



Constituents from oak bark (*Quercus robur* L.) inhibit degranulation and allergic mediator release from basophils and mast cells *in vitro*



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ABSTRACT

Ethnopharmacological relevance: Oak bark has been used since ancient times in European ethnomedicine because of its adstringent, antimicrobial and hemostatic features, e.g. as a remedy for the treatment of wounds and skin diseases.

Purpose: Oak bark tannins are considered as bioactive natural products, interacting with surface proteins of mucous membranes and might be beneficial for the treatment of allergic diseases. This study investigated the effect of an oak bark decoction (OBD) and isolated tannin fractions on the degranulation capacity and cytokine/chemokine release from rat basophilic cells and human mast cells *in vitro*, which are essential for the initiation of early- and late-phase allergic reactions.

Methods and methods: By chromatographic separation on Sephadex® LH-20 high- and low-molecular weight tannins were separated from OBD and the tannin composition analyzed by HPLC(DAD)-MSⁿ. Then, the OBD and its fractions were tested in degranulation (β -hexosaminidase activity) of allergen-specific-activated basophilic cells in a photometric assay.

Results: The OBD and the high-molecular tannin fraction showed a dose-dependent inhibition of cell degranulation. Furthermore, the OBD and particularly its high molecular weight tannin fraction exhibited an inhibitory activity on the IL-8-, IL-6- and TNF- α -secretion from stimulated human mast cells, detected and quantified by ELISA.

Conclusion: The OBD and its high-molecular weight tannins revealed an impact on allergic mediator release of basophilic cells and human mast cells and thereby provide a rationale for the topical treatment with OBD preparations.

1. Introduction

Since ancient times, remedies from oak bark have been applied in the European ethnomedicine. The old herbal book of *Leonhard Fuchs* from the 16th century has already described the use of oak bark, because of its adstringent and hemostatic features for the treatment of wounds, against ulcers and gynecological disorders (Fuchs, 2001). Beyond, oak bark was applied in traditional herbal medicine, e.g. as a bath supplement for the treatment of skin diseases, weeping and itching eczema, foot sweat, burns, hemorrhoids and internally for the therapy of mouth rot, heartburn and diarrhea (Blaschek et al., 2016).

According to the European Pharmacopoeia (Ph. Eur. 8, 2014) oak bark can be obtained from three different species, namely *Quercus robur* L., *Q. petrae* (Matt.) Liebl. and *Q. pubescens* WILLD., whereby the bark of 10–15 year old trees is preferentially utilized. Because of its high tannin content (up to 20%; Blaschek et al., 2016), oak bark possesses astringent, desiccating, emplastic, antimicrobial and anti-inflammatory activities, among others (Schilcher et al., 2010). Nowadays oak bark and its hydro-alcoholic extracts are mainly applied in phytotherapy and in anthroposophic medicine (Meyer, 2005), e.g. for the treatment of eczema, scrofula, menorrhagia, haemorrhoidal ailments, varices, and the formation of fissures and rhagades of the skin and mucous

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membranes (Blaschek et al., 2016), as well as skin inflammations and allergic diseases.

Allergies are hypersensitivity reactions of the immune system towards distinct and generally innocuous environmental substances. The latter are classified as allergens and have the capability to bind to immunoglobulin(Ig)E antibodies that are associated with their specific receptors (FcεRI) on the surface of basophils and/or mast cells (Gould and Sutton, 2008). The allergen cross-linking of the IgE-FcεRI complex may initiate an “early-phase” allergic reaction by degranulation of preformed histamine and other soluble mediators, stored in small granules, that challenge a rapid increase of vascular permeability and contraction of smooth muscle cells at the allergen-exposed target organs, especially the nose or the lung (Pearlman et al., 1999). Following activation, mast cells and basophils in parallel release inflammatory mediators such as interleukin(IL)-6 and -8, or tumor necrosis factor(TNF)-α at sites of allergen exposition and thereby orchestrate the late-phase of the allergic reaction. Once recruited, the activated granulocytes and lymphocytes may cause significant damage to the surrounding tissue thereby promoting health risks and severe long-term illnesses, like chronic rhinitis or asthma (Macfarlane et al., 2000). Allopathic target-based treatments of allergic diseases comprise antihistamines, mast cell stabilizers, glucocorticoids, anti-leucotriene agents and bronchodilators that essentially suppress the symptoms of acute- and late-phase allergic reactions through its specific mode of action. Although effective conventional drugs are available, approximately 30% of allergic patients in Europe use complementary therapies, because of therapy failure or to avoid side-effects (Mainardi et al., 2009).

Since little is known from the literature regarding anti-allergic and anti-inflammatory effects of tannin rich herbal preparations, the objective of the current investigation was to monitor the *in vitro* activity of a traditional aqueous oak bark decoction (OBD) on the secretion of allergic mediators from basophilic cells and human mast cells. Furthermore, OBD fractionation yielded low- and high-molecular weight tannin constituents which were also assessed by *in vitro* assays, to target the active principle of the extract.

2. Materials and methods

2.1. Plant material and preparation of the oak bark decoction (OBD)

Oak bark (pH. Eur.) was collected from *Quercus robur* L. and air dried at the Sonnenhof GmbH (Bad Boll, Germany) in March 2012. A voucher specimen was identified by our in-house laboratory via a microscopic investigation (Rahfeld, 2011) and deposited at the WALA Heilmittel GmbH (voucher number: A070352). The dried oak bark was milled (particle size < 5 mm) and 120 g were processed according an official procedure (GHP=German Homoeopathic Pharmacopoeia, 2014, specification: 23 a), comprising addition of water (1200 g) and heating for 30 min at reflux temperature. Afterwards the hot decoction was filtered over a filter cloth. Samples were immediately taken from the fresh decoction and frozen at −80 °C until investigation. The dry matter of the decoction was determined by evaporation of a small aliquot (5 ml) in an open crucible, on a boiling water bath (30 min), then heating in a drying cabinet (3 h, 105 °C) and weighing out after cooling in a desiccator.

2.2. Separation of the low- and high-molecular tannin constituents

Tannin constituents were separated following a literature procedure (Engels et al., 2009). Thus, from the OBD (A, 500 ml; see separation scheme, Fig. 1) the water was distilled off by vacuum rotary evaporation at 38 °C bath temperature. The residue obtained was dried for 1 h *in vacuo* at p < 5 mbar to yield a brown solid (10.64 g, 2.1% dry matter). The latter was treated with an EtOH/acetone mixture (10:1, v/v, 220 ml), sonicated for 5 min and filtered over a G3 glass frit by

vacuum suction and the residue was washed with acetone (50 ml) to yield an insoluble solid (4.31 g, 40.5% of the dry matter). From the unified filtrates the solvent was removed *in vacuo* and a light-brown foam was obtained (B, 6.11 g, 57.4% of the dry matter). From the latter 2.0 g were dissolved in EtOH (96 vol%, 30 ml) and loaded on a Sephadex® LH-20 (GE Healthcare Europe GmbH, Freiburg, Germany) flash column (25.0 g, column dimension: 200 mm×20 mm, i.d.), subsequently preconditioned with water (200 ml), EtOH/water (1/1, v/v, 100 ml) and EtOH (96 vol%, 300 ml). Fractionation was performed by flushing the column with EtOH (96 vol%, 500 ml) and acetone/water (1/1, v/v, 400 ml) under slight pressure. Solvent rotoevaporation and drying of the residues at 38 °C *in vacuo* (p < 5 mbar, 1 h) yielded a beige solid foam (C, 1.32 g, yield: 66.0% calculated on B) and a brown solid (D, 0.57 g, yield: 28.5% calculated on B), from the EtOH and the acetone/water fraction respectively. Both fractions were stored at −80 °C until investigation. For LC-MS analyses samples were dissolved in 10% MeOH/water (v/v) at a concentration of 1 mg/ml.

2.3. RP-HPLC(DAD)-ESI-MSⁿ analyses

Chromatographic analyses were carried out on an Agilent 1200 HPLC system (Agilent Technologies Inc., Palo Alto, USA), equipped with a binary pump, a micro vacuum degasser, an autosampler, a thermostatic column compartment and a UV–VIS diode array detector. A Sunfire® C18-reversed phase column (5 μm particle size, 150×2.1 mm i.d., Waters Corporation, Milford, MA, USA) was used for chromatographic separation at 25 °C and a flow rate of 0.21 ml/min. The UV detection of polyphenolic compounds (Fig. 2 and Table 1) was performed at 280 nm. The mobile phase consisted of HCOOH/H₂O 1/99 (v/v; mobile phase A) and acetonitrile/H₂O 9/1 (v/v; mobile phase B). Starting with 0% B for 5 min, a linear gradient was followed to 12.5% B at 40 min, then increasing to 25% B at 105 min, further increasing to 100% B at 110 min, continuing for 5 min, before re-equilibration to starting conditions. The injection volume of each sample was 10 μl. The LC system was coupled to an HCTultra ion trap (Bruker Daltonik GmbH, D-Bremen) with an ESI source operating in the negative ionization mode applying the following parameters: capillary voltage: +4000 V; dry gas flow (N₂) 9.00 l/min with a capillary temperature of 365 °C; nebulizer pressure 35 psi. Full scan mass spectra (mass range *m/z* 50–2500) of the HPLC eluates were recorded during chromatographic separation yielding [M-H][−] ions. To obtain further structural information, CID experiments were performed. MSⁿ data were acquired in the auto MS/MS mode. The instruments were controlled by Agilent ChemStation (B.01.03 (Agilent, Waldbronn, Germany) and EsquireControl V 6.1 (Bruker Daltonik GmbH, Bremen, Germany) software.

2.4. Cell cultures

RBL-2H3 (rat basophilic cells) cells were obtained from H. Pircher (Institute of Medical Microbiology and Hygiene, Division of Immunology, Freiburg, Germany) and grown in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Karlsruhe, Germany). HMC-1 (human mast cells) cells were kindly provided by J. H. Butterfield (Rochester, MN, USA) and grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen, Karlsruhe, Germany). Each medium was supplemented with 10% heat-inactivated fetal calf serum (FCS; PAA, Coelbe, Germany), 2 mM L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin (Invitrogen, Karlsruhe, Germany) and the cells were cultivated at 37 °C in a humidified incubator at a 5% CO₂/95% air atmosphere. Stock solutions of the dried OBD tannin fractions (B–D) were prepared in dimethyl sulfoxide (DMSO) and passed through a 0.22 μm sterile Nalgene nylon filter. The cell cultures (RBL-2H3 and HMC-1) were incubated with the sterile *Quercus* preparations at 6–100 μg/ml. Adequate amounts of DMSO (sol1-

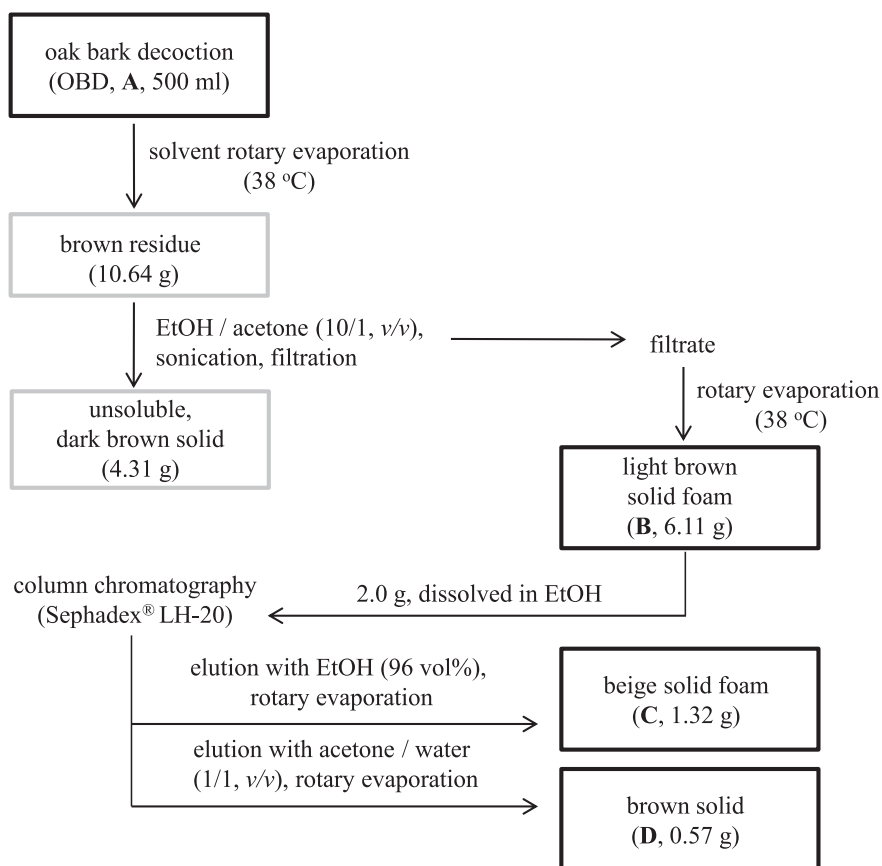


Fig. 1. Separation scheme for low- and high-molecular weight tannins from an OBD.

sol5) were used as controls (see Figs. 4–6). The vehicle DMSO alone (maximal concentration: 10 µl/ml medium) did not exhibit any impact on growth of the cells.

2.5. Assay for cell viability

Cell proliferation was determined using the water soluble tetrazolium (WST)–1 assay (Roche, Mannheim, Germany) according to the manufacturer's instructions. Cytotoxicity was expressed as a cell

viability ratio of treated compared to untreated cells (=100%). A sample was considered cytotoxic, if cell viability was below 70%. As positive cell viability control we used Triton-x 100 (0.5%; Sigma-Aldrich, Taufkirchen, Germany).

2.6. RBL-2H3 activation

For degranulation studies of RBL-2H3 cells a method according to literature was used (Huber et al., 2012). Briefly, 2×10^5 cells were

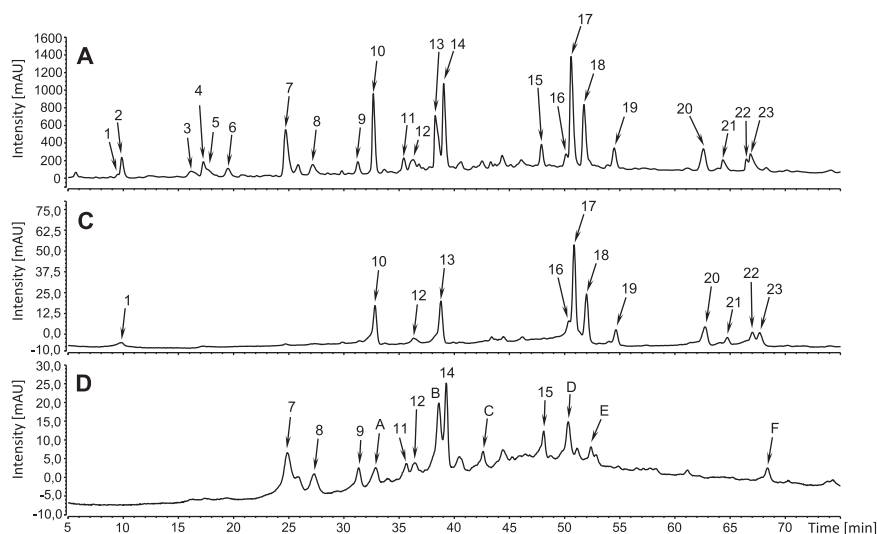


Fig. 2. (A) HPLC-DAD chromatograms at 280 nm of an OBD; (C) Sephadex® fraction, EtOH eluate, containing low-molecular weight tannins; (D) Sephadex® fraction, acetone eluate, containing high-molecular weight tannins (according to separation scheme Fig. 1). Compound assignment based on UV and MS-MSⁿ spectra (see Table 1). The fraction B is not shown because of an identical compound pattern to A.

Table 1

HPLC, UV and MS (ESI, negative ionization mode) data of phenolic compounds detected from an oak bark decoction (OBD).

Compound No.	t_R [min]	Peak Assignment	UV λ_{max} [nm]	MS ⁿ data [m/z]		
				Precursor ion (MS ¹)	MS ⁿ	Reference
1	9.5	gallic acid hexoside	232, 278	331 ^{a)}	MS ² : 169 , MS ³ : 125	Zhao et al. (2013), Duckstein and Stintzing (2011)
2	9.9	gallic acid	232, 272	169 ^{a)}	MS ² : 125	Zhao et al. (2013)
3	16.6	pedunculagin isomer	236, 274 ^{sh}	783 ^{a)}	MS ² : 481 , MS ³ : 437 , 419, 341, 329, 299	Duckstein et al. (2012)
4	17.1	gallic acid quinate	232, 274	687 ^{b)}	MS ² : 343 ^{a)} , MS ³ : 191 , MS ⁴ : 171	
5	17.8	grandinin / roburin E	234, 270 ^{sh}	1065 ^{a)}	MS ² : 1047, 1029, 975 , MS ³ : 931 , MS ⁴ : 913	Jourdes et al. (2011)
6	19.4	mongolinin A	234, 270 ^{sh}	1373 ^{a)}	MS ² : 1329 , MS ³ : 1285, 1039 , MS ⁴ : 995, 869	Ishimaru et al. (1988)
7	24.7	castalagin /vescalagin	238, 270 ^{sh}	933 ^{a)}	MS ² : 631 , MS ³ : 613 , 587, 569, 467	Glabasnia and Hofmann (2006), Jourdes et al. (2011)
8	27.1	procyanidin digallate	236, 276	593 ^{a)}	MS ² : 425 , MS ³ : 407 , MS ⁴ : 285, 282	Bicker et al. (2009)
9	31.1	valoneic acid dilactone	234, 264 ^{sh} , 372	469 ^{a)}	MS ² : 425 , MS ³ : 407 , 300 , MS ⁴ : 271	Mämmelä et al. (2000)
10	32.6	methyl gallic acid hexoside	234, 268	345 ^{a)}	MS ² : 183 , MS ³ : 168, 124	Méndez et al. (2004)
11	35.4	eugenigrandin A/ guajavin B	236, 270 ^{sh}	1221 ^{a)}	MS ² : 915 , MS ³ : 897, 871 , 569 , MS ⁴ : 509	König and Scholz (1994), Tanaka et al. (1992)
12	36.1	procyanidin dimer	236, 278	577 ^{a)}	MS ² : 559, 451, 425 , 407 , 289, MS ³ : 285, 283 , 282, 257, 243, MS ⁴ : 257	Zhao et al. (2013), Duckstein and Stintzing (2011)
13	38.4	(+)-catechin ^{c)}	236, 280	289 ^{a)}	MS ² : 271, 245 , MS ³ : 227, 203 , MS ⁴ : 187, 175, 160	Zhao et al. (2013)
14	39.2	acutissimin A/B	236, 272 ^{sh}	1205 ^{a)}	MS ² : 915 , MS ³ : 897, 871 , 613, 569	Stark et al. (2010)
15	47.7	acutissimin A/B isomer	236, ^{sh} 272	1205 ^{a)}	MS ² : 915 , MS ³ : 897, 871 , 613, 569,	Jourdes et al. (2011)
16, 17	50.5	methyl gallic acid hexoside	234, 270	497 ^{a)}	MS ² : 465, 345 , MS ³ : 183 , MS ⁴ : 168, 124	Lee et al. (2002)
18	51.9	taxifolin hexoside isomers	236, 292	931 ^{b)}	MS ² : 465 ^{a)} , MS ³ : 447, 339, 285 , MS ⁴ : 257, 241, 199, 175	Knittel et al. (2014)
19	54.5		234, 288			
20	62.6		234, 290			
21	64.4	ellagic acid pentoside	252, 360	433 ^{a)}	MS ² : 301 , MS ³ : 300, 257 , MS ⁴ : 229, 185	Fernandes et al. (2011)
22	66.4	ellagic acid rhamnoside	252, 362	447 ^{a)}	MS ² : 300 , MS ³ : 257, 244, 229, MS ⁴ : 201, 184	Mämmelä et al. (2000)
23	67.4	ellagic acid	252, 366	301 ^{a)}	MS ² : 257 , MS ³ : 229, 185	Duckstein and Stintzing (2011), Duckstein et al. (2012)
A	32.9	procyanidin dimer digallate	232, 274	881 ^{a)}	MS ² : 863, 755, 711 , 593, 543, 525, 467, 425, 407 , 289, MS ³ : 285, 283 , 282, MS ⁴ : 257	Agarwal et al. (2007)
B	38.6	catechin ellagic acid hexoside	234, 274	754 ^{a)}	MS ² : 593 , 425, 407, MS ³ : 289 , MS ⁴ : 245, 177	
C	42.6	unknown dihydro-acutissimin	234, 274	1207 ^{a)}	MS ² : 1163, 917 , MS ³ : 873, 855, 793, 721, 667	
D, F	50.3	mongolicinin A/B	234, 274	1175 ^{a)}	MS ² : 873 , 855, MS ³ : 829 , 811, 785, 721, 665	Fernandes et al. 2011
E	52.4	procyanidin dimer monogallate	234, 274	729 ^{a)}	MS ² : 577 , MS ³ : 559, 451, 425, 407 , 289, MS ⁴ : 285, 283, 282, 257	Agarwal et al. (2007)

^a [M-H]⁻.^b [2M-H]⁻; Bold characters indicate the base peaks in the MSⁿ spectra.^c (+)-Catechin was assigned by comparison with an analytical reference standard (Phytolab GmbH & Co. KG, Vestenbergsgreuth, Germany).

incubated with 0.5 µg/ml IgE mAb with antigen specificity for anti-2,4-dinitrophenyl (DNP) (SPE-7; Sigma-Aldrich, Taufkirchen, Germany) for 12–18 h at 37 °C in complete medium with 10% FCS. Non-IgE activated cells served as negative controls. The cells were then washed twice with Tyrode's buffer (130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, and 0.1% bovine serum albumine (BSA) in 100 mM 2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethansulfonsäure (HEPES), pH 7.4; all from Sigma-Aldrich, Germany). Before stimulation (30 min, 37 °C) with 20 ng/ml dinitrophenylated human serum albumin (DNP-HSA; Sigma-Aldrich, Taufkirchen, Germany), cells were treated (10 min, 37 °C) with extracts or fractions from the OBD or DMSO as solvent control as well as with a solution of azelastine as positive inhibition control (10⁻¹¹ M; Sigma-Aldrich, Taufkirchen, Germany).

2.7. β -hexosaminidase release assay

The degranulation activity of mast cells can be assessed by measuring the β -hexosaminidase activity and was conducted according to the literature (Huber et al., 2012). This enzyme is stored in the secretory granules of mast cells and basophilic cells and may be released like histamine. Total β -hexosaminidase was obtained by lysing

the cells with 0.1% Triton-X 100 prior to removing the supernatant for activity assessment. To determine the amount of β -hexosaminidase released by RBL-2H3 cells, 25 µl of supernatant and 50 µl of 1.2 mM β -hexosaminidase substrate (4-nitrophenyl-N-acetyl- β -D-glucosaminide; Sigma-Aldrich, Taufkirchen, Germany) in 0.1 M sodium acetate buffer (pH 4.5) were mixed in 96-well plates and incubated for 2 h at 37 °C. The reaction was completed by adding a 0.4 M glycine buffer (pH 10.7, 150 µl). The reaction product was measured at 405 nm using a microplate reader (Tecan, Crailsheim, Germany), and the relative percentage of β -hexosaminidase release [%] was calculated as follows: [release supernatant]/[(release supernatant+release pellet)] $\times$ 100.

2.8. Activation and measurement of cytokine and chemokine production from human mast cells (HMC-1)

HMC-1 cells (10⁵) were preincubated for 30 min with well-defined levels of test compound, medium alone (positive control for cytokine/chemokine production) or a solution of dexamethasone (10⁻⁵ M, positive control for inhibition; Sigma-Aldrich, Taufkirchen, Germany) followed by an additional 2.5 h activation period with 40 nM of the mitogen phorbol 12-myristate-13-acetate (PMA) and 1 µM of the calcium ionophore A23187 (all from Sigma-Aldrich, Taufkirchen,

Germany). Non-activated cells incubated with medium alone were used as negative controls. Cytokine and chemokine production was quantified in the supernatant of the respective cells using classical ELISA-Technology according to the manufacturer's specification (ebioscience, Frankfurt, Germany). The procedure was previously published by the authors (Huber et al., 2012). Limit of detection for human IL-8, IL-6 and TNF- α were 2.0, 2.0 and 4.0 pg/ml, respectively.

2.9. Data- and statistical analysis

For statistical analysis, data were processed with Microsoft Excel and SPSS software (IBM, Version 22.0, Armonk, New York, USA). Values are presented as mean $\pm$ SD for the indicated number of independent experiments (defined quantity is presented in figure legends). Statistical significance was determined by one-way ANOVA followed by Dunnett's post hoc pairwise comparisons. The data were all adjusted in relation to untreated control cells ($=100\% \pm$ SD). P values < 0.05 are considered as statistically significant.

3. Results

3.1. Separation of tannins from OBD and characterization by LC-MS/MSⁿ

For separation of low- and high-molecular weight tannins from the OBD (A) the latter was evaporated to dryness, the obtained residue dissolved in EtOH/acetone and the soluble material (B) was loaded on Sephadex[®] LH-20 (see separation scheme, Fig. 1). Subsequent elution with EtOH (96 vol%) and acetone/water (1/1, v/v) resulted in two fractions C and D (Fig. 1). For compound elucidation the OBD (A) and fractions B-D were analyzed by RP-HPLC(DAD)-ESI-MSⁿ. The phenolic constituents were ionized in the ESI negative mode and identified by their pseudo-molecular ions [M-H]⁻, their specific MS-fragmentation patterns and UV spectra (for examples, see Fig. S1, Appendix A. Supplementary material). By comparison with literature data and one reference compound ((+)-catechin **13**) 29 phenolic constituents could be tentatively assigned from the OBD and its fractions (see Fig. 2 and Table 1), belonging to the gallotannin-, ellagitannin-, procyanidin- and taxifolin-structure type (see structural formulas, Fig. 3). While in fraction C the low-molecular weight tannins were enriched (mainly the gallotannins **10** and **17**, catechin **13** and taxifolin hexosides **18–20**), in fraction D preferably the high-molecular weight tannins were found (e.g. castalagin/vescalagin **7**, acutissimin **14** and the ellagicatechin **B**), see Fig. 2C and D, respectively. Several of the compounds assigned were found for the first time in *Q. robur* L., e.g. the taxifolin hexosides **18–20**. Until now, the latter were only reported from *Q. mongolica* var. *grosseserrata* (Ishimaru et al., 1988).

3.2. Oak bark tannins inhibit degranulation capacity of basophilic cells

In a first step we investigated the influence of an OBD (A) and its fractions (B-D) on β -hexosaminidase release of IgE/DNP specific-activated basophilic cells in comparison to controls (Fig. 4A). The incubation with antigen-specific IgE molecules alone (pos) increased the degranulation capacity compared to non-activated control cells (neg). The presence of different concentrations of the OBD (0.058–0.58 mg/ml) and its tannin fraction D (12–100 μ g/ml) revealed a dose-dependent inhibition of basophilic degranulation capacity that was comparable to the used dosage of the synthetic anti-allergic compound azelastine (Az). Fractions B and C demonstrate no dose-dependent effect on the parameters monitored. The inhibition of the degranulation capacity of basophils by A and its fraction D was not due to cytotoxicity since no effects on basophilic cell viability was detected in the mentioned concentration range (Fig. 4B).

3.3. Oak bark tannins modulate mediator release of human mast cells

To analyze whether OBD (A) and its fractions (B-D) modulate the release of mediators that orchestrate “late-phase” allergic reactions from human mast cells, we stimulated the cells with PMA/A23187 and analyzed IL-8 (Fig. 5A), IL-6 (Fig. 5B) and TNF- α (Fig. 5C) exposition in the supernatant. The results demonstrate that the OBD diminished the release of all analyzed mediators in a dose-dependent fashion and at high dose the inhibition rate was comparable to the dexamethasone (Dex) levels used. The OBD fractions overall demonstrate inhibitory capacity on cytokine and chemokine secretion from human mast cells, but solely the OBD and fraction D demonstrate a dose-dependent inhibition of IL-8 and IL-6 not provoked by solvent-based DMSO. Cell viability data (Fig. 6) revealed that high concentrations of the investigated oak bark samples reduce viability, however, comparable to values of non-activated control cells (with exception of oak bark decoction 1.7 mg/ml). In conclusion, the results indicate an impact of OBD and its fraction D on allergic mediator release of human mast cells.

4. Discussion

In this study we analyzed the influence of constituents from OBD on mediator release of basophils and mast cells *in vitro*, which are essential for the initiation of early- and late-phase allergic reactions. The results demonstrate that the OBD and its high-molecular weight tannins inhibit the degranulation of basophils and cytokine/chemokine release from mast cells at a level comparable to the synthetic drugs azelastine (antihistamine) and dexamethasone (glucocorticoid).

Tannins of diverse molecular weight were separated by chromatography from OBD (Fig. 1), based on their different interaction with Sephadex[®] LH-20 (Engels et al., 2009) and were later characterized by HPLC-DAD-MSⁿ-analyses (see Figs. 2, 3 and Table 1).

The degranulation effect of the OBD and its tannin fractions was measured by β -hexosaminidase release, since this enzyme is stored in the secretory granules and is simultaneously secreted with histamine. This assay has been widely used for monitoring the capacity of potential new drugs to block basophilic and mast cell activation and degranulation (Ortega Soto and Pecht, 1988; Aketani et al., 2001; Granberg et al., 2001). Mast cells are the main source of early-phase mediators in an allergic reaction. These mediators are formed and stored in cytoplasmic granules (Rivera and Gilfillan, 2006) and are responsible for triggering typical allergic symptoms, such as bronchoconstriction, vasodilatation, increased vascular permeability and exaggerated mucus production (Galli et al., 2005). In the present study dose-dependent inhibitory effects on β -hexosaminidase release of the OBD and its high molecular weight tannins were observed (Fig. 4A). As shown in Fig. 4B, this effect was not due to cytotoxicity (Fig. 4B). In a similar fashion, inhibitory effects of tannins from different plant species on mast cell degranulation (Yamada et al., 2012; Tokura et al., 2005) and histamine release are reported from literature (Kanoh et al., 2000; Kanda et al., 1998). However, to the best of our knowledge, anti-allergic properties of OBD have not been studied before.

To study the release of pro-inflammatory and chemotactic cytokines, e. g. TNF- α , IL-1 β , IL-6, IL-8, and IL-13, *in vitro* models utilizing PMA/A23187 for the activation of mast cells has been described in the literature before (Amin, 2012). The analysis of degranulation and cytokine release was done in different cell types since we would like to test IL-8 and TNF-alpha in combination because it has relevance for the late allergic response. This is not possible in RBL-2H3 cells since these cells do not express solid amounts of IL-8. Second, influence of the used preparations on an antigen-specific (RBL-2H3) and -unspecific (HMC-1) cell system would like to be tested because only RBL-2H3 basophilic cells express an IgE receptor on the surface and the human mast cell line HMC-1 do not. These

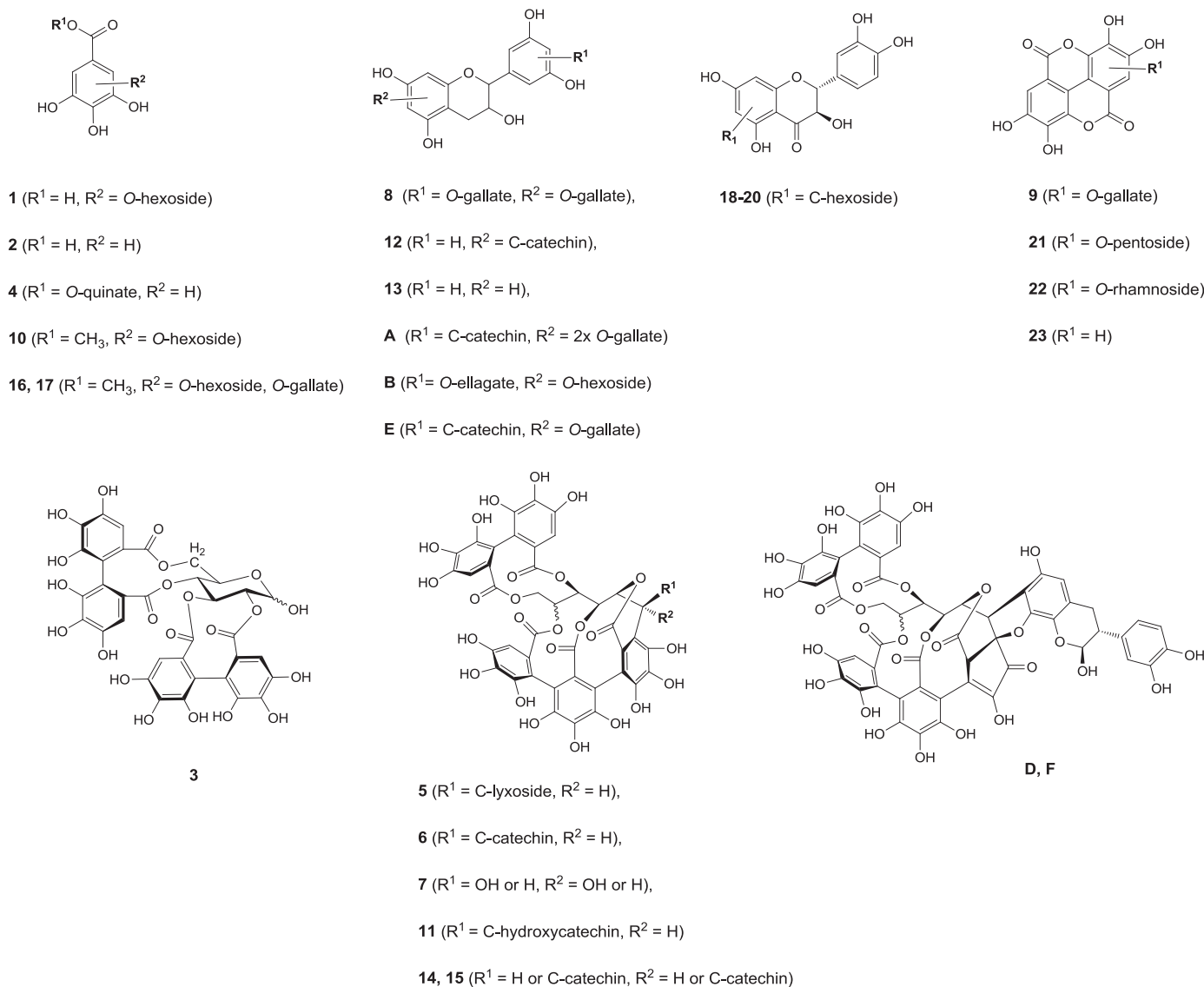


Fig. 3. Tentative structures of assigned compounds from OBD.

mediators have essential effects on permeability and penetrability of vascular endothelium, resulting in an increased migration of inflammatory cells (*e.g.* eosinophils, neutrophils and lymphocytes). In this way, desintegration of healthy tissue and promotion of allergen-specific immunopathology may be induced. The reduction of the aforementioned mediators is considered as an option for therapeutic intervention of late phase allergic symptoms (Amin, 2012). Experiments from the present study demonstrate that the OBD lowers the release of pro-inflammatory cytokines, particularly IL-8, IL-6 and TNF- α , from human mast cells in a dose-dependent manner (Fig. 5). Since the used solvent concentration of DMSO already showed an inhibitory effect on mediator release, only the OBD (A) and fraction D exhibit a potent inhibition of IL-8 and IL-6 production, comparable to the glucocorticoid dexamethasone at higher concentrations (Fig. 5A, B). A decreased TNF- α release, which was not exclusively influenced by DMSO, was also seen for the OBD and fraction D at high dose (Fig. 5C). However, DMSO strongly interferes with the TNF- α release in this *in vitro* assay and obviously masks the pharmacological impact of the OBD tannins. Our study indicates that DMSO itself exhibited an anti-inflammatory effect. It is supported by evidence from the literature that DMSO displays anti-inflammatory properties, even though mechanistic studies are rare (Jacob and Herschler, 1986; Santos et al., 2003). Thus, future studies avoiding DMSO as solvent appear to be worthwhile.

Tannins and tannin-containing plant extracts have been shown before to inhibit allergic mediator (Kano et al., 2000; Nagano et al., 2012; Yamada et al., 2012) and TNF- α production *in vitro* (Kim et al., 2013; Granica et al., 2015). Also a previous study reported that vescalagin (7), a specific tannin constituent of oak bark, prevented cytokine release and inflammation (Chang et al., 2013). Taking into account, our data demonstrate that the OBD high molecular weight tannins solely have the capacity to diminish the secretion of IL-8, IL-6 and potentially TNF- α from human mast cells. The twofold effect of OBD on the release of early- and late-phase allergic mediators may be advantageous for a therapeutic invention, since conventional antihistamines are merely effective against early-phase allergic symptoms and do not prevent the release of late-phase allergic cytokines (Holgate and Polosao, 2008).

5. Conclusion

Based on our observations which provide evidence that high-molecular tannins attenuate mast cell degranulation and cytokine expression, we conclude that OBD might be a therapeutic remedy for treatment of allergic reactions. Since high-molecular tannins show a strong affinity to surface proteins, forming stable complex structures (Dobrev et al., 2014), OBD is conceivable for topical applications, *e.g.*

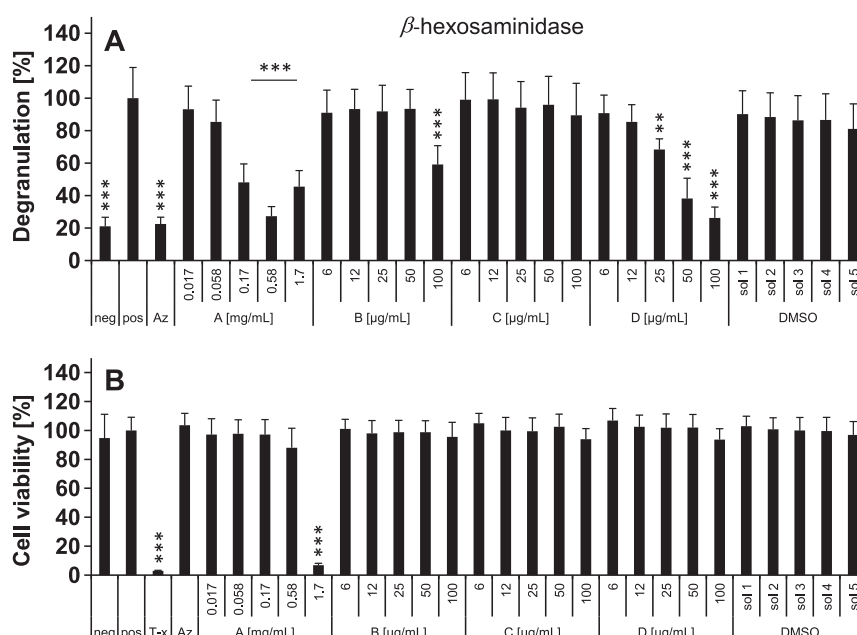


Fig. 4. Effects of OBD and its fractions on the viability and degranulation of basophilic cells. RBL-2H3 cells, sensitized with DNP-specific IgE antibody, were incubated with medium alone (pos), various concentrations of the OBD (A) or fractions B-D before stimulation with DNP-HSA. Adequate DMSO dilutions were used as solvent controls (sol1-sol5). Azelastine (Az; 10^{-11} M) was used as inhibition control, Triton-X100 (T-x; 0.5%) served as control for cell death. Data were adjusted in relation to untreated control cells (pos=100% $\pm$ SD), non-sensitized cells incubated with medium alone were used as negative controls (neg). (A) β -hexosaminidase release was analyzed and (B) cell viability of basophilic cells was assessed. Data are presented as mean $\pm$ SD of five (viability) and six (degranulation) representative experiments. The asterisks indicate significant differences from stimulated cells alone (**P < 0.01, ***P < 0.001).

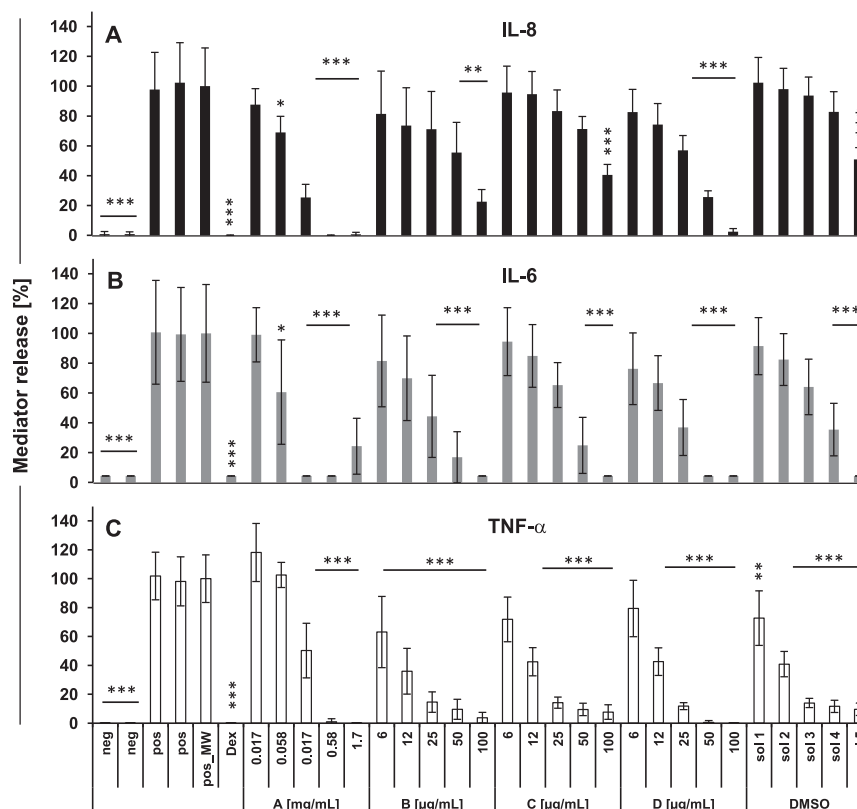


Fig. 5. Effects of OBD on IL-8, IL-6 and TNF- α from human mast cells. HMC-1 cells were stimulated with PMA and A23187 after preincubation with medium alone (pos), various concentrations of the OBD (A), fractions (B-D) or dexamethasone (Dex; 10^{-5} M). Adequate DMSO dilutions were used as solvent controls (sol1-sol5). Mean values were composed out of the two positive controls (pos_MW; =100% $\pm$ SD) and data were adjusted. Non-stimulated cells incubated with medium alone were used as negative controls (neg). Supernatants were analyzed for human IL-8 (A), IL-6 (B) and TNF- α (C). Data are presented as means $\pm$ SD of five independent experiments. The asterisks indicate significant differences from untreated PMA/A23187-stimulated cells alone (*P < 0.05, **P < 0.01, ***P < 0.001).

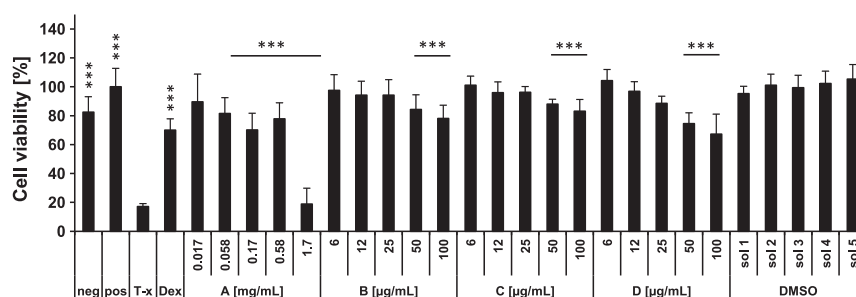


Fig. 6. Effects of OBD on viability of human mast cells. After pre-treatment, with medium alone (pos), various concentrations of the OBD (A), fractions B-D or dexamethasone (Dex; 10^{-5} M), HMC-1 cells were activated with PMA and A23187. Adequate DMSO dilutions were used as solvent control (sol1–sol5). Triton-X100 (T-x; 0.5%) was used as control for cell death. The data were adjusted in relation to activated but untreated control cells (pos=100% $\pm$ SD). Non-activated cells incubated with medium alone were used as negative controls (neg). Data are presented as means $\pm$ SD of six independent experiments. The asterisks represent significant differences from PMA/A23187-stimulated cells alone (***) $P < 0.001$.

on nasal mucous membranes. But presumably, by a systemic application, concentrations of OBD tannins needed for inhibition of mast cell degranulation will not be achieved *in vivo* due to their low bioavailability (Serrano et al., 2009).

Taken together, the OBD, a traditional herbal remedy, might provide a complementation to conventional synthetic anti-allergic drugs. The molecular action pathways of the oak bark tannins, their bioavailability and pharmacology await further studies, to broaden the spectrum of their pharmaceutical application.

Conflict of interests

All authors from WALA Heilmittel GmbH (P.L., M.H., S.M., A.H., K.G., C.B., M.K., F.C.S.) state that they are employees of this company. C.G. has been consultant of WALA Heilmittel GmbH in affairs not related to this project. M.G.-K., F.G., R.H. and C.S. have no conflict of interest. Consumables for the *in vitro* experiments were financially supported by WALA Heilmittel GmbH.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.jep.2016.10.027>.

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Appendix A. Supplementary Material

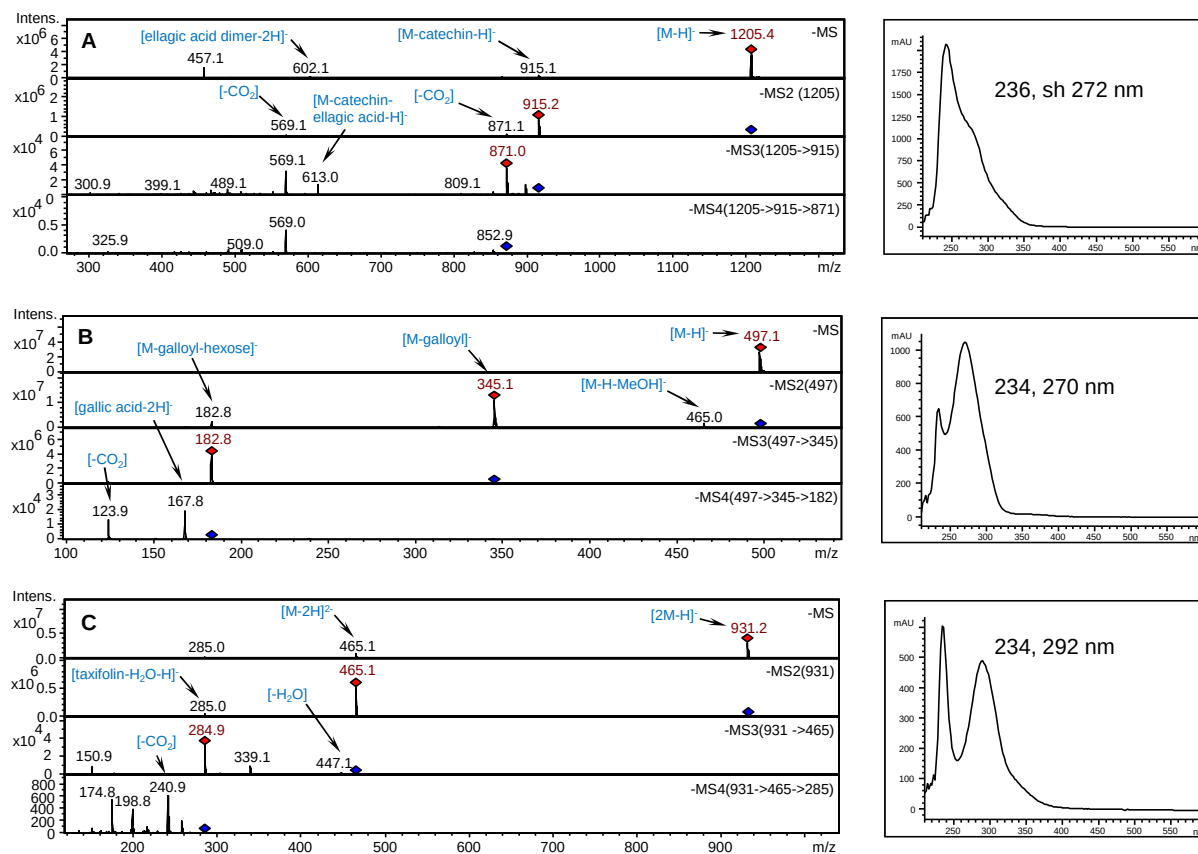


Fig. S1. (A) MS⁻, MS²⁻ and MS³⁻ data of acutissimin A **14** ($C_{56}H_{38}O_{31}$, MW = 1206.88), (B) methyl gallic acid hexoside gallate **16** ($C_{21}H_{22}O_{14}$, MW = 498.39) and (C) the taxifolin hexoside isomers **18-20** ($C_{21}H_{22}O_{12}$, MW = 466.39) as obtained in the negative ionization mode. At the right, UV spectra of the pursuant compounds are shown at their specified retention times (see Fig. 2).