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Ethnobotany, chemical constituents and biological activities of the flowers of *Hydnora abyssinica* A.Br. (Hydnoraceae)

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Hydnora abyssinica A.Br. (Hydnoraceae), a holoparasitic herb, is for the first time recorded for Abyan governorate of South Yemen. Flowers of this species were studied for their ethnobotanical, biological and chemical properties for the first time. In South Yemen, they are traditionally used as wild food and to cure stomach diseases, gastric ulcer and cancer. Phytochemical analysis of the extracts showed the presence of terpenes, tannins, phenols, and flavonoids. The volatile components of the air-dried powdered flowers were identified using a static headspace GC/MS analysis as acetic acid, ethyl acetate, sabinene, α -terpinene, (+)-*D*-limonene and γ -terpinene. These volatile compounds that characterize the odor and taste of the flowers were detected for the first time in a species of the family Hydnoraceae. The flowers were extracted by *n*-hexane, dichloromethane, ethyl acetate, ethanol, methanol and water. With exception of the water extract all extracts demonstrated activities against Gram-positive bacteria as well as remarkable radical scavenging activities in DPPH assay. Ethyl acetate, methanol and water extracts exhibited good antifungal activities. The cytotoxic activity of the extracts against FL cells, measured in neutral red assay, was only weak ($IC_{50} > 500 \mu\text{g/mL}$). The results justify the traditional use of the flowers of *Hydnora abyssinica* in South Yemen.

1. Introduction

The small family Hydnoraceae comprises two holoparasitic genera: *Hydnora* and *Prosopanche*. The genus *Hydnora* is distributed from southern Africa to the southern Arabian Peninsula, and in opposite the genus *Prosopanche* is located in Central and South America (Heywood et al. 2007; Musselman and Visser 1989; Naumann et al. 2013). The hypogeous herb *Hydnora abyssinica* A.Br., frequently referred to by its younger synonym *H. johannis* Becc. (African Plant Database 2015), is the most widespread species of the genus and is distributed from southern Africa, across East Africa to the Arabian Peninsula (Collenette 1999; Musselman 1992, 2000; Musselman and Visser 1987, 1989; Visser and Musselman 1986; Wood 1997), where it is the only representative of that genus (Musselman 1996).

Most previous studies were focused on botany and systematics of the family Hydnoraceae (Naumann et al. 2013; Nickrent et al. 2002), the genus *Hydnora* (Maass and Musselman 2004; Nickrent et al. 1997) and its rare species (Maass and Musselman 2001, 2004; Musselman 1992, 1998, 2000; Musselman and Visser 1987, 1989; Seymour et al. 2009). Only a few studies targeted the biological activities (Bolin et al. 2009; Nethathe and Ndip 2011; Saadabi and Ayoub 2009; Sparg et al. 2000; Yagi et al. 2011, 2012). The previous studies of biological activities of *Hydnora abyssinica* were directed on the antibacterial (Saadabi and Ayoub 2009; Yagi et al. 2012), antifungal (Saadabi and Ayoub 2009) and antischistosomal properties (Sparg et al. 2000). No isolation of chemical components was reported for the genus *Hydnora* except the investigations of Burger et al. (1988) and Yagi et al. (2012). The main chemical constituents of the roots of the plant are recognized as tannins, phenols and flavonoides (Yagi et al. 2012).

In South Yemen, like in other developing countries, traditional medicine including herbal medicine is an important part of health

care (Al-Fatimi 1999, Al-Fatimi et al. 2005, 2007, 2013; Ali et al. 2013; Schopen 1983). In this country, the flowers of *H. abyssinica* have traditionally been used as wild food and to cure stomach diseases, gastric ulcer and cancer. For our investigations, the rare parasite plant *H. abyssinica* was selected based on ethnobotanical information from its native locality and on its specific morphological characters. Whereas all previous reports focused on the roots of the *Hydnora* species including *H. abyssinica*, we report here for the first time about the flowers of the plant.

2. Investigations, results and discussion

2.1. Ethnobotanical survey

In South Yemen, Abyan province, in the districts of Lawdar and Dathina, the people collect the flowers of *Hydnora abyssinica* after the rain and thunder. They think that thunder after the rain is responsible for the emerging of the plant flowers that grow on the roots of *Acacia* species, mostly on *Acacia tortilis* (Forssk.) Hayne. The inhabitants eat the flowers of wild plants as food fresh or grilled. For medicinal therapy they grind the dried flowers, apply them with milk or water to cure different stomach diseases, gastric ulcer and cancer. The collected flowers form a distally 4-lobed, fleshy, outside orange-brownish, inside reddish to orange or pinkish tube of up to 12 cm length and 3–4 cm diameter; the apically hooded lobes are 2.5–3.9 cm long and 1.5–3.9 cm wide (Fig. 1), whereas the roots and the subterranean fleshy, subglobular fruits are not used in South Yemen. The Arabic local names of the plant in Lawdar and Dathina are: Nabeekh, Fateekh and Tarateef. They refer to the growth type of the plant emerging from the soil surface.

Traditional medicinal use of roots or fruits of this parasitic plant is reported for different countries (Belayneh et al. 2012; Miller and Morris 1988; Yagi et al. 2012). In Sudan the roots are traditionally



Fig. 1: *Hydnora abyssinica*. A: Flower emerging from soil shortly before anthesis; B: flowers dug out from soil before anthesis at different stages of development; C: longitudinally sectioned flower at anthesis, the four perianth lobes free, os = osmophores, an = anthers, st = stigma, ov = ovary; D: flower at anthesis; E: flower with perianth removed above the 4-lobed stigma; F: flower with perianth removed at the level of the anthers and sectioned longitudinally offering free view down to the stigma. – Scale bars (in B and C) = 10 cm.

used for the treatment of dysentery, diarrhea, cholera and swelling tonsillitis (Yagi et al. 2012). In Oman, the fruits but not the roots of *H. abyssinica* are used as food and as tanning agent (Miller and Morris 1988). In Eastern Ethiopia, the plant is used for treatment of haemorrhage, diarrhea, wound and mouth infections (Belayneh et al. 2012). On the contrary, in South Yemen we documented the use of the *H. abyssinica* flowers as wild food and folk medicine.

2.2. Botanical study

The systematic position of the family Hydnoraceae was long disputed and it was either considered as allied with the holoparasitic family Rafflesiaceae, or otherwise with the non-parasitic Aristolochiaceae. Molecular phylogenetic analysis by Nickrent et al. (2002) and Naumann et al. (2013) confirmed the close affinity with the Aristolochiaceae and their placement in the early diverging angiosperm order Piperales.

The species of *Hydnora* are all chiefly hypogaeous, root-parasitic herbs, with an extremely reduced vegetative body, which has no leaves or bracts and consists of a massive, fleshy horizontal rhizome like root covered with haustorial roots as wartlike outgrowth (Musselman and Visser 1989; Nickrent et al. 2002). The actinomorphic flowers are solitary, arise directly from the horizontal roots and are the only part of the plant appearing from the soil above ground (Bolin et al. 2009; Fig. 1). The subglobose fruit develops hypogaeously and finally splits irregularly, releasing numerous seeds below ground. Currently, seven species in two lineages are recognised in the genus *Hydnora*. One lineage includes the species parasitising plants of the families Burseraceae and Fabaceae,

which are, besides the most widespread *H. abyssinica* treated here (Musselman and Visser 1987, 1989; Visser and Musselman 1986), *H. esculenta* Jum. & H. Perrier, *H. sinandevu* Beentje & Q. Luke (Beentje and Luke 2001). The other lineage includes those species parasitising exclusively on species of the genus *Euphorbia* (Euphorbiaceae), *H. africana* Thunb., *H. longicollis* Welw., *H. triceps* Drège & E. Mey. (Maass and Musselman 2004; Tennakoon et al. 2007) and *H. visseri* Bolin, E. Maass & Musselman (Musselman and Visser 1989; updates by Bolin et al. 2011).

Reports of *H. africana* from Dhofar, South Oman (Miller and Morris 1988), are erroneous for *H. abyssinica* (Musselman 1996 under *H. johannis*). In the Arabian Peninsula, *H. abyssinica* is restricted to the southwestern part (South West Saudi Arabia, North and South Yemen and South Oman). Due to their chiefly hypogaeous existence, the species of the genus are rather inconspicuous and their distribution, and in particular their frequency of occurrence, is probably incompletely known. In the flora of Yemen, *Hydnora abyssinica* was known so far from the governorates of Taiz (Wood 1997), Ibb (Al-Khulaidi 2000) and Lahj (Deflers 1895). The first author collected the species also in the governorate of Abyan, in the districts of Al-Awadhel (Lawder) and Dathina, and this is its first report for the Abyan governorate. We observed that the roots attached with *H. abyssinica* were from *Acacia tortilis*.

2.3. Chemical investigation of the dried flowers of *Hydnora abyssinica*

2.3.1. Identification of volatile components by static headspace GC/MS analysis

Static headspace GC/MS analysis was used to examine the volatile organic compounds released by flowers of *H. abyssinica*. The compounds were identified on the basis of their EI mass spectra compared with the corresponding data in the NIST 11, the FFNSC2 library (Shimadzu) and the MassFinder 4.0 software which also consider the Kovats retention indices.

Table 1: Relative composition of volatile components of the powder of *H. abyssinica* flowers determined using Static Headspace GC/MS Analysis

No.	Compound	Retention Time (min)	Relative Composition (%)
1	Acetic acid	2.48	69.9
2	Ethyl acetate	2.64	7.0
3	Sabinene	7.42	8.7
4	α -Terpinene	8.12	3.8
5	D-Limonene	8.32	4.4
6	γ -Terpinene	8.80	6.2

In total, 6 volatile compounds were identified (Table 1). Two oxygenated non-terpene volatile compounds dominated the volatile blend with 77 %: acetic acid (69.9 %) and ethyl acetate (7.0 %). The amount of the remaining four hydrocarbon monoterpenes was 23 %: sabinene (8.7 %), *D*-limonene (4.4 %), α -terpinene (3.8 %) and γ -terpinene (6.2 %). Possibly due to lack of photosynthesis, no oxygenated terpenes could be found.

The dominant compound acetic acid is the general biosynthetic precursor of terpenes in plants (Evans 2002). Acetic acid was found as major compound among other natural products in Cheddar cheese (Gogus et al. 2006). Possibly, this major amount of acetic acid/ethyl acetate identified in the flower is responsible for the cheese like taste of the flower of *H. abyssinica*. α -Terpinene is the intermediate precursor leading to γ -terpinene (Alonso and Croteau 1991) and γ -terpinene those for p-cymene in flowers of other non-parasitic plants (Aschmann et al. 2011). However, p-cymene is not formed in *H. abyssinica* flowers due to the absence of photosynthesis. The monoterpenes α - and γ -terpinene have a cyclohexadiene ring structure similar to limonene and they also smell like lemon (Rudbäck et al. 2012; Tomiyama et al. 2012). They are

used as flavoring additives in foods and beverages, as fragrances in cosmetics, and as scent in household products (Gomes-Carneiro et al. 2005). As well as many monoterpenes which function as odor components of plant flowers (Evans 2002), the detected volatile components are responsible for the odor of the flowers and may act as pheromones to attract insects.

The identified six volatile compounds were found for the first time in a species of *Hydnoraceae*. In opposite to our results, Burger et al. (1988) found other 31 odor volatile components in the flowers of *H. africana* grown on Euphorbiaceae. This difference might be in relation to the different studied *Hydnora* species, the geographical origin and the host plant.

2.3.2. Phytochemical screening of the extracts

Phytochemical screening of the different extracts of *H. abyssinica* flowers revealed the presence of phenols like flavonoides and tannins in the polar extracts (ethyl acetate, ethanol and methanol), while the nonpolar *n*-hexane and dichloromethane extracts contained lipids, triglycerides, monoterpenes, essential oils and fixed oils. Fatty acids were the main constituents of the nonpolar extracts of the flowers of *H. abyssinica*. By CC of the methanol and the ethyl acetate extract α -linolenic acid, β -sitosterol and catechin could be isolated (detailed results not demonstrated). In a previous study the following compounds have been isolated from the roots: cirsiolol, *trans*-3'-5'-dihydroxy-4'-7'-dimethoxydihydroflavonol, vanillin, protocatechuic acid, catechin, stigmasterol, oleic acid, myristic acid and palmitic acid (Yagi et al. 2011).

The content of tannins justifies the use of *H. abyssinica* as tanning agents in Oman (Miller and Morris 1988) and in East Africa (Leemann 1933). In contrast, in South Yemen the people use the leaves of *Acacia* species, the host of *H. abyssinica*, for this purpose. Phenolics are also main components of the roots (Nethathe and Ndip 2011; Saadabi and Ayoub 2009; Yagi et al. 2012). Probably, the flavonoides are responsible for the pale orange color of the *H. abyssinica* flowers and cause the different colors for each external and internal part of the parasitic plant. Whereas Nethathe and Ndip (2011) described the presence of alkaloids in the roots we could not found alkaloids in the flowers.

2.4. Biological activities

2.4.1. Antimicrobial activity

The results of the antibacterial and antifungal screening of the different extracts of the flowers of *H. abyssinica* against five bacteria and two fungi are summarized in Table 2 (inhibition zones in the agar diffusion assay) and Table 3 (MIC values). An inhibi-

Table 2: *In vitro* antimicrobial activity of the extracts of *H. abyssinica* flowers

Extracts (2 mg/disc)	Zone of inhibition (mm)						
	Gram (+) bacteria			Gram (-) bacteria		Yeasts	
	<i>S.a.</i>	<i>B.s.</i>	<i>M.f.</i>	<i>E.c.</i>	<i>P.a.</i>	<i>C.a.</i>	<i>C.k.</i>
<i>n</i> -Hexane	15	10	-	-	-	10	-
Dichloromethane	15	15	15	10	-	10	10
Ethylacetate	25	15	15	10	10	15	15
Ethanol	15	15	10	-	-	10	10
Methanol	20	15	15	10	10	15	15
Water	10	10	-	8	-	15	15
Ampicillin	26	28	31	NT	NT	NT	NT
Gentamicin	NT	NT	NT	15	18	NT	NT
Nystatin	NT	NT	NT	NT	NT	23	25

S.a.: *Staphylococcus aureus* ATCC 29213; *B.s.*: *Bacillus subtilis* ATCC 6059; *E.c.*: *Escherichia coli* ATCC 25922; *P.a.*: *Pseudomonas aeruginosa* ATCC 27853; *M.f.*: *Micrococcus flavus* SBUG 16; *C.a.*: *Candida albicans* ATCC90028; *C.k.*: *Candida krusei* ATCC 90878; Values are inhibition zone diameter (mm); conc. 2 mg/disc; -: no inhibition; N.T.: not tested; negative controls did not show any activity.

Table 3: MIC values of the antibacterial activity of the extracts of *H. abyssinica* flowers

Extracts	MIC (μ g/disc)	
	<i>Staphylococcus aureus</i>	<i>Bacillus subtilis</i>
<i>n</i> -Hexane	1000	>1000
Dichloromethane	1000	>1000
Ethylacetate	250	>1000
Ethanol	1000	>1000
Methanol	250	>1000

tion zone > 15 mm in the agar diffusion assay was considered as a high antimicrobial activity.

Dichloromethane, ethylacetate, ethanol and methanol extracts exhibited antibacterial activity against all three Gram-positive bacteria. Both ethyl acetate and methanol extract exhibited the strongest inhibitory effect against *Staphylococcus aureus* (MIC: 250 μ g/disc), followed by *n*-hexane, dichloromethane and ethanol extracts (MIC: 1000 μ g/disc) (Table 3). Non extract was remarkable active against the Gram-negative bacteria (Table 2). Ethyl acetate, methanol and water extracts showed moderate activity against the two yeast strains tested, *C. albicans* and *C. krusei*.

Until now, antimicrobial activities have already been shown for root extracts of *H. abyssinica* (Yagi et al. 2012: water extracts!) and *H. africana* (Nethathe and Ndip 2011; Saadabi and Ayoub 2009).

The antimicrobial properties of the extracts can be related to the presence of phenols, flavonoids, tannins and terpenes. The antimicrobial activity of the compounds identified in the flowers of *H. abyssinica* is well known (catechin: Liao et al. 2012; linolenic acid: Fagali and Catalá 2008; sabinene, γ -terpinene: Espinosa-García and Langenheim 1991; Giweli et al. 2012; Tomiyama et al. 2012; α -terpinene: Rudbäck et al. 2012).

2.4.2. Antioxidant activity

The DPPH scavenging activities of *H. abyssinica* extracts are summarized in Table 4. The order of the DPPH scavenging activity at 500 μ g/ml was found to be methanol extract (90.6 %) > ethyl acetate extract (82.2 %) > dichloromethane extract (80.8 %)

Table 4: Free radical scavenging activities of the extracts of *H. abyssinica* flowers

Extracts	Radical scavenging activity (%) of different extract concentrations				
	10 μ g/mL	50 μ g/mL	100 μ g/mL	500 μ g/mL	1000 μ g/mL
Dichloromethane	55.4	58.6	85.2	89.8	92.9
Ethylacetate	68.3	72.8	80.5	82.2	85.5
Ethanol	60.8	74.5	79.4	80.3	81.5
Methanol	78.3	84.5	89.5	90.6	91.5
Water	6.0	17.6	28.29	50.5	69.7
Ascorbic acid	87.0	96.9	96.9	97.1	97.3

(Table 4). Also at a lower concentration of 100 μ g/ml, all tested extracts showed strong antioxidant activities above 80 % except water extracts (Table 4). At the concentration of 50 μ g/mL only ethyl acetate and methanol were active.

The detected radical scavenging activities can be explained by the presence of polyphenolic compounds such as catechins, tannins and flavonoides in the extracts, that are known as strong antioxidant (Evans 2002; Ji et al. 2011). Also the identified volatile compounds α - and γ -terpinene are known as good antioxidant agents (Ozturk 2012; Rudbäck et al. 2012).

2.4.3. Cytotoxic activity

With IC_{50} values between 500 and 600 $\mu\text{g/mL}$ dichloromethane, ethylacetate and methanol extracts show only low or lacking cytotoxicity against FL cells in neutral red assay (IC_{50} value 578.3, 587.8, 535.4 $\mu\text{g/mL}$ resp.). Yagi et al. (2012) evaluated the cytotoxicity of water and ethanol extracts of roots as moderate. By determination of cell number they found IC_{50} values of $> 50 \mu\text{g/mL}$ against a human mouth epidermoid carcinoma cell line (KB cells, determination of cell number) and no effects on a non-cancer human fetal lung cell line (MRC-5). On the other hand, i.m. or p.o. application of pulverized roots (2, 10, or 20 % in food for 8 weeks) or ethanol extract of roots (50, 100, 200 or 400 mg/kg/d for 2 weeks) into rats induced signs of hepatotoxicity. The toxic effects were caused only by higher concentrations and were stronger for the extract than for the powder (Yagi et al. 2011). The results let assume that the ethnomedicinal use of *H. abyssinica* in normal amounts is safe. Possibly the use of flowers and their extracts could be safer than those of roots. Nevertheless *in vivo* investigations also of the flowers are necessary.

2.5. Conclusion

Plants from traditional medicine are a promising source of new drugs, cosmetics and healthy food (Avrelja and Walter 2010; Evans 2002; Gomes-Carneiro et al. 2005). The results show that the flowers of *H. abyssinica* have remarkable antioxidant and moderate antimicrobial properties with weak cytotoxic activity. We assume that the flowers of *H. abyssinica*, powder or extracts can be used safely as a potential source of natural antioxidant agents and for the treatment of antibacterial infections. The results justify the traditional uses of the plant flowers as wild food and ethnomedicine in South Yemen. To the best of our knowledge, our results about the chemical and biological properties of the flowers of *H. abyssinica*, have been not reported before. Further studies should aim on different biological examinations and the chemical isolation, purification and characterization of more active principles of *H. abyssinica*. This would provide a better understanding of the antimicrobial and antioxidant mechanism. These findings highlight that the Yemeni flora yield promising sources for antimicrobial and antioxidant natural products according to the Yemeni Traditional Medicine.

3. Experimental

3.1. Plant material

Flowers of *Hydnora abyssinica* A.Br. were collected in Lawder, Abyan province, South Yemen, in July 2010 at the flowering stage (Fig. 1) from the root of *Acacia tortilis* (Forssk.) Hayne. The plants were identified and authenticated by Dr. N. Kilian (Botanischer Garten und Botanisches Museum Berlin-Dahlem, Freie Universität Berlin) and by Dr. M. Al-Fatimi (Pharmacognosy Department, Aden University). Voucher specimens (MAF-HYD 129) are deposited at the personal herbarium of the first author and in the herbarium of the Botanischer Garten und Botanisches Museum Berlin-Dahlem, Freie Universität Berlin. The study area is situated between Lawder (Al-Awadhel) and Modyah (Dathina), rural districts located in South Yemen, in the east and north of Abyan (Al-Awadhel and Dathina districts) and is an area of dry tropical climate.

3.2. Extraction

The collected flowers of *H. abyssinica* were allowed to air dry and afterwards pulverized in a grinder. Thirty grams of the fine pulverized materials were successively extracted with 300 mL of *n*-hexane, 300 mL of dichloromethane, 300 mL of ethyl acetate, 300 mL of ethanol, 300 mL of methanol and 300 mL of water at room temperature for 8 h. After that the extracts were concentrated under reduced pressure at 40 °C, freeze dried and stored in exsiccator until use.

3.3. Determination of chemical components

3.3.1. Determination of volatile components

The dried flowers of *Hydnora abyssinica* were powdered to determine the volatile components. Static headspace GC-EIMS measurements were performed on a QP-2010 Ultra (Shimadzu Corporation, Kyoto, Japan) by using the following conditions: electron energy 70 eV, detected mass range m/z 50-500; source temperature 200 °C; column: ZB-5MS (Phenomenex, 30 m x 0.25 mm, 0.25 μm film thickness), injector temperature 220 °C, interface temperature 300 °C, carrier gas Helium, column flow 1.02 mL/min, constant flow mode, column temperature program: 40 °C for 1 min,

then raised to 300 °C at a rate of 10 °C/min and then hold on 300 °C for 5 min. Static headspace conditions: split injection (split ratio 1:5), incubation temperature 40 °C; incubation time 30 min; syringe temperature 45 °C; injection volume 500 μL .

3.3.2. Phytochemical screening of the extracts

The screening of the chemical constituents was carried out using chemical reagents and thin layer chromatography (TLC) methods according to the methodology suggested by Wagner (2009).

3.4. Determination of antimicrobial activities

The following bacterial strains were employed: *Staphylococcus aureus* (ATCC 29213), *Bacillus subtilis* (ATCC 6059), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Micrococcus flavus* (SBUG 16). As fungal strains the yeasts *Candida krusei* (ATCC 90878) and *Candida albicans* (ATCC 90028) were used.

The antimicrobial activities of the extracts (2 mg extract per disc) were determined using the agar diffusion method according to Al-Fatimi et al. (2007). Ampicillin, gentamicin and nystatin were used as positive, the solvents as negative controls. Inhibition zone diameters around each of the disc include diameter of the disc (6 mm). An average zone of inhibition was calculated for the three replicates. An inhibition zone of 15 mm or greater was considered as good antimicrobial activity.

Minimal inhibitory concentrations (MICs) were determined by the agar diffusion technique as described by Rajbhandari and Schöpke (1999). The highest concentration of extract tested during the experiment was 1 mg/disc. The MIC corresponds to the lowest concentration of the test extract able to inhibit any visible microbial growth.

3.5. Determination of radical scavenging activity

The free radical scavenging activity was measured by using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay as described by Brand et al. (1995) and Al-Fatimi et al. (2007). The reaction mixture contained 500 μL of test extract and 125 μL of DPPH in ethanol. Different concentrations of test samples (10, 50, 100, 500 and 1000 $\mu\text{g/mL}$) were prepared while the concentration of DPPH was 1 mM in the reaction mixture. After incubation of reaction mixture at 37 °C for 30 min the absorbance was measured at 517 nm. Percent radical scavenging activity was determined by comparison with an ethanol treated control group. Ascorbic acid was used as positive control. The DPPH radical concentration was calculated using the following equation:

$$\text{scavenging effect (\%)} = 100 - \frac{A_0 - A_1}{A_0}$$

where A_0 was the absorbance of the control reaction and A_1 was the absorbance in the presence of the test sample.

3.6. Determination of cytotoxicity

The cytotoxicity was measured by the neutral red uptake assay (Lindl and Bauer 1989; Al-Fatimi et al. 2007) using FL-cells, a human amniotic epithelial cell line. Only living cells are able to take up and accumulate neutral red. Cells were cultivated in a 96-well microtiter plate (10^5 cell/mL EAGLE-MEM, Sifin, Berlin, D, 150 μL per well) at 37 °C in a humidified 5 % carbon dioxide atmosphere. The EAGLE-MEM was completed by glutamine (0.10 g/L), HEPES (2.38 g/L), penicillin G (105 IE/L), streptomycin sulphate (0.10 g/L) and fetal calf serum (Gibco, 80.0 mL/L). After 24 h 50 μL of the extract sample or medium with equal amounts of solvent (control) were added. After a further incubation for 72 h cells were washed three times with phosphate buffered saline solution (PBS). One hundred microliters of neutral red solution (SERVA, 0.3 % in EAGLE-MEM) were added per well. The cells were then incubated for 3 h at 37 °C, followed by another three times washing with PBS. 100 μL of a solution of acetic acid (1 %, v/v) and ethanol (50 %, v/v) in distilled water were added. After shaking for 15 min, the optical density was measured at 540 nm with an ELISA-Reader HT II (Anthos Labtec Instruments Salzburg, A). The mean of four well measurements for each concentration in three independent assays was determined. Etoposide (Alexis Biochemicals) was used as positive control.

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