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ORGAN TOXICITY AND MECHANISMS

Raising awareness of new psychoactive substances: chemical analysis and in vitro toxicity screening of 'legal high' packages containing synthetic cathinones

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Abstract The world's status quo on recreational drugs has dramatically changed in recent years due to the rapid emergence of new psychoactive substances (NPS), represented by new narcotic or psychotropic drugs, in pure form or in preparation, which are not controlled by international conventions, but that may pose a public health threat comparable with that posed by substances listed in these conventions. These NPS, also known as 'legal highs' or 'smart drugs', are typically sold via Internet or 'smartshops' as legal alternatives to controlled substances, being announced as 'bath salts' and 'plant feeders' and is often sought after for consumption especially among young people. Although NPS have the biased reputation of being safe, the vast majority has hitherto not been tested and several fatal cases have been reported, namely for synthetic cathinones, with pathological patterns comparable with amphetamines. Additionally, the unprecedented speed of appearance and distribution of the NPS worldwide brings technical difficulties in the development of analytical procedures and risk assessment in real time. In this study, 27 products commercialized as 'plant feeders' were chemically characterized by gas chromatography–mass spectrometry and nuclear magnetic resonance spectroscopy. It was also evaluated, for the first time, the in vitro hepatotoxic effects of individual synthetic cathinones, namely methylone, pentedrone, 4-methylethcathinone (4-MEC) and 3,4-methylenedioxypyrovalerone (MDPV). Two commercial mixtures ('Bloom' and 'Blow') containing mainly cathinone derivatives were also tested, and 3,4-methylenedioxymethamphetamine (MDMA) was used as the reference drug. The study allowed the identification of 19 compounds, showing that synthetic cathinones are the main active compounds present in these products. Qualitative and quantitative variability was found in products sold with the same trade name in matching or different 'smartshops'. In the toxicity studies performed in primary cultured rat hepatocytes, pentedrone and MDPV proved to be the most potent individual agents, with EC_{50} values of 0.664 and 0.742 mM, respectively, followed by MDMA ($EC_{50} = 0.754$ mM). 4-MEC and methylone were the least potent substances, with EC_{50} values significantly higher (1.29 and 1.18 mM, respectively; $p < 0.05$ vs. MDMA). 'Bloom' and 'Blow' showed hepatotoxic effects similar to MDMA ($EC_{50} = 0.788$ and 0.870 mM, respectively), with cathinones present in these mixtures contributing additively to the overall toxicological effect. Our results show a miscellany of psychoactive compounds present in 'legal high' products with evident hepatotoxic effects. These data contribute to increase the awareness on

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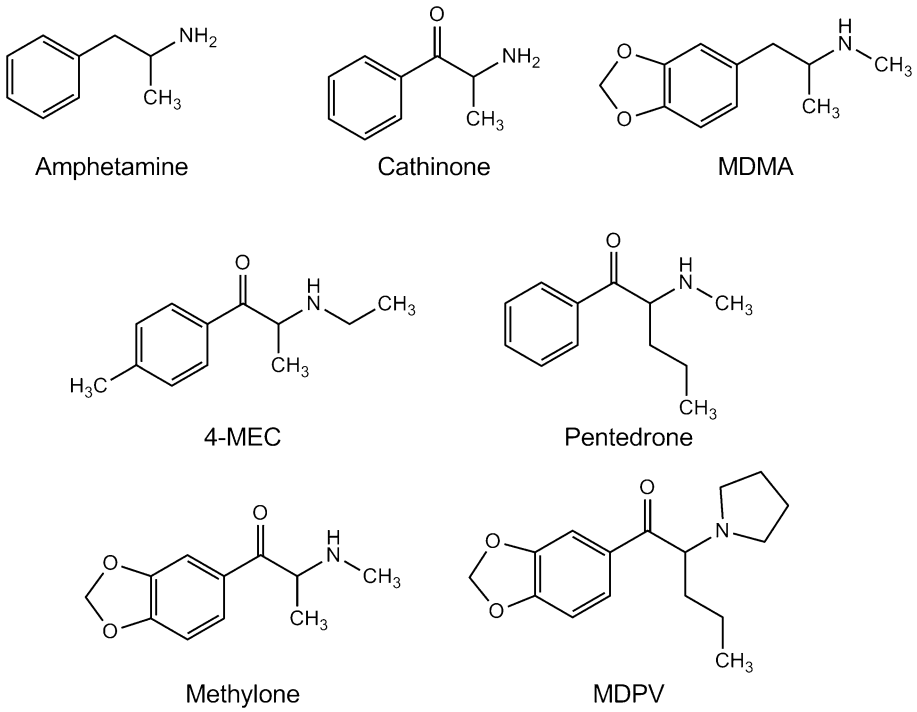

Fig. 1 Chemical structure of amphetamine, cathinone, MDMA and synthetic cathinones (those used in the in vitro toxicity studies)

the real composition of 'legal high' packages and unveil the health risks posed by NPS.

Keywords Synthetic cathinones · Chemical characterization · GC–MS · NMR · Primary rat hepatocyte cultures · Hepatotoxicity

Introduction

A large number of novel psychoactive substances (NPS) have appeared on the European market in recent years, being easily purchased via Internet websites or through specialized stores—the so-called 'smartshops' or 'headshops' (Arunotayanun and Gibbons 2012; EMCDDA 2012, 2013; Prosser and Nelson 2012). Although these substances are sold in packages labeled 'not for human consumption', they are intentionally marketed as replacements for illegal drugs, such as ecstasy (3,4-methylenedioxymethamphetamine, MDMA) or cocaine (Arunotayanun and Gibbons 2012; Prosser and Nelson 2012). In fact, the chemical structures of most of these NPS are redrawn from controlled psychoactive molecules in order to create alternative psychoactive compounds, aiming to circumvent the drug legislation, being then sold under the common name of 'legal highs', or with the label of inoffensive products, as 'bath salts' or 'plant feeders' (Baumann et al. 2013).

The chemical structure of cathinone, the main active principle of the plant *Catha edulis* Forsk (family celestracea), has been used as a prototype for the development of several derivatives through different radicals' substitutions and/or combinations. Like MDMA and cocaine, both cathinone and synthetic derivatives are able to exert their stimulant effects through the interaction with monoamine membrane transporters, namely dopamine, serotonin and noradrenaline transporters, leading to the synaptic accumulation of these biogenic amines (Kelly 2011; Valente et al. 2014). In fact, cathinone derivatives are closely related to the phenethylamine class, differing only by the presence of a β-keto group in the aliphatic chain, therefore often called bk-amphetamines (Fig. 1). Due to this structural relationship, synthetic cathinones exhibit central nervous system stimulant and sympathomimetic effects similar to those observed with amphetamine, and some derivatives have even a direct analog, as for example methylone, which is the β-keto analog of MDMA (Fig. 1) (Kelly 2011; Valente et al. 2014). The illusory idea of being 'safe' substances, due to their legal status in many countries, dramatically increased the popularity of these substances, especially among adolescents and young adults (Coppola and Mondola 2012a). The dizzying speed with which these substances appear and are distributed throughout the world has been a subject of major concern, especially in recent years. In Europe, more than 230 NPS were reported to the EMCDDA (European Monitoring Centre for Drugs and Drug Addiction) between 2005 and 2012, of which more than 30 belong to the class of synthetic cathinones (EMCDDA 2013). Cathinone derivatives, along with synthetic cannabinoids, represent currently about two-thirds of all substances notified since 2005, but the data also

include legal derivatives of phenethylamines, piperazines, tryptamines and other substances without defined class. The speed at which new drugs appear in the market implies technical difficulties in the development of analytical procedures and risk assessments in real time. Thus, a biased, but frequently sustained claim among sellers and consumers is that NPS are more pure than illegal drugs and carry less health risks. Importantly, information regarding the composition and purity of these products is still very scarce in the scientific literature. Therefore, an objective of this study was to chemically characterize products apparently containing cathinones marketed in Portuguese 'smartshops', prior to the approval of Decree 54/2013 (Portuguese Government 2013), prohibiting the marketing of these products. Nuclear magnetic resonance (NMR) spectroscopy and gas chromatography–mass spectrometry (GC–MS) were applied to the characterization of the real composition of these products.

It is also important to note that the vast majority of these NPS has not been yet tested on animals or humans, albeit these substances have been associated with numerous cases of toxicity and deaths, with clinical patterns similar to those of classical drugs of abuse, as it has been extensively reported for synthetic cathinones (Borek and Holstege 2012; Carbone et al. 2013; James et al. 2011; Kovacs et al. 2012; Lusthof et al. 2011; Murray et al. 2012; Pearson et al. 2012; Schifano et al. 2012; Warrick et al. 2012; Wood et al. 2010; Valente et al. 2014). Despite the scarcity of experimental data on the pharmacological and toxicological properties of these 'legal highs', due to the structural similarities between cathinone derivatives and amphetamines like MDMA (Fig. 1), identical mechanisms of action and effects are predictable.

As the liver is acknowledged to be one of the main targets of toxicity for amphetamine-like compounds (reviewed in Carvalho et al. 2010, 2012) and acute or fulminant hepatic failure has been described in cases of synthetic cathinone intoxication (Penders et al. 2012; Borek and Holstege 2012; Fröhlich et al. 2011), primary cultures of rat hepatocytes were chosen for the in vitro toxicological assessment of individual synthetic cathinones and of two commercial mixture products. As far as we know, this is the first time that potential hepatotoxic effects of cathinone derivatives are screened in vitro.

Materials and methods

Reagents

All chemicals used were of analytical grade. Methanol was obtained from Fisher Chemicals (Loures, Portugal). Caffeine, trifluoroacetic anhydride (TFAA), William's E medium, insulin solution from bovine pancreas, hydrocortisone hemisuccinate, collagenase from *Clostridium histolyticum* type IA (≥600 CDU/mg solid), bovine serum albumin (fraction V), ethylene glycol-bis(β-aminoethyl)-*N,N,N′,N′*-tetraacetic acid (EGTA), gentamicin, dexamethasone, trypan blue solution, Triton X-100 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibiotic mixture of penicillin/streptomycin, fetal bovine serum (FBS), trypsin 0.05 %-EDTA and Hank's buffered salt solution (HBSS) were obtained from Gibco-Invitrogen (Barcelona, Spain), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was obtained from VWR (Leuven, Belgium).

The synthetic cathinones methylone, pentedrone, 4-MEC (4-methylethcathinone) and MDPV (3,4-methylenedioxypyrovalerone) (HCl salts) were purchased online from the Sensearomatic website (http://sensearomatic.net), during March 2013. All salts were fully characterized by mass spectrometry, NMR and elemental analysis, and purity was greater than 98 %. MDMA (HCl salt) was extracted and purified from high purity MDMA tablets provided by the Portuguese Criminal Police Department, as described elsewhere (Dias da Silva et al. 2013a).

Chemical characterization studies

Samples

Twenty-seven products sold as 'plant feeders', apparently containing cathinones, were purchased in three different Portuguese 'smartshops' during 2012 and 2013, 22 being powders and 5 being tablets (Table 1). All products were acquired prior to the approval of Decree 54/2013 (Portuguese Government 2013) prohibiting the marketing of these products.

GC–MS analysis

After homogenization, methanolic extracts of all samples and standards solutions were prepared at a concentration of 1 mg/mL and 2 µL were directly injected into the GC–MS apparatus. Analysis after derivatization with TFAA was performed according to the method developed by Silva et al. (2010) with adaptations. Standards and samples were prepared at a concentration of 10 µg/mL by dilution of initial methanolic extract (1 mg/mL) and were evaporated to dryness under nitrogen flow. Fifty microliters of ethyl acetate and fifty microliters of TFAA were added to the dried residue. Incubation was performed at 70 °C for 30 min. After cooling to room temperature, the extract was dried under nitrogen flow. The obtained residue was dissolved

Table 1 Main features of 'legal highs' acquired for analysis

Sample no.	Product name	Smartshop	Supplier	Lot	Product color	Chemical composition indicated on the label	Price (€) per gram
1	Bloom	'Smartshop'1 (Porto)	Aurafeel	N/A	Yellowish	Ketones, vegetable extracts and glucose	36
2	Bloom	'Smartshop' 1 (Porto)	Aurafeel	2012 45X19P	White	94 % Ketones, 5 % caffeine and 1 % glucose	36
3	Bloom	'Smartshop' 1 (Lisbon)	Aurafeel	2012 45X19P	White	94 % Ketones, 5 % caffeine and 1 % glucose	36
4	Blast	'Smartshop' 2 (Porto)	Aurafeel	N/A	White	89 % Ketones, 10 % caffeine and 1 % glucose	36
5	Blast	'Smartshop' 1 (Lisbon)	Aurafeel	2012 46X12P	White	100 % Ketones	36
6	Rush	'Smartshop' 1 (Porto)	Aurafeel	N/A	Yellowish	89 % Ketones, 10 % caffeine and 1 % glucose	30
7	Rush	'Smartshop' 1 (Lisbon)	Aurafeel	N/A	White	89 % Ketones, 10 % caffeine and 1 % glucose	30
8	Invader (Crabby)	'Smartshop' 2 (Porto)	Aurafeel	2012 47X2P	White	100 % Ketones	37
9	Invader (Cyclop)	'Smartshop' 2 (Porto)	Aurafeel	2012 45X2P	White	100 % Ketones	37
10	Bliss	'Smartshop' 1 (Porto)	Aurafeel	2013 X0619P	White	95 % Ketones, 5 % caffeine and 1 % glucose	30
11	Bliss	'Smartshop' 2 (Porto)	Aurafeel	2012 37014P	White	100 % Ketones	30
12	Bliss	'Smartshop' 1 (Lisbon)	Aurafeel	2012 42X16P	White	100 % Ketones	30
13	Charlie	'Smartshop' 1 (Porto)	Aurafeel	2012 40X17P	White	100 % Ketones	37
14	Charlie	'Smartshop' 1 (Porto)	Aurafeel	2012 35017P	White	100 % Ketones	37
15	Charlie	'Smartshop' 1 (Lisbon)	Aurafeel	2012 35017P	White	100 % Ketones	37
16	Blow	'Smartshop' 1 (Porto)	N/A	N/A	Yellowish	Ketones, vegetable extracts and glucose	38
17	Blow	'Smartshop' 2 (Porto)	Aurafeel	N/A	White	94 % Ketones, 5 % caffeine and 1 % glucose	38
18	Blow	'Smartshop' 1 (Lisbon)	Aurafeel	N/A	White	94 % Ketones, 5 % caffeine and 1 % glucose	38
19	Kick	'Smartshop' 1 (Porto)	Aurafeel	N/A	White	Ketones, vegetable extracts and glucose	30
20	Kick	'Smartshop' 2 (Porto)	Aurafeel	2012 36015P	White	94 % Ketones, 5 % caffeine and 1 % glucose	30
21	Kick	'Smartshop' 1 (Lisbon)	Aurafeel	2012 38015P	White	94 % Ketones, 5 % caffeine and 1 % glucose	30
22	MMB	N/A	N/A	N/A	White	N/A	N/A
23[a]	M	'Smartshop' 1 (Porto)	Aurafeel	N/A	Pink	Ketones, dicalcium, phosphoates, magnesium and stearate	16
24[a]	M	'Smartshop' 1 (Lisbon)	Aurafeel	N/A	Pink	Ketones, dicalcium, phosphoates, magnesium and stearate	16
25[a]	Bliss	'Smartshop' 2 (Porto)	Aurafeel	N/A	Pink	160 mg Ketones, 120 mg lactose, 100 mg corn starch, 50 mg calcium stearate, 20 mg magnesium stearate, 20 mg E124, 6 mg E132 and 4 mg E142	14
26[a]	Bloom^{+}	'Smartshop' 1 (Porto)	Aurafeel	N/A	Pink	100 mg Aminoalkyl benzofurans, 120 mg lactose, 100 mg corn starch, 50 mg calcium stearate, 20 mg magnesium stearate, 20 mg E128 and 5 mg E142	20

Table 1 continued

Sample no.	Product name	Smartshop	Supplier	Lot	Product color	Chemical composition indicated on the label	Price (€) per gram
27[a]	Bloom^{+}	'Smartshop' 1 (Lisbon)	Aurafeel	N/A	Pink	100 mg Aminoalkyl benzofurans, 120 mg Lactose, 100 mg corn starch, 50 mg calcium stearate, 20 mg magnesium stearate, 20 mg E128 and 5 mg E142	20

[a] Samples of 1–22 were acquired in the form of powders, and the others are tablets

in 100 µL of ethyl acetate, and 2 µL was injected into the GC–MS apparatus.

GC–MS analysis was performed with a Varian CP-3800 gas chromatograph (USA) equipped with a Varian Saturn 4000 Ion Trap mass selective detector (USA) and a Saturn GC–MS workstation software version 6.8. The GC was equipped with a VF-5MS (30 m × 0.25 mm × 0.25 µm) capillary column (Varian). A CombiPAL automatic autosampler (Varian) was used for all experiments. The carrier gas was helium C-60 (Gasin, Portugal), at a constant flow of 1 mL/min. Injections were performed in split mode, with a ratio of 1/40. The injector port was heated to 250 °C. The initial column temperature of 80 °C was held for 1 min, followed by a temperature ramp of 20 °C/min to 250 °C held for 2 min and 10 °C/min to 300 °C held for 20 min. The ion trap detector was set as follows: transfer line, manifold and trap temperatures were 280, 50 and 180 °C, respectively. The emission current was 10 µA, and the maximum ionization time was 2,500 µs. Ionization was maintained off during the first 4 min to avoid solvent overloading. Total separation run time was 36 min. For analysis of derivatized compounds, the GC–MS conditions were properly adapted. Injections were performed in splitless mode. The injector port was heated to 250 °C; the oven temperature was adjusted to 100 °C held for 1 min, followed by a temperature ramp of 15 °C/min to 300 °C held for 10 min. The emission current was 30 µA, and the maximum ionization time was 2,500 µs. Total separation run time was 20 min.

All mass spectra were acquired in electron impact (EI) mode at 70 eV and the mass ranged from 40 to 600 m/z. The analysis was performed in full scan mode.

NMR analysis

The samples (approximately 30–50 mg) were dissolved in 500 µL of methanol-d4 (Cambridge Isotope Laboratories, Inc.). The NMR spectra were recorded using a Bruker Avance 400 spectrometer with a frequency of 400 MHz for ^{1}H and 100 MHz for ^{13}C. The structure's identification with the respective assignment of the proton and carbon signals was based on the analysis of NMR spectra obtained by 1D (^{1}H, ^{13}C, APT) and 2D (including the COSY, HMBC and HSQC experiments) techniques. ^{1}H and ^{13}C NMR data assignments are described in supplementary material (Table S2).

In vitro toxicity screening study

Animals

Adult male Wistar rats weighing 280–320 g were used. Portuguese General Directorate of Veterinary Medicine licensed animal experiments. Housing and experimental treatment of animals were in accordance with the Guide for the Care and Use of Laboratory Animals from the Institute for Laboratory Animal Research (Research IFLA 1996). Surgical procedures for the isolation of hepatocytes were performed under isoflurane anesthesia and carried out between 10.00 and 11.00 a.m.

Isolation and primary culture of rat hepatocytes

Hepatocyte isolations were performed by the collagenase perfusion method as described previously (Carvalho et al. 2004). The initial viability of isolated hepatocytes was always above 85 %, as estimated by the trypan blue exclusion test. A suspension of 5 × 10^{5} viable cells/mL in complete culture medium (William's E medium supplemented with 10 % FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 10 µg/mL gentamicin, 2 ng/mL insulin and 5 nM dexamethasone) was seeded in 96-well plates (BD Biosciences, Oxford, UK). Cells were incubated at 37 °C with 5 % CO_2, overnight for cell adhesion.

Cell viability assay

Twenty-four hours after cell seeding, the culture medium was replaced by serum-free medium followed by exposure of cells for 48 h at 37 °C to the individual cathinone derivatives as well as to 'Bloom' (sample 1) and 'Blow' (sample 16). Thirteen different concentrations of methylone,

Table 2 Active ingredients and their relative proportions detected in 27 'legal highs' acquired in three distinct 'smartshops' after analysis by GC–MS (Table S1) and NMR (Table S2)

Sample No.	Product name	Smartshop	Active substances identified by GC-MS and NMR (%)
1	Bloom	'Smartshop' 1 Porto	Methylone (43%), 4-MEC (29%), Pentedrone (25%), Dimethocaine (<1%) Isopentedrone (<1%)
2	Bloom	'Smartshop' 1 Porto	Methedrone (34%), Pentedrone (29%), Ethcathinone (24%), Caffeine (13%), Isopentedrone (<1%)
3	Bloom	'Smartshop' 1 Lisbon	Methedrone (36%), Pentedrone (24%), Caffeine (20%), Ethcathinone (19%), Isopentedrone (<1%)
4	Blast	'Smartshop' 2 Porto	Flephedrone (87%), Caffeine (13%)
5	Blast	'Smartshop' 1 Lisbon	Flephedrone (78%), Caffeine (22%)
6	Rush	'Smartshop' 1 Porto	Buphedrone (87%), Caffeine (13%), Methylamine [b]
7	Rush	'Smartshop' 1 Lisbon	Pentedrone (76%), Caffeine (23%), Isopentedrone (<1%), Methylamine [b]
8	Crabby	'Smartshop' 2 Porto	3,4-Dimethylmethcathinone (3,4-DMMC) (>99%)
9	Cyclop	'Smartshop' 2 Porto	3,4-DMMC (>99%)
10	Bliss	'Smartshop' 1 Porto	Methedrone (89%), Pentedrone (5%), 3,4-DMMC (4%), Caffeine (<1%), Isopentedrone (<1%),
11	Bliss	'Smartshop' 2 Porto	Methedrone (>99%)
12	Bliss	'Smartshop' 1 Lisbon	Methedrone (>99%)
13	Charlie	'Smartshop' 1 Porto	Buphedrone (80%), Ethcathinone (19%), Caffeine (<1%)
14	Charlie	'Smartshop' 1 Porto	Buphedrone (52%), Ethcathinone (48%),
15	Charlie	'Smartshop' 1 Lisbon	Buphedrone (69%), Ethcathinone (31%),
16	Blow	'Smartshop' 1 Porto	4-MEC (86%), MDPV (14%), 3-Methylethcathinone (3-MEC) (<1%),
17	Blow	'Smartshop' 2 Porto	4-MEC (83%), MDPV (8%), Caffeine (8%), 3-MEC (<1%)
18	Blow	'Smartshop' 1 Lisbon	4-MEC (85%), MDPV (9%), Caffeine (5%), 3-MEC (<1%),
19	Kick	'Smartshop' 1 Porto	Pentedrone (89%), Isopentedrone (11%), Methylamine [b]
20	Kick	'Smartshop' 2 Porto	Buphedrone (80%), Caffeine (20%)
21	Kick	'Smartshop' 1 Lisbon	Buphedrone (84%), Caffeine (16%)
22	MMB	N/A	Dimethocaine (89%), Alpha-methyltryptamine (AMT) (11%),
23 [(*)]	M	'Smartshop' 1 Porto	4-Fluoroamphetamine (>99%)
24 [(*)]	M	'Smartshop' 1 Lisbon	4-Fluoroamphetamine (>99%)
25 [(*)]	Bliss	'Smartshop' 2 Porto	Methylone (>99%)
26 [(*)]	Bloom [+]	'Smartshop' 1 Porto	6-(2-Aminopropyl)benzofuran (6-APB) (90%), 4-(2-Aminopropyl)benzofuran (4-APB) (10%),
27 [(*)]	Bloom [+]	'Smartshop' 1 Lisbon	6-(2-Aminopropyl)2,3-dihydrobenzofuran (6-APDB) (93%), 6-APB (6%), 4-APB (<1%)

pentedrone, 4-MEC and MDPV (0, 0.05, 0.1, 0.2, 0.5, 0.8, 1.0, 1.2, 1.6, 2.0, 2.5, 3.0, 4.0 and 5.0 mM) were prepared in fresh serum-free culture medium. MDMA was used for comparison purposes, in the same concentration range. GC–MS analysis revealed that 'Bloom' and 'Blow' are mixtures of cathinones (Table 2), and therefore, solutions were prepared in such a way that the range of concentrations corresponds to the major cathinone derivative present in each product (methylone and 4-MEC, respectively). These serial dilutions covered a broad range of concentrations, so that a complete concentration–response relationship could be recorded allowing robust computation of the data. Each individual plate also included four replicates of negative death control (i.e., no test agents) and four replicates of positive controls (1 % Triton X-100).

The cytotoxic effects of MDMA and cathinone derivatives, individually and as mixture products sold in 'smartshops', were determined using the MTT reduction assay. The mitochondrial reductases of viable cells reduce the MTT dye to purple formazan crystals that can be spectrophotometrically quantified. Therefore, the assay results represent mitochondrial function and, consequently, cell viability (Mosmann 1983). Briefly, cells were incubated with a 0.5 mg/mL solution of MTT for 1 h 20 min at 37 °C in a humidified 5 % CO_2 atmosphere. The intracellular formazan crystals formed through mitochondrial succinate dehydrogenase were then dissolved with 100 µL/well of DMSO and detected at 550 nm in a 96-well plate reader (PowerWaveX; Bio-Tek, Winooski, VT, USA). To reduce inter-experimental variability, data were normalized on a plate-by-plate basis and scaled between 0 % (negative controls) and 100 % effect (positive controls). Results were graphically presented as percentage of cell death versus log concentration (mM). Data were obtained from five independent experiments, with each test plate containing quadruplicates of increasing concentrations of the tested drugs.

Regression modeling

Curves of normalized MTT data were constructed and analyzed using the best-fit approach (Scholze et al. 2001). In the present study, the logit function was employed: $Y = \theta_{min} + (\theta_{max} - \theta_{min})/(1 + \exp(-\theta_1 - \theta_2 \times \log(x)))$, where θ_{min} and θ_{max} are the minimal and maximal observed effects, respectively; x is the concentration of the test drug; θ_1 is the parameter for the location, and θ_2 is the slope parameter (Silva et al. 2013a). All of the nonlinear regression models describe sigmoidal concentration–response relationships, and the plots were constructed using the GraphPad Prism 6 (version 6.0c) for Mac OS X. MTT data are presented as mean ± 95 % confidence interval (CI). The EC_{50} values were determined for each individual drug and mixture, allowing for comparison between drugs.

Calculation of predicted mixture effects

In this study, we evaluated the toxicity of two 'real' mixture products, and therefore, range concentrations of each cathinone in the mixture were estimated by GC–MS. A range of test concentrations was obtained through serial dilutions, so that the ratio between each constituent was maintained. Parameters derived from nonlinear fits of individual cathinone concentration–response data, in the MTT reduction assay, were used to compute the predicted mixture effect curves assuming additive joint responses. The expected effects were calculated using the concepts of concentration addition (CA) and independent action (IA) approaches, as described in Payne et al. (2000) and used as a reference for the assessment of combination effects in terms of synergism (if the observed effects are greater than additive predictions), additivity (if the experimental mixture outcomes equal the prediction) and antagonism (if the experimental joint effects fall short of additivity) (Kortenkamp 2007).

Results

Chemical characterization of 'legal high' products containing synthetic cathinones

A total of 27 'legal high' products were purchased in three distinct 'smartshops' ('smartshop' 1 in Porto and Lisbon and 'smartshop' 2 in Porto), and all of them were packed in sealable silver foil bags or, occasionally, in see-through plastic bags. The packages featured the trade name, the alleged composition, the product quantity and the dosage as 'plant feeders', along with the warnings 'not for human consumption'. Some products presented also its batch number but no other information was provided. The majority of powder products (86 %) had a white color, but some presented a yellowish color (Table 1), whereas the predominant color of the tablets was pink. Noteworthy, color variation was observed in products with the same trade name, either acquired at the same 'smartshop' (e.g., sample 1 and 2) or in different 'smartshops' (e.g., samples 1 and 3; 6 and 7; or 16, 17 and 18). Considering the amount of 1 g for all products, the prices for the powders varied between 30 and 38 €, while the price of tablets varied between 14 and 20 € per package. Regarding the composition, the ingredient names printed on the packaging are presented in Table 1 and the spelling mistakes were not corrected (e.g., phosphoates which should read phosphates). With the exception of samples 22, 26 and 27, all others had printed 'ketones', presumably indicative of the presence of cathinone derivatives. Samples 26 and 27 ('Bloom^{+}') stated the presence of aminoalkyl benzofurans, compounds with a structure similar to amphetamine analogs, namely MDMA, with the particularity of the 3,4-methylenedioxy group be replaced by a benzofuran ring. Most powder products also referred the presence of caffeine and glucose.

Methanolic extracts were directly analyzed by GC–MS, resulting in different chromatographic profiles, showing that the marked products are mixtures of structurally similar compounds with different retention times. Initially, potential identifications were made through the analysis of the fragment pattern found in the mass spectra of each compound and their trifluoroacetic derivative, and by comparison of *SWGDRUG* mass spectral library. We were able to unequivocally identify methylone, 4-MEC, pentedrone, MDPV and caffeine in commercial products by comparing retention times and mass spectra of each chromatographic peak with the respective standards underivatized and derivatized with TFAA (see Supplementary Table S1). Due to the lack of standards to confirm or reject the other identifications, NMR 1D (^{1}H, ^{13}C, APT) and 2D (COSY, HMBC and HSQC) experiences were performed for a correct structural elucidation. Supplementary Table S2 shows the chemical skeleton with the respective NMR assignment for the major psychoactive substances found in the analyzed 'plant feeders' in methanol-d_4. NMR analysis confirmed the chemical structure of each compound while allowing the distinction of positional isomers, such as the 3-MEC (3-methylethcathinone) and 4-MEC isomers in 'Blow' samples and 4-APB and 6-APB in 'Bloom^{+}' tablets, through the differences observed in the ^{1}H NMR pattern of aromatic moiety. Additionally, NMR analysis allowed the identification of methylamine, a reagent usually used in the synthesis of *N*-methyl cathinones and not detected in the GC–MS analysis due to its volatility.

The products' active ingredients identified by GC–MS and NMR analyses are summarized in Table 2. A total of 19 different substances were detected, with the vast majority belonging to the class of synthetic cathinones. Substances of other classes were also found, namely dimethocaine (cocaine derivative), alpha-methyltryptamine (from the tryptamine class), as well as other substances derived from the phenethylamines class, namely the derivatives of benzofurans 4-APB (4-(2-aminopropyl)benzofuran), 6-APB (6-(2-aminopropyl)benzofuran) and 6-APDB (6-(2-aminopropyl)2,3-dihydrobenzofuran)). In total, 11 distinct cathinone derivatives were identified, with isopentedrone, pentedrone and buphedrone as the most frequent, while flephedrone and methylone were the least common. Caffeine was a common substance in these products. The number of compounds present in each 'legal high' showed to be inconsistent. In fact, some products present only one active principle (e.g., samples 8, 9, 11, 12, 23, 24 and 25), while most products are mixtures of psychoactive substances with a high number of constituents, as for example sample 1, 2 and 3 ('Bloom') or sample 10 ('Bliss') that

have five different components each. Of the 27 analyzed products, only 7 (26 %) contained a single psychoactive substance; 8 (30 %) were composed by two; 5 (18 %) presented three; 3 (11 %) presented four; and 4 (15 %) had five different psychoactive substances. The 27 analyzed samples correspond to a total of 12 different commercial products, though 19 different compositions were found, revealing the existence of a compositional variability.

Concentration-dependent toxicity of individual cathinone derivatives in primary cultured hepatocytes

In this study, the potential hepatotoxic effects of cathinone derivatives were screened, for the first time in vitro. In the MTT assay, all tested individual cathinone derivatives yielded reproducible effects, inducing cell death in a concentration-dependent manner. The data were obtained through five independent experiments, using independently prepared serial dilutions of all tested compounds. The cytotoxicity curves for each of the tested drugs, including the upper and lower 95 % CI, are displayed in Fig. 2. Complete concentration–response curves were obtained for all cathinone derivatives under study. The shape, slope and plateau of the curves for the tested individual cathinones were relatively similar. A summary of the best-fit regression model parameters for each individual compound is presented in Table 3.

Significant differences were observed in the EC_{50} values. Since cathinone derivatives hold structural similarities with MDMA, this drug was chosen to draw comparisons at the hepatotoxic potency level. MDMA presented an EC_{50} value of 0.754 mM. Methylone, with an EC_{50} of 1.18 mM, shares a similar potency of cytotoxic effects with 4-MEC (EC_{50} of 1.29 mM), but with significantly lower potency than MDMA ($p < 0.05$). In turn, no significant differences were found between mean EC_{50} values of MDPV (0.742 mM) or pentedrone (0.664 mM) and MDMA, meaning that these three substances have similar cytotoxic potencies. Although EC_{50} values of pentedrone and MDPV were not significantly different, the first derivative appears to be most cytotoxic at high concentrations, whereas MDPV showed to be the most cytotoxic at low concentrations (Fig. 2).

Additive toxicity is observed in cathinone mixtures obtained in 'smartshops'

Another main aim of this work was to investigate potential interactions between synthetic cathinones and evaluate the predicted effects using the mathematical CA and IA models. In order to produce the data necessary for calculating predictions of mixture effects, extensive concentration–response curve analyses of all individual agents that compose the mixtures had to be carried out. 'Bloom' and 'Blow' samples used were specifically chosen as GC–MS analysis revealed that they are composed by mixtures of individual cathinones herein tested. A summary of the best-fit regression model parameters for individual and combined drugs is presented in Table 3.

Based on information of the concentration–response curves of all individual agents in terms of shape, slope and maximal effects, a theoretical mixture study was performed to assess the joint toxic effects of these two 'real' mixture products ('Bloom' and 'Blow') in primary rat hepatocytes. In order to correctly assess combination effects of chemicals in terms of additivity, synergism or antagonism, it is crucial to formulate a hypothesis about the expected effect of the mixture. Two models for the calculation of expected additive mixture effects were applied: concentration addiction (CA) and independent action (IA) (Dias da Silva et al. 2013a; Payne et al. 2000). To our knowledge, these models have never been applied to synthetic cathinone compounds.

As shown in Fig. 3, 'Bloom' and 'Blow' presented potent in vitro hepatotoxic effects, with cathinones present in these products contributing additively to the overall toxicological effects. The two additive models produced very similar curves, and the slope for each prediction model was identical, both ranging the same order of magnitude from minimal to maximal mortality. The overlapping between predicted and observed effect confirms that both models are suitable for predicting the toxic effects induced by these mixtures, with a best prediction for concentrations below 1 mM. Supporting this observation, the EC_{50} values for predicted curves according to the IA (0.923 mM) and CA (0.979 mM) models are similar to that obtained for 'Bloom' mixture (0.788 mM) ($p > 0.05$). The same findings were obtained for 'Blow' mixture, with the EC_{50} value (0.870 mM) not differing significantly from those ascertained from IA or CA prediction curves (EC_{50} of 1.009 and 1.109 mM, respectively). Nevertheless, in the 'Blow' mixture, at higher concentrations, the predicted curves are slightly displaced to the right, i.e., lower concentrations of mixture are required than those provided by the mathematical models for the same effects, revealing a slight synergistic effect.

Discussion

For a proper application of the law, the forensic laboratory must be able to correctly identify the compounds present in the NPS. The process of detection and identification of these new substances is difficult to attain because many of them exhibit identical chemical, chromatographic and spectral properties. Furthermore, the absence of good quality standards at a reasonable price and reference spectral data will increase the complexity of identification, being hardly achieved through

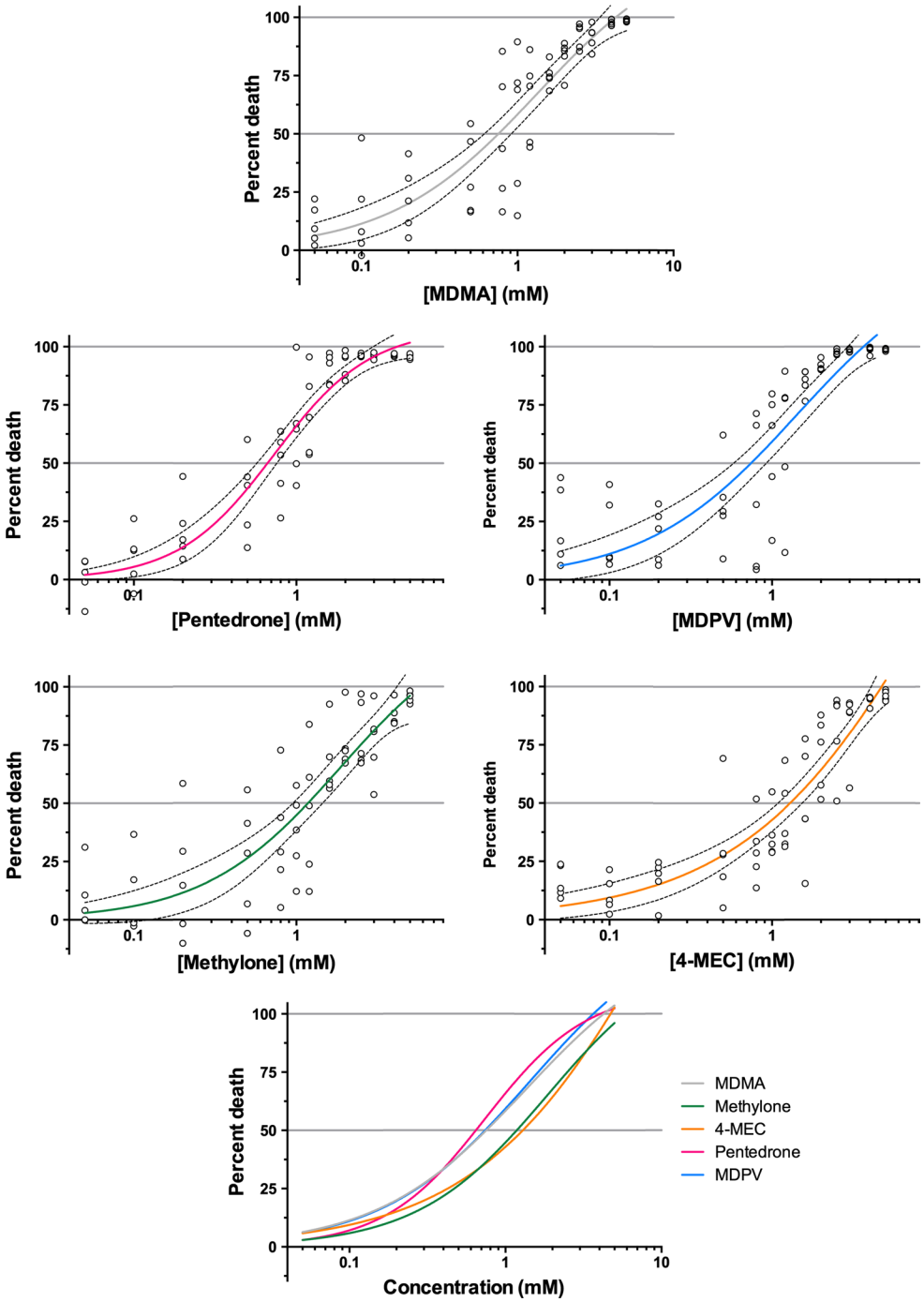

Fig. 2 Regression models for the cytotoxicity effects of individual cathinones, using isolated rat hepatocytes as a model at 37 °C. The *gray line* shows the EC_{50} and EC_{max} for each *response curve,* and the *dashed lines* show the 95 % CI belt of the fit. Data were normalized to negative (untreated) and positive (1 % Triton X-100) controls. Data were obtained from five independent experiments run in triplicate

routine analytical protocols (Davis et al. 2012; Zuba and Byrska 2013). GC–MS is an analytical method used in most laboratories for qualitative and quantitative analysis of diverse psychoactive drugs, including the 'legal highs' (Zuba and Byrska 2013; Brandt et al. 2010, 2011; Camilleri et al. 2010; Davies et al. 2010, 2012; Baron et al. 2011; Elie et al. 2013; Daeid et al. 2014; Leffler et al. 2014). Taking into account the numerous potential identifications due to the lack of certified standards to unequivocally identify the psychoactive substances, the existence of a wide variety of isomers, and identical fragmentation patterns of many NPS under EI ionization conditions, the derivatization proved to be an effective methodology to confirm the identities of compounds, allowing us to obtain more specific structural information. In addition, this method allowed us to obtain cleaner chromatograms and better separations with increased resolution and response, as well as the identification of the molecular ion of certain compounds such as MDPV (see Supplementary Table S1). Additionally, NMR

Table 3 Parameters derived from nonlinear fits of single agents and 'legal highs' mixtures concentration–response data to the asymmetric logit function, in the MTT reduction assay, for primary hepatocytes

Compounds	Estimated parameters for the best-fit regression model				EC_{50} (mM)	Fraction in the mixture
	Regression model	θ_1	θ_2	θ_{max}		
MDMA	Logit	−0.270	2.11	135	0.754	n/a
Methylone	Logit	−0.647	2.40	130	1.18*	0.439
4-MEC		−1.78	1.64	296	1.29*	0.289
Pentedrone		0.457	3.38	108	0.664	0.272
'Bloom'		0.177	3.42	110	0.788	1.000
4-MEC	Logit	−1.78	1.64	296	1.29*	0.965
MDPV		−0.332	2.13	142	0.742	0.0347
'Blow'		0.125	4.39*	108	0.870	1.000

$n = 5$; * $p < 0.05$ for each product versus MDMA

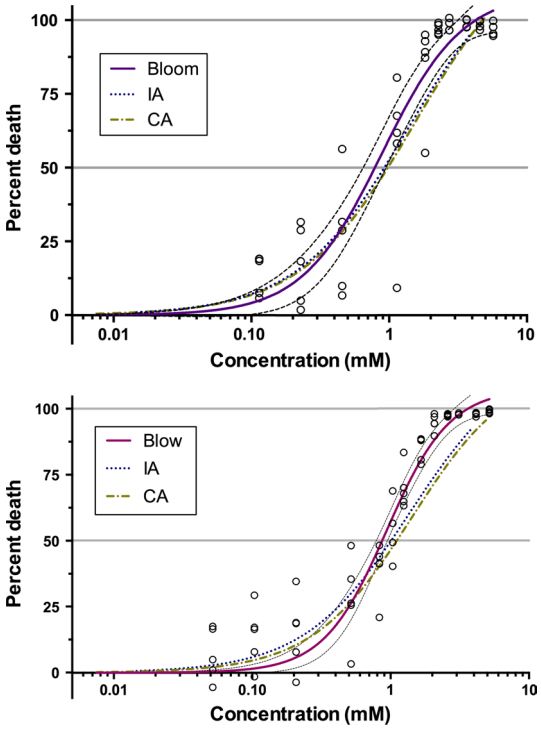

Fig. 3 Predicted and observed effects of 'Bloom' and 'Blow' mixtures and their individual agents in MTT assay. *Circles* represent individual data points of the 'Bloom' and 'Blow' mixtures. The *dashed lines* show the prediction according to concentration addition (CA) and independent action (IA). The *gray lines* show the EC_{50} and EC_{max} for each *response curves*. Experimental data derive from five independent experiments run in triplicate

has proved to be a valuable tool for the molecular structure confirmation and to allow the positional isomers distinction, as well as the identification of compounds not detected in the GC–MS, such as methylamine. The combination of GC–MS and NMR experiments proved to be an effective methodology to unequivocally identify the molecular structure of the emerging new drugs present in 'plant feeders', even in the absence of certified standards.

For our study, a total of 27 'legal high' products were purchased in three distinct 'smartshops', acquiring repeating units of some of them randomly chosen, in order to ascertain the possible existence of qualitative and/or quantitative variability between theoretically identical products, as described in the literature (Brandt et al. 2010; Davies et al. 2010; Zuba and Byrska 2013). In fact, the existence of a great compositional variability was the most important finding of this study, that revealed 19 different substances, the vast majority belonging to the class of synthetic cathinones (58 %), despite also having been detected substances belonging to other classes such as cocaine, tryptamine or amphetamine analogs. Caffeine was a common active ingredient in these products, most likely added intentionally for being a cheap substance and improving the stimulant effects, having been detected in approximately 17 % of the products. A recent study performed by Zukiewicz-Sobczak et al. (2012) analyzing the composition of 'legal highs' revealed that synthetic cathinones were also the most frequent class of compounds (47 %) followed by cannabinoids (45 %), being piperazines (7.5 %) and phenethylamines (3.8 %) the less recurrent classes. In that study, MDPV was found to be the most common cathinone, and caffeine was also the psychoactive substance most commonly present. Another study, conducted by Zuba and Byrska (2013) on 'legal highs' seized in Polish 'headshops', showed that substances from different chemical classes were present, but active components belong to two major classes: cathinones and piperazines. The most common active ingredients detected in 'legal highs' seized were in descending order: MDPV, caffeine, butylone, TFMPP (3-trifluoromethylphenylpiperazine), lidocaine, 4-MEC, mephedrone, pFPP (para-fluorophenylpiperazine), BZP (benzylpiperazine) and MDPBP (3,4-methylenedioxy-α-pyrrolidinobutiophenone). It is important to note that in our study, a preselection of 'legal highs' products was made, with only those labeled as 'bath salts' or 'plant feeders' being acquired.

The number of compounds present in each 'legal high' showed to be inconsistent. It might be thought that the price of the products would be related with the number of

psychoactive compounds, though, according to our results, this is not the case, as samples 8 and 9 containing only one active ingredient have roughly the same retail price (37 €/g of product) as, for example, sample 1 that has five different active compounds (36 €/g of product). On the other hand, according to Table 1, it should be noted that there is a considerable difference between the price of powders and tablets products, as tablets were about half the price compared with the powders. This variability could be related to the product purity, leading us to consider the hypothesis that the powders are more potent than the products sold as tablets. Comparing the chromatographic profile of a powder and a tablet, in the same concentration and prepared in same conditions, it was found that the amount of cathinones in powder samples is significantly higher, with the chromatographic signal of the tablet samples being about two to four times lower. This finding associated with the fact that the powders are sold at a significantly higher price, gives emphasis to the hypothesis of being actually more potent products.

Great evidence resulting from chemical characterization studies of commercial 'legal highs' products is consistent with a huge variability and qualitative inconsistency. Color variation in products with the same trade name was initially found, raising the possibility of a different composition. This hypothesis was supported through the chemical analysis of our suspicious samples ('Bloom', 'Rush' and 'Blow'), revealing differences in their compositions. Even in cases where sample colors were similar, clearly distinct compositions were found, particularly in samples of 'Bliss' (10, 11 and 12), 'Charlie' (13, 14 and 15) and 'Kick' (19, 20 and 21). The analyses of two samples of 'Rush' and three samples of 'Kick', acquired in stores with the same name but located in different places, showed an utterly different composition (see Supplementary Figure S1). Furthermore, the same product ('Kick') sold in distinct 'smartshops' located in the same city presented a different composition. Our results also revealed that the same mixture could also be sold as products with different trade names, as is the case of samples 6, 20 and 21 (Table 2). This suggests that the distributors are unaware of what they are actually selling, being only responsible for packaging and distribution. In accordance, the study performed by Zuba and Byrska (2013) showed a high variability in the content of the products, having detected ten different qualitative compositions in the 'legal high' products named 'Coco', with some products containing a single component, whereas others were mixtures. Even when the name was the same and packages were identical, the content was different. This variability could be explained by the fact that the analyzed products belong to different lots, as happened with samples 13 and 14 ('Charlie'). However, there are different lots whose composition is the same, as for example samples 11 and 12 ('Bliss') or 20 and 21 ('Kick'). Nonetheless, it is not possible to draw further conclusions, since the lot number is unavailable for the majority of the analyzed samples, which corroborates the carelessness in the handling of these products.

In addition to the qualitative variability exploited above, in most cases, it was also noted inconsistency between the 'legal high' composition obtained by GC–MS and NMR analyses and the one labeled on the package. For instance, the '100 % ketones' composition of some samples labeled as such was not confirmed after analysis (e.g., sample five possesses cathinones and caffeine). This type of discrepancy has also been verified by other researchers (Baron et al. 2011; Brandt et al. 2010; Davies et al. 2012).

Variations in the contents of similarly labeled products containing psychoactive substances of variable potency and adverse effects are a serious threat for users and increase the risk of acute harm and toxicity associated with their use. This inconsistency also hampers the assessment of the clinical state of the patient and consequently the appropriate treatment. When one substance is replaced by another or by a mixture or the amount is higher than that labeled, the ensuing effects can be significantly different. This variability could affect the onset and duration of action, the multiplicity of effects and respective interactions, among others, which could ultimately lead to severe intoxications. All these discrepancies reported throughout the study corroborate the lack of control associated with these new substances, showing unequivocally their hazardous nature. Moreover, it is common for users to consume, deliberately or not, mixture of 'legal highs' in order to enhance their effects, making them even more unpredictable, which in turn hampers the evaluation of the risk of toxicity and injury associated with this type of consumption.

The liver is a remarkably vulnerable target for the toxicity of amphetamine-type compounds toxicity because these drugs are extensively metabolised in this organ generating very reactive species, which may induce oxidative damage that results in hepatocytes death. Other mechanisms have been proposed including the oxidation of biogenic catecholamines massively released after drug consumption that can promote oxidative stress leading to hepatic cell death; mitochondrial impairment and apoptosis; drug-induced hyperthermia, which may aggravate the drug direct oxidative effects in the liver cell; and drug–drug interactions from polydrug abuse (reviewed in Carvalho et al. 2010, 2012). In view of structural and pharmacological mechanism of action similarities between amphetamines and cathinone derivatives, and the fact that these drugs are also extensively metabolized in the liver (Meyer et al. 2010; Khreit et al. 2013; Kamata et al. 2006; Zaitsu et al. 2009; Valente et al. 2014) and hyperthermia is a toxicological effect that has been associated with the consumption of different

cathinone derivatives (Borek and Holstege 2012; Fröhlich et al. 2011; Garrett and Sweeney 2010; Levine et al. 2013; Lusthof et al. 2011; Penders et al. 2012; Regunath et al. 2012; Rojek et al. 2012; Warrick et al. 2012; Wikstrom et al. 2010; Valente et al. 2014), we can hypothesize that identical mechanisms may be involved in the hepatotoxicity of these emergent NPS. However, the loophole to this kind of information is still huge.

In this work, we reveal, for the first time, the potential hepatotoxicity of 'legal highs' containing cathinone derivatives. Pentedrone and MDPV proved to be the most powerful hepatotoxic individual agents, closely followed by MDMA, meaning that these two synthetic cathinones and MDMA have similar potencies. Although EC_{50} values of pentedrone and MDPV were not significantly different, the first derivative appears to be more cytotoxic at high concentrations, whereas MDPV showed a higher cytotoxicity at lower concentrations. Due to the presence of the *N*-pyrrolidinyl ring (Fig. 1), MDPV is more lipophilic than other cathinones, showing greater ability to cross barriers (Coppola and Mondola 2012a, b), which may in part explain the high cytotoxic effects observed for MDPV at lower concentrations. In contrast, 4-MEC and methylone presented the least hepatotoxic profile, with EC_{50} values significantly higher than MDMA, being identified a less effective individual drugs to induce cell death, when compared to MDMA in the present experimental model.

It could be argued that the observed hepatotoxic effects occur at relatively high concentrations of the tested drugs when compared to the low micromolar concentrations found in blood of fatal intoxication victims (Wood et al. 2010; Lusthof et al. 2011; Spiller et al. 2011; Kovacs et al. 2012; Rojek et al. 2012; Carbone et al. 2013). However, similarly to other drugs like MDMA, whose levels were found to be up to 18 times higher in the liver (Garcia-Repetto et al. 2003; de Letter et al. 2006), the drug concentrations that the hepatocytes are actually exposed to may be much higher than those found in blood.

Drug abuse commonly takes place in combination, and therefore, a more realistic scenario is to investigate combinations of abused agents. Furthermore, as we have demonstrated in this work, drugs available in 'smartshops' are rarely pure, so the possibility of contamination or mixture of cathinones is more realistic. The in vivo relevance of such interactions can be clearly demonstrated by several reports of human intoxications involving cathinone derivatives (Warrick et al. 2012; Boulanger-Gobeil et al. 2012). For this reason, we also tested two commercial 'plant feeders' samples, 'Blow' (sample 16) that is composed of 4-MEC, MDPV and 3-MEC (minor component), and 'Bloom' (sample 1) composed of pentedrone, 4-MEC, methylone (as major components), isopentedrone and dimethocaine (as minor components, present in <1 %) (Table 2). Of note, 3-MEC and isopentedrone are most likely byproducts of synthesis since they are also present in standards. These two commercial mixtures showed hepatotoxic effects similar to MDMA. The use of CA and IA models to predict mixture effects requires a complete characterization of the concentration–response curves of individual mixture components in terms of shape, position and maximal effects. The MTT assay proved to be a successful method to meet these requirements, allowing high throughput with minimal variability and complete curves covering a wide concentration–response range. In our study, both mathematical models adopted for the prediction of mixture effects seem to be appropriate for the assessment of additive joint effects of cathinone mixtures tested in this in vitro model. As in our results, CA and IA models often generate undistinguishable additivity expectation (Payne et al. 2000, 2001). In the case of the 'Blow' mixture, at higher concentrations, the predicted curves are slightly displaced to the right, revealing a weak synergistic effect. Due to structural similarity between the tested agents, it is expected that they use alike pharmacological and detoxification pathways, possibly with competition for the same metabolic enzymes, and thus interacting with the metabolism of each other, which could explain the deviation from the additivity. Notwithstanding, we acknowledge the fact that other substances that were not detected, and consequently not considered in the calculations, might be present in the tested mixture. Based on the additivity postulations, these substances, even when present at undetectable and ineffective concentrations, can contribute significantly to the overall mixture toxicity (Silva et al. 2002, 2013b; Dias da Silva et al. 2013b). The effective demonstrations of additivity unequivocally heighten concerns about health risks.

In synopsis, these data contribute to increase the consciousness on 'legal highs' real composition and disclose the health risks posed by NPS. GC–MS, in combination with NMR, was shown to be a powerful tool for the unequivocal identification of a great variety of NPS in products sold as 'plant feeders' at 'smartshops'. The observed inconsistency in both qualitative and quantitative compositions corresponds to a high risk factor in the NPS phenomenon and leads to serious health concerns as users are unaware of active dose, time of duration and even pharmacological effects. Moreover, the present study demonstrates, for the first time, that synthetic cathinone mixtures exhibit cytotoxic potential corresponding to those of its individual components, detaining thus additive effects. In view of these results, the evaluation of mixture effects of synthetic cathinones is extremely important from a toxicological point of view, since most 'legal highs' users, consciously or not, take a wide variety of cathinones and other substances on a single session. Understanding the impact of drug interactions can provide valuable information that

may help explain lethal intoxications, as well as facilitate the diagnosis and treatment of nonfatal cases. After being given the first step for assessing the synthetic cathinones hepatotoxicity, additional studies on the molecular mechanisms by which synthetic cathinones cause liver damage are warranted in order to develop risk management procedures to decrease their morbidity and mortality.

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