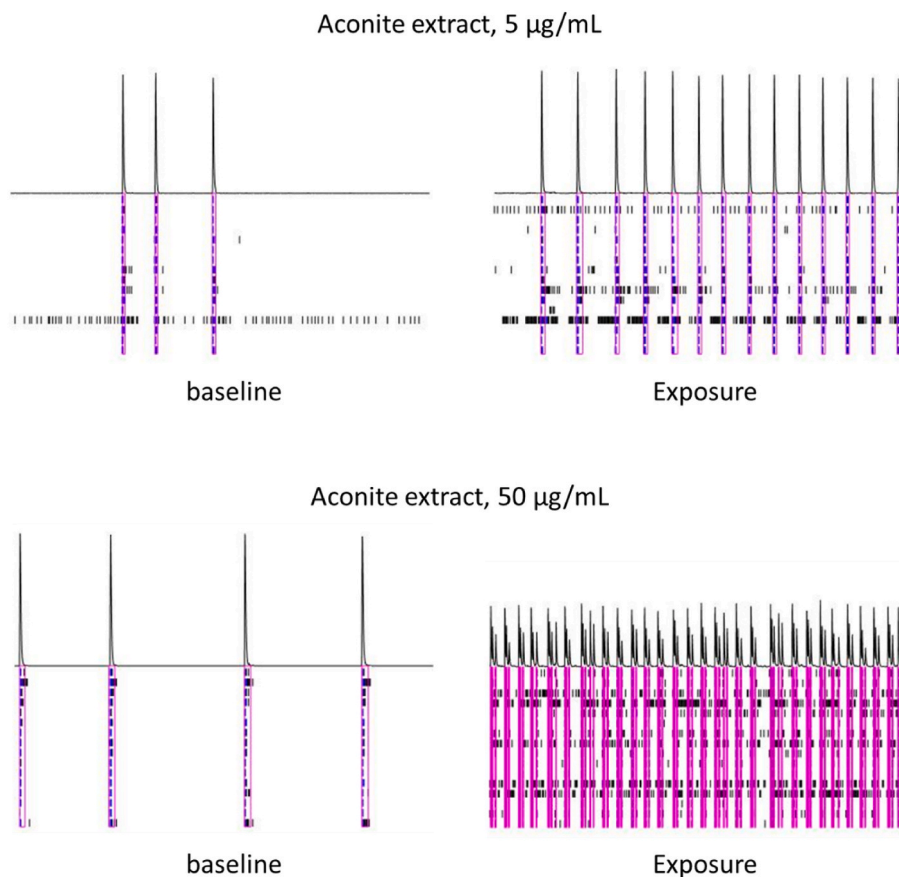## Aconite extract, 50 μg/mL

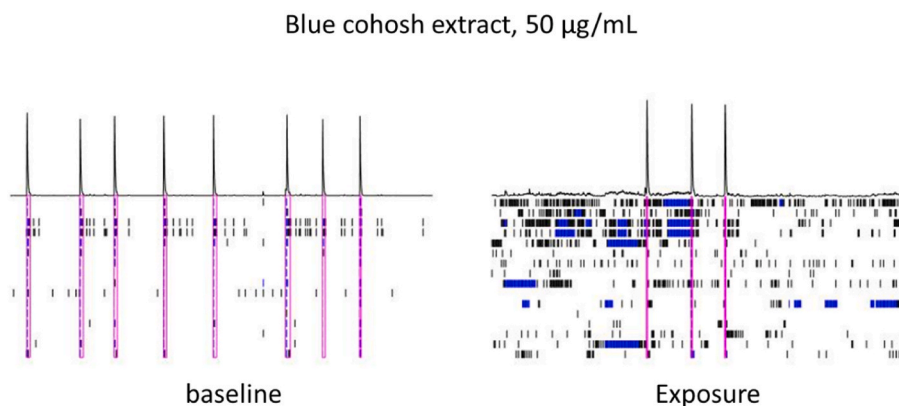**Fig. 9. Raster plot showing the activity of the neuronal network in a single, representative well before (baseline) and after exposure to aconite extract at 5 μg/mL (top) and 50 μg/mL (bottom).** Activity is recorded at 16 different electrodes and is depicted in black (spikes), blue (bursts) and purple (network burst). The trace on top of the recording shows the cumulative activity recorded at the 16 electrodes. Plots show 90s fragment before (baseline, left) and after (exposure, right) exposure to the botanical extract. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Blue cohosh extract, 50 μg/mL

Fig. 10. Raster plot showing the activity of the neuronal network in a single, representative well before (baseline) and after exposure to blue cohosh extract at 50 μg/mL. Activity is recorded at 16 different electrodes and is depicted in black (spikes), blue (bursts) and purple (network burst). The trace on top of the recording shows the cumulative activity recorded at the 16 electrodes. Plots show 90s fragment before (baseline, left) and after (exposure, right) exposure to the botanical extract. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Many different activity parameters can be derived from MEA recordings. For clarity, we focus on a selection of 9 parameters that have previously proven most important for assessing changes in neuronal activity. These include number of spikes, number of bursts, burst duration, number of spikes per burst, number of network bursts, network burst duration, number of spikes per network burst, mean inter-spike interval (ISI) within network bursts, and area under cross correlation as a measure for synchronicity.

Since each well of the MEA plate contains a self-organized and spontaneously active neuronal networks, there can be quite some variation in baseline activity between wells (see for example the raster plots in Figs. 3, 5, 7, 9–12). The acute effects of botanical extracts on spontaneous activity were therefore determined using a paired-comparison of the baseline activity with activity following exposure for each individual well, i.e., a treatment ratio per well. A custom-made MS Excel macro was used to calculate this treatment ratio of different metric
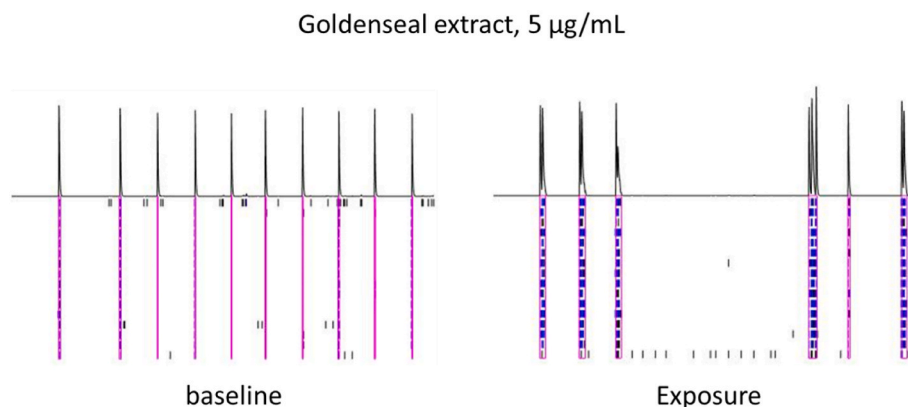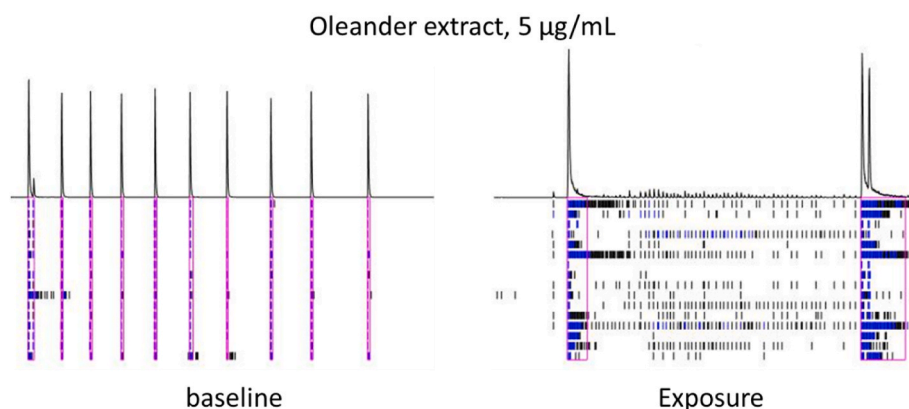
Goldenseal extract, 5 μg/mL

Fig. 11. Raster plot showing the activity of the neuronal network in a single, representative well before (baseline) and after exposure to goldenseal extract at 5 μg/mL. Activity is recorded at 16 different electrodes and is depicted in black (spikes), blue (bursts) and purple (network burst). The trace on top of the recording shows the cumulative activity recorded at the 16 electrodes. Plots show 90s fragment before (baseline, left) and after (exposure, right) exposure to the botanical extract. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Oleander extract, 5 μg/mL

Fig. 12. Raster plot showing the activity of the neuronal network in a single, representative well before (baseline) and after exposure to oleander extract at 5 μg/mL. Activity is recorded at 16 different electrodes and is depicted in black (spikes), blue (bursts) and purple (network burst). The trace on top of the recording shows the cumulative activity recorded at the 16 electrodes. Plots show 90s fragment before (baseline, left) and after (exposure, right) exposure to the botanical extract. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

parameters (parameter$_{exposure}$/parameter$_{baseline}$) of each well, for DMSO controls and following exposure to botanical extracts. Thereafter, treatment ratios were normalized to solvent controls (DMSO, set at 100% and normalized per plate). Next, experiments (N) are combined and results are presented as percentage change $\pm$ standard error of the mean (SEM) compared to DMSO control. In this way, possible differences between isolations, cultures, or plates are eliminated. See Gerber et al. (2021) for further details on the analysis of MEA data. For each experimental condition, data represent average values of primary cultures derived from 9 to 36 wells (n) and 2–4 plates (N) from $\geq$2 independent preparations. Experimental values that exceeded mean $\pm$ 2$\times$ SD (of their respective condition) were considered to be outliers (4.1% outliers) and therefore excluded from further analysis.

For the MEA data analysis, benchmark response (BMR) cut-offs were set at 25%, which is based on the average variation in all pooled DMSO control experiments ($n = 290$, $N = 37$). Effects that are smaller than the BMR are considered to be of limited toxicological relevance, even if significantly different from control. Statistical analyses were performed using SPSS (IBM SPSS Statistics, version29.0.0.0). Data were tested for significant effects using one-way ANOVA with significance values adjusted for multiple tests with Dunnett's post-hoc test. A p-value $\leq$0.05 was considered statistically significant.

3. Results

3.1. Effects on cell viability

Prior to specific neurotoxicity testing we evaluated the ability of the botanical extracts to induce cytotoxicity. Mitochondrial activity, as a measure of cell viability, was assessed after exposure of cortical cultures for 7 days to 50 μg/mL of the botanical extracts. Exposure to extracts derived from aconite, aristolochia, comfrey, ephedra, Asian ginseng, green tea, kratom, and usnea did not induce any significant change in mitochondrial activity. Exposure to ashwagandha, blue cohosh, kava, and oleander extracts induced a minor increase in mitochondrial activity up to 107–111% of DMSO controls, and just above the BMR of 5%, possibly indicative of mild cell stress. However, exposure to goldenseal, milk thistle, tripterygium, and yohimbe extracts induced a profound decrease in mitochondrial activity, indicative of decreased cell viability (Fig. 1).

The extracts that induced cytotoxicity following 7 days exposure at 50 μg/mL were also tested at the lower dose of 5 μg/mL (n = 15–40 wells). At this lower dose, milk thistle, tripterygium, and yohimbe extracts no longer decreased mitochondrial activity. However, the goldenseal extract still induced a profound decrease in mitochondrial activity to 63% of DMSO controls, indicative of decreased cell viability (not shown).

Since the evaluation of the neuroactive potency involves acute

Table 3

List of tested botanicals extracts and the acute effects on neuronal activity.
If known, the possible neuroactive Mode of Action (MoA) and the suspected active ingredient are listed.

Standardized Common Name	Effect on neuronal activity	Possible Neuroactive Mode of Action (active ingredient; refence)
Ashwagandha	None	None reported
Comfrey	None	None reported
Asian ginseng	None	None reported
Aristolochia fangchi	Inhibition	Inconclusive, possibly inhibition of calcium influx (aristolochic acids)
Ephedra	Inhibition	Inhibition of dopamine and norepinephrine membrane transporters (DAT and NET), α- and β-adrenergic receptor agonist (ephedrine; Dawson and Moffatt, 2012; Bowyer et al., 2000; Calvert et al., 2015).
Tea (decaffeinated)	Inhibition	glutamate receptor antagonist (L-theanine; Anas Sohail et al., 2021)
Milk thistle	Inhibition	Inhibition of calcium influx and glutamate release (silymarin; Lu et al., 2020)
Usnea	Inhibition	None reported for usnea lichen or usnic acid
Kava	Strong inhibition	GABA receptor modulation and inhibition voltage-dependent Na$^+$ and Ca^{2+} channels (kavalactones and flavokavains; Romm et al., 2010)
Kratom	Strong inhibition	μ-opioid receptor partial agonist (mitragynine; Suhaimi et al., 2016; Karunakaran et al., 2022)
Tripterygium	Strong inhibition	Possibly inhibition of voltage-gated Na + channels (triptolide, celastrol, demethylzeylasteral, and wilforgine; Xu et al., 2023)
Yohimbe	Strong inhibition	Direct (Nah and McCleskey, 1994; Watanabe et al., 1987) and indirect (via α2-adrenoceptor antagonism; Papa et al., 2022) inhibition of calcium channels (Yohimbine)
Aconite	Hyperexcitation	Activation of voltage-gated Na$^+$ channels (aconitine; Wang and Wang, 2003)
Blue cohosh	Excitation (intensi-fied bursting)	Nicotine-like effects (N-methylcytosine, and anagyrine; Slater et al., 2003; Green et al., 2010)
Goldenseal	Biphasic: excitation up to near-complete cessation	Acetylcholinesterase inhibition (berberine, palmatine, hydrastine, and canadine; Senol Deniz et al., 2023) and modulation of K$^+$ and Ca^{2+} channels (berberine; Amssayef and Eddouks, 2023)
Oleander	Biphasic: excitation up to near-complete cessation	Inhibition of Na$^+$/K$^+$-ATPase and increased intracellular Ca^{2+} concentrations (oleandrin and other cardiac glycosides; Botelho et al., 2019).

exposure, we also tested if these four extracts could decrease mitochondrial activity already after 24 h exposure. Exposure to 50 μg/mL induced a limited decrease in mitochondrial activity for extracts of goldenseal and milk thistle, whereas tripterygium, and yohimbe extracts induced a profound decrease in mitochondrial activity indicative of decreased cell viability (Table 2). At the lower dose of 5 μg/mL the extracts induced no or only very minor effects on mitochondrial activity, indicating that cell viability is not affected at the low dose following 24 h exposure.

3.2. Botanical extracts with little neuroactive potential

Exposure to extracts from ashwagandha, comfrey, and Asian ginseng

evoked little change in neuronal activity (Fig. 2).

None of the neuronal activity parameters were affected to a degree that exceeds the BMR of 25% following exposure to ashwagandha extract at 5 μg/mL. At 50 μg/mL only minor increases in (network) burst duration were observed (Supplemental Fig. S1).

Similarly, neuronal activity parameters were not affected by exposure to comfrey extract at 5 μg/mL. At 50 μg/mL only a minor decrease in the number of network bursts was observed, which was paralleled by a minor increase in network burst duration (Supplemental Fig. S2).

Exposure to the Asian ginseng extract at 5 μg/mL did not evoke changes in neuronal activity parameters, although minor increases in the (network) burst duration, the number of network bursts, and the mean inter-spike interval (ISI) within network bursts were observed at 50 μg/mL (Supplemental Fig. S3). The example raster plot shows the neuronal activity of a well before (baseline) and after exposure to Asian ginseng extract at 50 μg/mL, highlighting that changes in neuronal activity are limited (Fig. 3).

3.3. Inhibitory botanical extracts

Exposure to aristolochia, ephedra, green tea, milk thistle, and usnea extracts resulted in inhibition of neuronal activity (Fig. 4).

Exposure of neuronal cultures to 5 μg/mL aristolochia extract evoked a minor increase in (network) burst duration. At 50 μg/mL, however, a profound decrease in the number of spikes, the number of (network) bursts and the Area Under Cross-Correlation was observed as well as a minor increase in burst duration (Supplemental Fig. S4). The example raster plot shows the neuronal activity of a well before (baseline) and after exposure to aristolochia extract at 50 μg/mL, highlighting the inhibition in the number of spikes and the number of (network) bursts (Fig. 5).

Neuronal activity parameters were not affected by exposure to ephedra extract at 5 μg/mL. However, at 50 μg/mL, exposure to the ephedra extract strongly reduced the number of spikes, the number of (network) bursts and the Area Under Cross-Correlation (Supplemental Fig. S5).

The green tea study material induced a minor decrease in the number of network burst following exposure at 5 μg/mL. At the high dose of 50 μg/mL, the inhibitory effects of it were strongly exacerbated and also included a strong reduction in the number of spikes, (network) bursts and in the number of spikes per burst. Also, the area under cross correlation was reduced, indicating a reduction in synchronicity (Supplemental Fig. S6).

Following exposure to milk thistle extract at 5 μg/mL a minor increase in mean inter-spike interval (ISI) within network bursts was observed. At 50 μg/mL, milk thistle extract evoked a clear reduction in the number of spikes, the number of bursts, network burst duration, the number of spikes per network bursts, and the area under cross correlation (Supplemental Fig. S7).

Usnea extract at 5 μg/mL did not affect any of the neuronal activity parameters to a degree that exceeds the BMR. At 50 μg/mL, usnea extract strongly reduced the number spikes, (network) bursts and the area under cross correlation (Supplemental Fig. S8).

The kava, kratom, tripterygium, and yohimbe extracts provided induced a very profound inhibition of neuronal activity (Fig. 6).

Exposure of cortical cultures to the kava extract induced a profound reduction in all neuronal activity parameters, except burst duration, already at 5 μg/mL. At 50 μg/mL, neuronal activity was completely abolished (Supplemental Fig. S9).

The kratom extract also already affected neuronal activity at the dose of 5 μg/mL. It reduced the number spikes, (network) bursts, the mean inter-spike interval (ISI) within network bursts, and the area under cross correlation, with a minor increase in the number of spikes per network burst. Comparable to the kava extract, the kratom extract at 50 μg/mL completely abolished neuronal activity (Supplemental Fig. S10).

Exposure of cortical cultures to tripterygium extract at 5 μg/mL did

not evoke significant changes in neuronal activity, except for a minor decrease in the number of spikes. At 50 μg/mL however, tripterygium extract strongly reduced the number spikes, number of (network) bursts, and the area under cross correlation, which was paralleled by an increase in (network) burst duration and mean inter-spike interval (ISI) within network bursts (Supplemental Fig. S11).

Cultures exposed to the yohimbe extract at 5 μg/mL showed a modest reduction in the number of spikes, (network) bursts, mean inter-spike interval (ISI) within network bursts, and the area under cross correlation. At 50 μg/mL these inhibitory effects exacerbated resulting in a profound reduction of all neuronal activity parameters, although the inhibition of burst duration did not reach significance (Supplemental Fig. S12). The example raster plot shows the neuronal activity of a well before (baseline) and after exposure to yohimbe extract at 50 μg/mL, highlighting the strong inhibition in the number of spikes and the number of (network) bursts (Fig. 7).

3.4. Botanical extracts with a specific or excitatory neuronal activity phenotype

Although most selected botanical extracts inhibit neuronal activity, the aconite, blue cohosh, goldenseal, and oleander extracts induce specific and/or excitatory neuronal activity phenotypes (Fig. 8).

Exposure of cortical cultures to aconite extract at 5 μg/mL induced a strong increase in the number of spikes and (network) bursts, the burst duration, mean inter-spike interval (ISI) within network bursts, and the area under cross correlation. At 50 μg/mL the number of (network) bursts and mean inter-spike interval (ISI) within network bursts were still increased, but the network burst duration, the number of spikes per (network) burst and the area under cross correlation were strongly reduced (Supplemental Fig. S13), indicative for a larger number of less intense (network) bursts. The example raster plot shows the neuronal activity of a well before (baseline) and after exposure to aconite extract at 5 μg/mL and at 50 μg/mL, highlighting the strong and dose-dependent increase in neuronal activity, in particular the number of (network) bursts (Fig. 9).

Blue cohosh extract at 5 μg/mL evoked little effect except for a minor reduction in the number of network bursts. At 50 μg/mL, however, there was a remarkable increase in (network) burst duration, and the number of spikes per (network) burst, although the number of (network) bursts was strongly reduced (Supplemental Fig. S14). The example raster plot shows the neuronal activity of a well before (baseline) and after exposure to blue cohosh extract at 50 μg/mL, highlighting the strong increase in burst duration (Fig. 10).

At the high dose of 50 μg/mL, exposure to the goldenseal extract resulted in the near complete cessation of neuronal activity. At the low dose of 5 μg/mL, the number of (network) bursts was also reduced, although the (network) burst duration and the number of spikes per (network) burst were markedly increased, suggestive of a specific activity phenotype that affects the intensity of the (network) bursts (Supplemental Fig. S15). The example raster plot shows the neuronal activity of a well before (baseline) and after exposure to the goldenseal extract at 5 μg/mL, highlighting the increase in (network) burst duration (Fig. 11).

Oleander extract, at the low dose of 5 μg/mL, strongly decreased the number of spikes and (network) bursts as well as the area under cross correlation. However, the duration of the remaining (network) bursts and the number of spikes per (network) burst were strongly increased also suggestive for a specific activity phenotype that affects the intensity of the (network) bursts. Comparable to what was observed following exposure to the high dose of Goldenseal extract, exposure to 50 μg/mL oleander extract resulted in a near complete cessation of neuronal activity (Supplemental Fig. S16). The example raster plot shows the neuronal activity of a well before (baseline) and after exposure to oleander extract at 5 μg/mL, highlighting the strong reduction in the number of (network) bursts, which is paralleled by an increase in

(network) burst duration (Fig. 12).

4. Discussion

Our results clearly demonstrate the neuroactive potential of these select botanical extracts in in vitro cortical cultures. The acute effects on neuronal activity are unlikely due to cytotoxicity as effects on cell viability after 24 h exposure appear limited to the highest dose of tripterygium and yohimbe extracts. Given potential ADME differences between in vitro and in vivo exposures, it is yet unclear if such effects on neuronal viability also occur in vivo. However, the finding that extracts of goldenseal, milk thistle, tripterygium, and yohimbe can induce a profound decrease in mitochondrial activity in rat primary (cortical) neurons following 7 days of exposure to 50 μg/mL (Fig. 1), which is indicative of decreased cell viability, warrants further investigation considering the widespread use of botanical extracts.

From the tested botanicals, only the ashwagandha, comfrey, and Asian ginseng extracts were without effect on neuronal activity (Fig. 2, Table 3). Most extracts inhibit neuronal activity (Table 3). However, when comparing the various neuronal activity heatmaps (Figs. 4 and 6), different patterns can be detected. For example, green tea catechins, usnea and kratom extracts reduced the number of spikes and (network) bursts, with kratom being particularly potent. Milk thistle, kava, and yohimbe extracts not only potently reduced the number of spikes and (network) bursts but also decreased the (network) burst duration and/or number of spikes per (network) burst. Aristolochia, ephedra, and tripterygium extracts also reduced the number of spikes and (network) burst, but this decrease was paralleled by a modest increase in (network) burst duration and/or number of spikes per (network) burst. In contrast to the inhibitory effects observed for most botanical extracts, exposure to the extracts of aconite, blue cohosh, goldenseal, and oleander induces specific excitatory phenotypes (Fig. 8). These distinct heatmap patterns may be due to differences of the extracts in the mode of action, the potency, or both.

Combined these data indicate that select botanicals modulate neuronal network activity in vitro with very distinct heatmap patterns and raster plot phenotypes. The distinct patterns in combination with the (limited) knowledge on neuroactive botanical constituents suggest a large number of possible underlying targets, likely including inhibition of Ca^{2+} and Na^+ channels, glutamate receptor antagonism, μ-opioid receptor agonism, Na^+ channel activation, cholinergic modulation, and inhibition of Na^+/K^+-ATPase. Ultimate identification of the responsible mechanisms will require additional in vitro testing, including dose-response testing of the individual constituents and specific mixtures thereof.

Dose-response testing will not only aid in categorization and rank-ordering the different botanicals, but it will also be an important first step in the attempt to relate these in vitro findings to human exposure levels and identification of possible health effects. Currently, this is however not possible as there is insufficient information available in literature on well-characterized extracts, and toxicodynamic and toxicokinetic considerations, to perform IVIVE and PK modeling on constituents for selected botanicals based on in vitro results to allow for comparisons to in vivo human dose and exposure levels. Despite these limitations, our data highlight the applicability of integrated screening approaches, like MEA recordings of neuronal activity in cortical cultures, for hazard identification and potency ranking of botanicals with diverse and often still unknown modes of action.

CRediT authorship contribution statement

Regina G.D.M. van Kleef: Formal analysis, Investigation, Writing – review & editing. **Michelle R. Embry:** Conceptualization, Writing – review & editing. **Constance A. Mitchell:** Conceptualization, Writing – review & editing. **Remco H.S. Westerink:** Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that there are no known competing financial interests or personal relationships that could have appeared influence the editorial process.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2024.114438.

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