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Screening of herbal extracts for activation of the human peroxisome proliferator-activated receptor

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The peroxisome proliferator-activated receptors play a pivotal role in metazoan lipid and glucose homeostasis. Synthetic activators of PPARα (fibrates) and PPARγ (glitazones) are therefore widely used for treatment of dislipidemia and diabetes, respectively. There is growing evidence for herbal compounds to influence nuclear receptor signalling e.g. the PPARs. We recently reported carnosic acid and carnosol, both being diterpenes found in the labiate herbs sage and rosemary, to be activators of PPARγ. The subsequent screening of a variety of ethanolic extracts, obtained from traditionally used herbs, for PPAR activation, led to an exceptionally high hit rate. Among 52 extracts nearly the half significantly activated PPARγ and 14 activated PPARα in addition, whereas three of them were pan-PPAR activators, which also activated PPARδ. The most active extracts, for which a concentration dependent effect could be shown, were the extracts of *Alisma plantago aquatica* (ze xie/european waterplantain), *Catharanthus roseus* (madagascar periwinkle), *Acorus calamus* (sweet calamus), *Euphorbia balsamifera* (balsam spurge), *Jatropha curcas* (barbados nut), *Origanum majorana* (marjoram), *Zea mays* (corn silk), *Capsicum frutescens* (chilli) and *Urtica dioica* (stinging nettle). The results of the present study provide a possible rationale for the traditional use of many herbs as antidiabetics.

1. Introduction

Peroxisome proliferator – activated receptors (PPAR) are nuclear receptors playing a crucial role in lipid and glucose homeostasis. Being activated by free fatty acids as endogenous *bona fide* ligands, the three PPAR subtypes α, γ and δ (which is also referred to as β) function as lipid sensor in metazoan organisms (Desvergne and Wahli 1999).

Being activated, PPAR mainly acts as a transcription factor, which after binding with the respective response elements on the DNA, enhances the expression of genes of primary metabolism, leading to the production of e.g. lipoproteinlipase (LPL) or membranous glucose transporters (GLUT), allowing thus cellular adaptation of energy consumption to the nutrients supply. The diversity in the effectiveness of the three PPAR subtypes is mainly attributed to the differential tissue expression of PPAR. PPARα, being mainly expressed in the liver, leads to an increase in hepatic fatty acid uptake from circulating lipoprotein, resulting in lower serum levels of LDL. This effect is clinically used in the fibrate type of lipid lowering drugs. PPARγ however, with major expression in adipose tissue, leads to increased cellular uptake and storage of both free fatty acids and glucose in the respective tissue. By lowering free fatty acid and adipocyte mediator serum levels like TNFα, PPARγ activation overcomes lipotoxicity and insulin resistance resulting in an increased cellular glucose uptake, an effect which is taken benefit of in the treatment of type 2 diabetes with glitazone type of PPARγ agonists.

Many herbs are traditionally used over centuries for treatment of diabetes. Because of the growing evidence for herbal compounds to influence nuclear receptor signalling e.g. the PPARs (Huang et al. 2005; Rau et al. 2006), a library of traditionally used herbal extracts in diabetes therapy has been screened in an explorative approach for their effect on PPAR.

2. Investigations, results and discussion

Fifty two herbs, selected on the base of their traditional use in the treatment of diabetes in different cultural areas of Africa, America, China or Europe (Hiller and Melzig 2003), have been tested *in vitro* for their potential to activate PPAR. For nearly half of these traditionally used herbs both preclinical and clinical evidence for either glucose or lipid lowering efficacy is provided in literature. Our investigation revealed that PPAR agonistic principles are inherent in many of the herbal extracts tested, since a statistically significant activation of PPAR was detected in roughly half of the test candidates (see Table). While all the 25 hits of this screening showed PPARγ agonistic activity (Fig. 1), only the half (fourteen) displayed in addition a significant activation of PPARα (Fig. 1) and only three did also moderately activate PPARδ (*Theobroma c., Catharanthus r. and Jatropha c.*). However none of the

Table: Botanical and vernacular names, plant family, drug names (indicating the extracted plant part), DER (drug extract ratio) and PPAR subtype activation of the selected herbs

Name	Vernacular	Family	Drug	DER	PPAR-activator
Alisma plantago-aquatica L.	ze xie	Alismataceae	Alismatis rhizoma[1]	11.9	α/γ
Allium cepa L.	onion	Alliaceae	Allii cepae bulbus[2]	2.0	no
Allium sativum L.	garlic	„	Allii sativi bulbus[3]	5.5	no
Allium ursinum L.	bear garlic	„	Allii ursini herba[4]	3.7	no
Angelica archangelica L.	angelica	Apiaceae	Angelicae radix[4]	4.7	γ
Daucus carota L.	carrot	„	Dauci carotae flos[s]	4.5	no
Catharanthus roseus (L.) G. Don	madagascar periwinkle	Apocynaceae	Vincae roseae herba[6]	5.5	α/γ/δ
Ilex paraguariensis St.-Hil.	mate	Aquifoliaceae	Mate folium[4]	3.2	no
Acorus calamus L.	calamus	Araceae	Calami rhizoma[7]	6.1	α/γ
Eleutherococcus senticosus (Rupr. & Maxim.) Maxim.	siberian ginseng	Araliaceae ginseng	Eleutherococci radix[4]	20.1	α/γ
Panax ginseng C. Meyer	ginseng	„	Ginseng radix[4]	4.3	$\gamma_{n.s.}$
Calendula officinalis L.	marigold	Asteraceae	Calendulae flos[4]	5.1	no
Cynara scolymus L.	artichoke	„	Cynarae herba[4]	8.6	no
Senecio *fuchsii* C.C. Gmel.	wood ragwort	„	Senecionis f. herba[6]	6.5	no
Silybum marianum (L.) Gartn.	blessed milkthistle	„	Cardui mariae fructus[4]	11.5	α/γ
Tabebuia impetiginosa (Martius ex DC.) Standley	lapacho	Bignoniaceae	Tabebuiae cortex[4]	11.9	γ
Betula pendula Roth.	birch	Betulaceae	Betulae cortex[4]	5.7	no
			Betulae folium[8]	4.4	no
Opuntia ficus-indica (L.) P. Mill.	barbary fig	Cactaceae	Opuntiae f.i. platycladus[6]	5.3	no
Codonopsis pilosula (Franch.) Nannf.	dang sheng	Campanulaceae	Codonopsidis radix[1]	1.8	no
Vaccinium myrtillus L.	whortleberry	Ericaceae	Myrtilli folium[4]	5.2	γ
Euphorbia balsamifera Ait.	balsam spurge	Euphorbiaceae	Euphorbiae stipites[6]	5.2	γ
Jatropha curcas L.	barbados nut	„	Stipes jatrophae[6]	8.0	α/γ/δ
Galega *officinalis* L.	professor-weed	Fabaceae	Galegae herba[4]	6.1	no
Phaseolus vulgaris L.	bean pod	„	Phaseoli fruct.sine sem.[4]	16.3	no
Trigonella foenum-graecum L.	fenugreek	„	Foenugraeci semen[4]	6.6	no
Gentiana lutea L.	gentian	Gentianaceae	Gentianae radix[4]	2.9	γ
Hamamelis virginiana L.	witchhazel	Hamamelidaceae	Hamamelidis folium[4]	4.0	no
Juglans regia L.	walnut	Juglandaceae	Juglandis nucis cortex[7]	5.4	no
Syzygium cumini (L.) Skeels	cumin	Myrtaceae	Cumini fructus[4]	10.8	γ
Lavandula angustifolia P. Mill.	lavender	Lamiaceae	Lamiaceae Lavandulae flos[4]	5.1	γ
Origanum majorana L.	marjoram	„	Majoranae herba[4]	5.7	α/γ
Cinnamomum verum J. Presl.	cinnamon	Lauraceae	Cinnamomi cortex[7]	18.7	γ
Olea europaea L.	olive	Oleaceae	Oleae folium[4]	6.9	γ
Harpagophytum procumbens (Burch.) DC.	devil's claw	Pedaliaceae	Harpagophyti radix[4]	3.8	no
Armeria maritima (P. Mill.) Willd.	thrift seapink	Plumbaginaceae	Armeriae m. herba[6]	14.6	α/γ
Zea *mays* L.	corn silk	Poaceae	Maydis stigmata[8]	21.6	α/γ
Adiantum capillus-veneris L.	common maidenhair	Pteridaceae	Capilli veneris herba[4]	14.9	$\alpha/\gamma_{n.s.}$
Agrimonia eupatoria L.	churchsteeples	Rosaceae	Agrimoniae herba[4]	3.8	y
Sanguisorba ancistroides (Desf.) Ces.	di yu	„	Sanguisorbae radix[1]	4.0	no
Sorbus aucuparia L.	sorb	„	Sorbi aucupariae fruct.[4]	1.7	no
Rosa canina L.	dog rose	„	Rosae pseudofructus[7]	2.8	no
Rubus idaeus L.	raspberry	„	Rubi idaei folium[4]	5.7	α/γ
Coffea *arabica* L.	coffee	Rubiaceae	Coffeae semen tosta[9]	5.4	no
Morinda citrifolia L.	indian mulberry	„	Morindae offic. radix	1.6	no
Citrus aurantium L.	orange	Rutaceae	Aurantii flos[4]	3.4	no
Populus nigra L.	poplar	Salicaceae	Populi gemmae[4]	4.8	γ
Capsicum frutescens L.	chili	Solanaceae	Capsici fructus[4]	4.9	α/γ
Lycium chinense P. Mill	chinese desert-thorn	„	Lycii radicis cortex[7]	10.1	no
Theobroma cacao L.	cacao	Sterculiaceae	Cacao cortex	13.1	α/γ/δ
Camellia sinensis (L.) O. Kuntze	tea	Theaeceae	Theae folium fermenta[10]	4.0	no
Urtica dioica L.	stinging nettle	Urticaceae	Urticae folium[7]	8.3	α/γ
Curcuma longa L.	turmeric	Zingiberaceae	Curcumae longae rhizoma[4]	11.2	$\gamma_{n.s.}$

Origin of drugs [1] local pharmacy; [2] Timbu; [3] Ostmann; [4] Caelo; [5] collected from a population near Frankfurt; [6] supplied by the Botanical Garden Frankfurt; [7] Klenk; [8] Bombastus; [9] Jacobs; [10] ParkLane. Herbs are ordered alphabetically by families. Subscript $_{n.s.}$ indicates effect is not significant. Nomenclature, where applicable, is in accordance to PLANTS Database of the Natural Resources Conservation Service of the US Department of Agriculture (http://plants.usda.gov/index.html)

extracts tested reached the maximum induction observed with the reference, even at the considerably high concentration of 100 mg/L, so it was not possible to further characterise the test extracts by EC_{50} values. Nevertheless it became apparent that the potential for PPARγ activation was generally stronger than that for activation of PPARα in the extracts activating both PPARγ and PPARα, whereas the potential for PPARδ activation, if present, was usually weaker. A significant dose dependency was observed for a subset of seven extracts on PPARγ and for three extracts on PPARα (Fig. 2), but for none of the extracts on PPARδ.

Remarkably, no extract was detected, which selectively activates PPARα alone, if any of the extracts was selective,

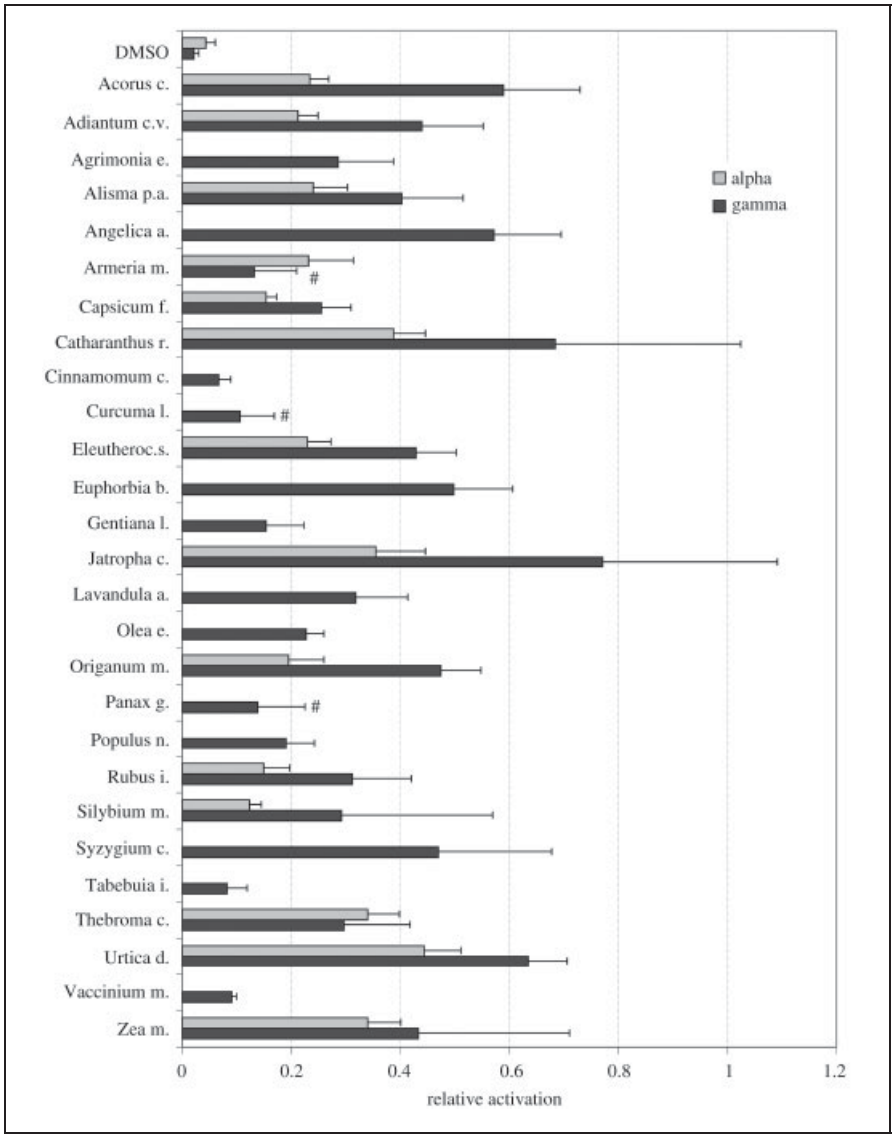

Fig. 1:
Extracts activating PPARγand PPARα
All extracts have been tested at a concentration of 100 mg/L except that of *Cinnamomum offic.*, which has been tested at 30 mg/L and *Curcuma l.*, tested at 20 mg/L. All values ($n \geq 3$) are significantly different from DMSO negative control with at least $P > 95\%$, except # indicating P less than 95%. Relative activation is based on the comparison to the effect of 1 μM pioglitazone chosen as reference for PPARγ and of 100 μM WY14,643 for PPARα. (the activation of reference was set 1). Transactivation of the PPARδ is not shown, as the three positive matches for this receptor subtype did not exceed a relative activation of 30% compared to 1 μM L165,041 chosen as reference. We also included *Armeria m.*, *Curcuma l.* and *Panax g.* in this figure, which owing to a high variance have not proven to be statistically significant. However though being not significant the observed effect is at least of interest for *Armeria m.*, because of its PPARα activity and for *Curcuma l.* because of its low test concentration and the reported PPARγ activity for curcuminoids

than for PPARγ (maybe with the exemption of *Armeria maritima*, since owing to high variance the significance of PPARγ could not be statistically proven). A literature research in PubMed database revealed reports for glucose or lipid lowering efficacy for 15 of the 25 positive matches. To mention only those hits, where a dose dependency has been shown for PPARγ agonistic activity, preclinical evidence is provided for *Catharanthus roseus* (Singh et al. 2001; Nammi et al. 2003; Kaleem et al. 2005), *Acorus calamus* (Parab and Mengi 2002), *Origanum majorana* (Lemhadri et al. 2004), *Capsicum frutescens* (Tolan et al. 2001; Gupta et al. 2002; Lee et al. 2003) and *Urtica dioica* (Bnouham et al. 2003; Farzami et al. 2003) to have hypoglycaemic or hypolipidemic effects, respectively. Moreover the hypoglycaemic effects of *Capsicum f.* are mentioned in a clinical report (Chaiyata et al. 2003) and preclinical data suggest capsaicin to be among the active principles (Tolan et al. 2004). However further investigations are necessary, to prove if these reported effects are PPAR mediated. On the other hand no reports could be found for *Alisma plantago-aquatica*, the spurge family herbs *Euphorbia balsamifera* and *Jatropha curcas* or for Maydis stigmata (from *Zea* mays) suggesting hypoglycaemic or -lipidemic properties. Thus, our investigation might provide a reasonable explanation for the traditional use of these herbal drugs.

Moreover the observations made in the present screening study are in agreement with the current knowledge regarding the mechanism of action of a number of traditionally used herbs. Taking *Cinnamomum verum* as an example, its antidiabetic effect is mainly attributed to phosphorylation of the insulin receptor, leading to an increased and prolonged effect of insulin on its receptor. This perception has been verified in the present screening study, as only a minor PPARγ agonistic activity has been observed, which of course might contribute to the antidiabetic effects of cinnamon, but seems to be to weak to explain the efficacy of doses down to one gram in a human study (Imparl-Radosevich et al. 1998; Khan et al. 2003). Similarly *Galega offic.*, whose biguanids are the precursor of the antidiabetic drug metformin, acts by inhibition of gluconeogenesis (Watanabe 1918; Vuksan and Sievenpiper 2005) and not by activation of PPARγ, as could be shown in this study.

Of course it should be kept clearly in mind, that screening a library, consisting of such complex mixtures as herbal extracts are, might lead to biased results due to non specific effects, increasing the possibility of high false positive rates. Frequent false positive results are reported for example for the screening of inhibitors of membrane receptors and could be expected for herbal extracts, containing typi-

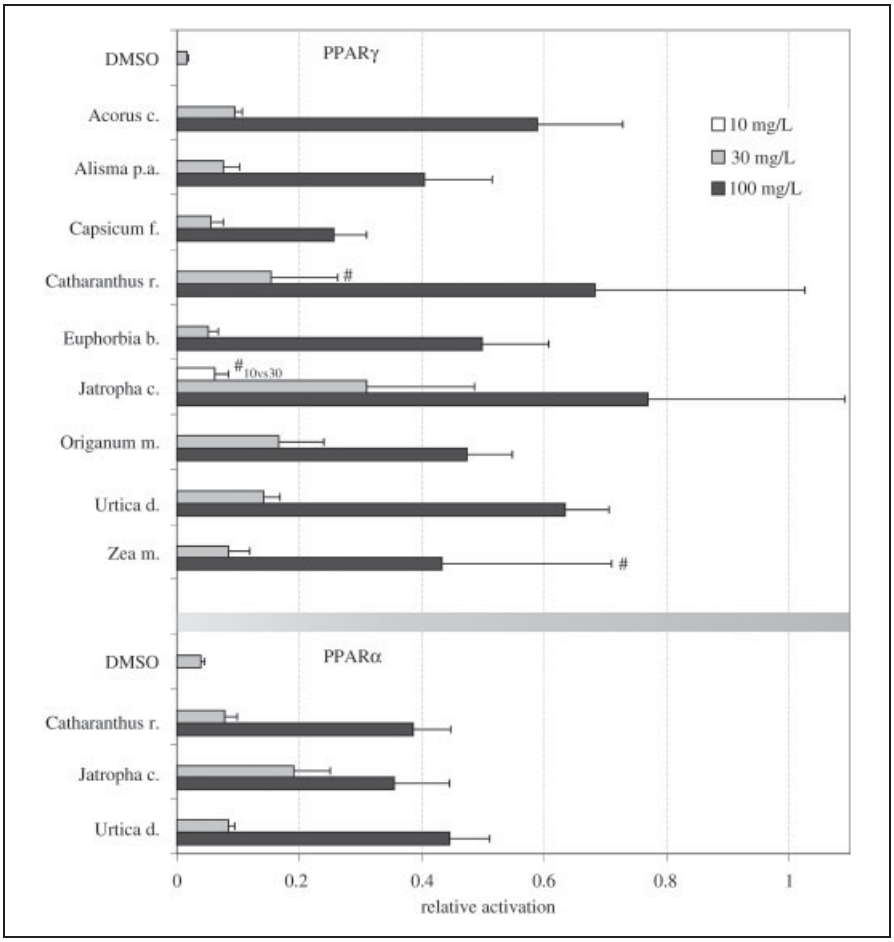

Fig. 2:
Extracts with concentration dependant PPAR agonistic effect
All extracts have been tested at the concentrations indicated. Values ($n \geq 3$) for different concentrations of a given extract are significantly different from DMSO negative control and from each other with at least $P > 95\%$ except # being not significant and $\#_{10vs30}$ indicating no significant difference between 10 mg/L and 30 mg/L value. Relative activation is based on the comparison to the effect of 1 μM pioglitazone chosen as reference for PPARγ (upper part of the figure) or to the effect of 100 μM WY14,643 chosen as reference for PPARα (lower part of the figure), respectively. The activation of both references was set 1. Though having not proven statistically significant concentration dependency, extracts of Catharanthus r. and Zea m. were included in this figure, *Catharanthus r.* for being one of the most potent PPARγ activators at 100 mg/L and *Zea m.* since the value at 30 mg/L concentration is significant, while that of the higher concentration is not, owing to high variance

cal non specific enzyme inhibitors e.g. polyphenols of the catechine or tannin type. In general the rate of false positive hits in a screening test is mainly influenced by the specificity of the mode of action of the assay used. The transactivation assay used in this study, involves a cellular membrane passage and several activation steps of specific target proteins. Furthermore the specificity is additionally guaranteed by using heterologous yeast Gal4-response element and chimeric proteins consisting of DNA-binding-domain fused to human PPAR ligand binding domain.

Thus there could be two reasonable explanations for the exceptionally high hit rate of roughly 50% observed in the present study. The first might be that PPAR agonistic principles are widespread as herbal ingredients, leading to the assumption, that a diet rich in herbs could play an important role in diabetes prevention. The second possible explanation might be that the preselection of herbs on the base of their traditional use, leads to such a high hit rate, which by far exceeds the hit rates observed with typical random high throughput screening tests and even those reported for computer aided drug discovery, hence indicating that there is more than just a grain of truth in traditional use of herbal drugs.

Taken together, the present study suggests for a variety of traditionally used herbs a significant activation of PPAR, however further investigations will have to prove to what extent this mechanism of action contributes to the effect of these herbs. Furthermore a screening approach has been used in this study, which is commonly estimated for being critical to be applied in testing more than one compound at once. However, in combination with a reasonable preselection the transactivation assay could lead to utilisable hits comparable both in time and convenience to established methods. Considering the complex chemistry of herbal compounds, the bioengineered production for example in callus culture of the respective herbs might lead to a sufficiently pure and economic production of isolated herbal substances and thus might provide a basis for a number of new compounds of natural origin with PPAR activity.

3. Experimental

The reference compounds as well as the extract preparation and propagation of the assay have been described elsewhere (Rau et al. 2006). In short, all extracts have been extracted with 80% ethanol using an ultra-turrax after previous incubation for at least two days in a dark glass bottle at room temperature. Afterwards the extracts have been cleared by filtering and evaporated to dryness at reduced pressure and 40 °C. Drug extract ratios have been calculated on basis of the dry mass of the crude drugs and the yield of extract. A voucher specimen of each drug has been deposited at the Institute of Pharmaceutical Chemistry, Johann Wolfgang Goethe University Frankfurt, Max-von-Laue-9, 60438 Frankfurt/Germany. The stock solution was prepared by dissolving all dry extracts in dimethylsulfoxide to about 10%. For testing the stock solution has been diluted with DMEM cell culture medium at least 1 : 1000, so that the final incubation solution had a concentration of 0.1% DMSO and a maximum concentration of 100 mg/L of the respective extract. If the extract was toxic to Cos7 cells, at this concentration, we chose a lower test concentration, which was tolerated by the cells. Cell culture was carried out using Cos7 cells maintained in DMEM at 10% CO_2 and 37 °C. The cells were seeded at a density of 30,000 per well in 96 well cell culture plates one day before transfection. Transfection was carried out with lipofectamine2000, a Gal4-driven luciferase reporter plasmid (pFR-Luc/Stratagene), the ligand binding domain of the respective human PPAR subtype cloned downstream of the Gal4-binding domain in the pFA-CMV/Stratagene expression vector and pRL-SV40/Promega for normalisation. Four hours after transfection, medium was changed to incubation medium, containing an appropriate extract concentration. Induction of the luciferase reporter gene was measured using DualGlo Luciferase Reporter System/Promega and a GENios Pro luminometer/Tecan according to the manufacturer's protocol the following day.

The Table indicates name, drug name, vernacular name, drug extract ratio (DER) and origin of the drug for each of the extracts tested.

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