Faculty of Biology, Chemistry and Geology, University of Bayreuth[1], and Faculty of Chemistry and Pharmacy, University of Regensburg[2], Germany

Structurally related immunological effects of triterpenoid saponins

M. Bernhardt[1], C. Sturm[2], K. H. Shaker[1], D. H. Paper[2], G. Franz[2] and K. Seifert[1]

The influence of the triterpenoid saponins **1**–**10** has been investigated on murine spleenocytes in the lymphocyte transformation test and on murine macrophages in an phagocytosis assay. The lymphocyte transformation test and the phagocytosis assay showed that the tested compounds have no stimulating effect. However, a significant inhibition of lymphocyte proliferation by the triterpenoid saponins **2**, **6** and **10** was demonstrated.

1. Introduction

Triterpenoid saponins belong to a class of substances exhibiting immunomodulatory and antitumoral effects [1]. Maharaj et al. [2] reported that orally administered saponins potentiate humoral immune responses in mice fed inactivated rabies vaccine. Chavali et al. [3] proved that saponins, when fed orally, enhanced cell proliferation *in vivo*, induced helper T-cell activation, and induced T-cell activity as well as T-independent B-cell stimulation by LPS (lipopolysaccharides). This and the fact that the investigated plants are extensively used in folk medicine prompted us to investigate the immunological properties of these triterpenoid saponins. The substances had been introduced to a validated screening system based on murine cell cultures.

However, there are two problems to be resolved. Endotoxins from bacterial membranes might cause false positive results [4] and also cytotoxic substances are lethal for the immunocytes on which the screening is based, and thereby simulate immunosupressive effects.

To overcome these problems the following screening system has been designed. First, the samples were tested on bacterial endotoxins in the limulus amoebocyte lysate test (LAL test) and cytotoxicity was determined against the murine cell line L 929 in the MTT test (MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). The endotoxin-free samples were afterwards examined at subtoxic concentrations for immunological properties in the lymphocyte transformation test (LTT) and for phagocytosis activity. Samples without any positive effects are investigated for inhibition of lymphocyte proliferation [5].

2. Investigations and results

Plants belonging to the genus *Fagonia* are often used in folk medicine, mainly for the treatment of various skin lesions. Additionally, *Fagonia indica* is claimed to be a remedy for cancer in its early stages [6–8]. We have isolated the ursolic acid saponins **1**, **3**–**5** and the oleanolic acid saponin **2** from whole plants of *Fagonia indica* [9, 10]. The triterpenoid glycoside **3** (matesaponin 1) has previously been obtained from the roots of *Aralia decaisneana* [11].

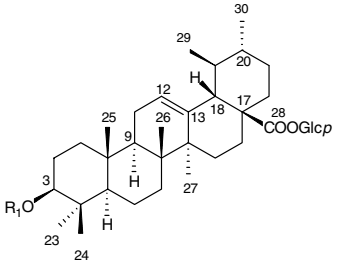
Extraction of the aerial parts of *Cornulaca monacantha* and chromatography of the crude mixture yielded the oleanolic acid saponins **6**–**8** [12] which have previously been isolated from *Bupleurum chinense* (**6**) [13], *Pisonia umbellifera* (**7**) [14] and *Meliosma lanceolata* (**8**) [15].

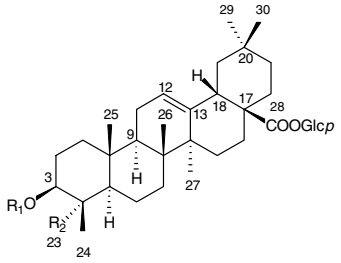
Extracts from various *Astragalus* species represent common drugs in traditional medicine, mainly used as remedies for the treatment of nephritis, diabetes, leukemia and uterine cancer [16, 17]. Cycloartane triterpenoid saponins isolated from several *Astragalus* species showed antitumor activity against some human cell lines and anti-HIV activity [18]. We have isolated from the whole plant of *Astragalus trigonus* the cycloartane glucoside **9** [12] which has previously been obtained from the roots of the same plant [19].

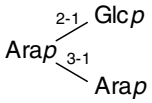
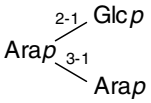
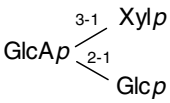
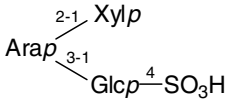
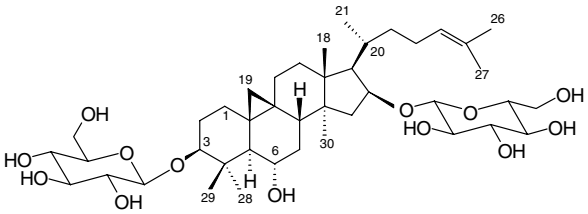
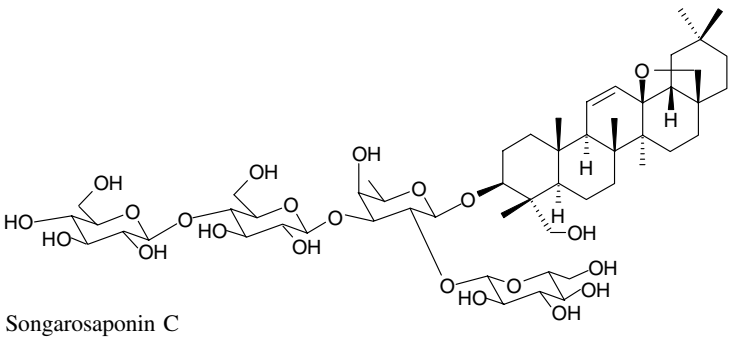
The leaves of *Verbascum densiflorum* are used in Europe against common cold. Cough will be reduced due to the presence of mucilage and saponins [20]. We have investigated a similar species *Verbascum songaricum* and were able to isolate the new triterpenoid glycoside songarosaponin C (**10**) [21].

The substances were examined for endotoxin contamination by means of the LAL test. The subtoxic concentrations of saponins were determined by the MTT test and were 100 μg/ml for **1**–**8** and 10 μg/ml for **9**, **10**. Effects on lymphocyte transformation and phagocytosis were investigated for the triterpenoid saponins **1**–**10**. In the lymphocyte transformation test and the phagocytosis assay none of the compounds showed any stimulating effect on murine cell cultures. However, significant inhibition of PWM-induced (PWM = pokeweed mitogen, B- and T-lymphozyte stimulator) lymphocyte proliferation was demonstrated for triterpenoid saponins **2**, **6** and **10**. Cyclosporin A, known for its immunosuppressive activity, was used as a reference compound and exhibited a stimulation index (SI) of 0.4 at a concentration (c) of 12 μg/ml (Fig. 1). The *Fagonia indica* compounds indicasaponin A (**1**) (SI = 12.7, c = 100 μg/ml) and saponin **3** (SI = 10.2, c = 100 μg/ml) showed only weak activity. Lower stimulation indices and consequently higher immunosuppressive activity were found for indicasaponin B (**2**) (SI = 2.6, c = 100 μg/ml) and the *Cornulaca monacantha* saponin **6** (SI = 0.6, c = 100 μg/ml). The highest activity (SI = 0.1, c = 10 μg/ml) was demonstrated for the monodesmosidic songarosaponin C (**10**). Fig. 2 shows the concentration dependent inhibition of PWM-induced proliferation of the triterpenoid saponins **2**, **6** and **10**.

The structural differences, as associated with the trisaccharide moiety of indicasaponin A (**1**) and the disaccharide unit of saponin **3** lead to a higher immunosuppressive activity. An even higher activity is shown by indicasaponin B (**2**) which differs from indicasaponin A (**1**) only in the position of the 29 methyl group of the aglycone. Indicasaponin A (**1**) has the 29 methyl group at position 19 with the aglycone ursolic acid. Indicasaponin B (**2**) has the 29 methyl group at position 20 with the aglycone oleanolic acid. A small modification of the structure leads to a significantly higher immunosuppressive activity of indicasaponin B (**2**) in comparison with indicasaponin A (**1**). The effect of the trisaccharide unit in position 3 of





	R_1		R_1	R_2
1	 Indicasaponin A	**2**	 Indicasaponin B	CH_3
3	Arap 3-1 Glcp	**6**	GlcAp 3-1 Xylp	CH_3
4	Arap 3-1 Glcp 4 SO_3H Indicasaponin C	**7**		CH_3
5	 Indicasaponin D	**8**	GlcAp 3-1 Xylp	CH_2OH
9				
10	 Songarosaponin C			

indicasaponin B (**2**) compared to the disaccharide unit in the *Cornulaca monacantha* saponin **6** also results in an increased activity. The *Verbascum songaricum* compound songarosaponin C (**10**) possesses the highest immunosuppressive activity and is more active than cyclosporin A *in vitro*.

3. Experimental

3.1. Endotoxin (LPS) determination

Endotoxin determination was performed with the Endosafe® test of Charles River Germany.

3.2. Cytotoxicity determination

10^4 L 929 cells (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) were seeded on 96-well microtiter plates and allowed to settle overnight. After 30 h cell culture with the test samples MTT (Sigma, Deisenhofen; 25 µl of 0.5% solution) was added and cultures were incubated for an additional 45–60 min. Formazan crystals were separated by centrifugation and dissolved in DMSO 100 µl/well. Absorbance was measured at 540 or 560 nm.

3.3. Animals

6–10 weeks old female DBA/2 mice were purchased from Charles River Wiga Germany.

3.4. Spleen cells

Cells were obtained from DBA/2 mice by passing aseptically removed spleens through a stainless steel mesh. Erythrocytes were deprived by hypotonic lysis (NH_4Cl-Tris buffer, pH 7.2)

3.5. Culture conditions

Tissue cultures were incubated at 37 °C in a 10% CO_2 atmosphere.

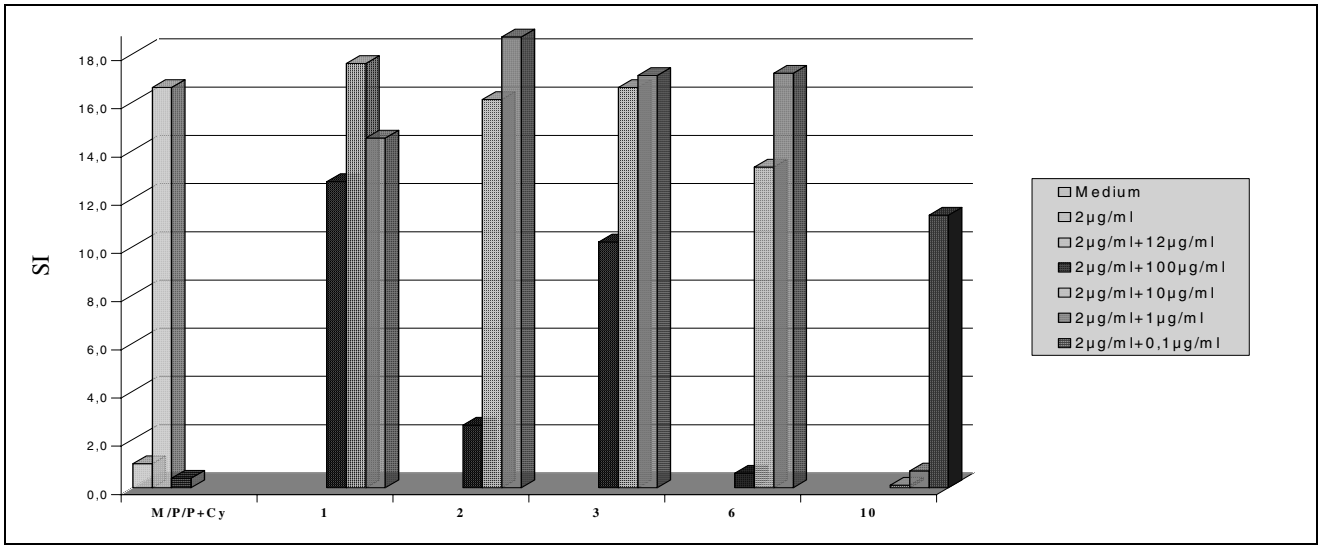


Fig. 1: PWM-induced lymphocyte proliferation for the saponins **1**–**3**, **6** and **10**. Cyclosporin A was used as reference compound. SI = stimulation index, M = medium, P = PWM, Cy = cyclosporin A

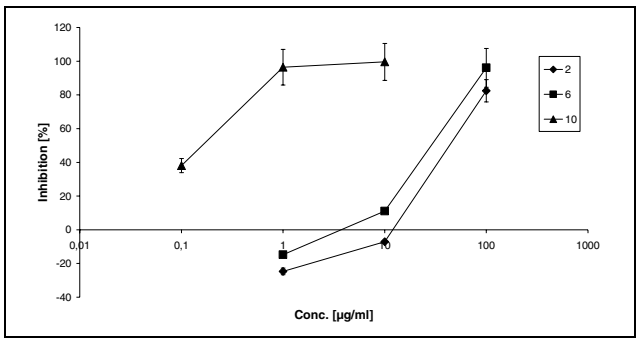


Fig. 2: Concentration dependent inhibition of PWM-induced lymphocyte proliferation in % of the saponins **2**, **6** and **10**

Dulbecco's modified eagles medium (DMEM; Life Technologies) was prepared with Ampuwa® (Fresenius AG, Bad Homburg) and supplemented with 3,7 g/l $NaHCO_3$, 10% heat inactivated fetal calf serum (Biochrom), 1 mM sodium pyruvate and 1% penicillin (10 000 U/ml)-streptomycin (10 000 µg/ml) (Life Technologies).
Macrophage cultivation medium was composed of DMEM 65%, horse serum 5% (Biochrom), 30% sterile L 929.

3.6. Lymphocyte transformation test (LTT) with murine spleenocytes

5×10^5 spleen cells/well were incubated with the test samples at a final volume of 200 µl/well for 40 h in white microtiter plates (Dynex Technologies). For an additional 4 h cells were labelled with BrdU (Cell proliferation ELISA, BrdU chemoluminescence; Roche Diagnostics Boehringer Mannheim GmbH). The labelling medium is removed, cells are fixed and DNA is denaturated. An enzyme linked antibody binds to the BrdU incorporated into the cellular DNA. The immune complexes are detected by a subsequent substrate reaction with luminol in the precence of H_2O_2. The emitted light is quantified by measuring the luminescence in a microtiter plate luminometer. Effects were calculated as stimulation indices (SI).

3.7. Phagocytosis assay

1×10^5 bone marrow macrophages were seeded on 96-well white microtiter plates and allowed to settle for 4 h in DMEM. Culture medium was sucked off and test samples were added. After coincubation for 15 h supernatants were removed and a solution with zymosan A and luminol was added (100 µg/well zymosan A dissolved in 200 µl 0.5 mM luminol solution). Phagocytosis was determined indirectly by measuring chemoluminescence with a microtiter plate luminometer for 2 h, comparing the AUCs and calculating phagocytosis indices (PI).

3.8. Inhibition of PWM-induced lymphocyte proliferation

The experiment was performed according to the LTT assay. To assess inhibition, cells were coincubated with test substances and PWM (2 µg/ml final concentration) The inhibition of lymphocyte proliferation is expressed in %.

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References

1 Plohmann, B.; Bader, G.; Hiller, K.; Franz, G.: Pharmazie **52**, 953 (1997)
2 Maharaj, J.; Froh, K. J.; Campbell, J. B.: Can. J. Microbiol. **32**, 414 (1986)
3 Chavali, S. R.; Francis, T.; Campbell, J. B.: Int. J. Immunopharmacol. **9**, 675 (1987)
4 Devlin, J. P.; Hargrave, K. D.: Tetrahedron **45**, 4327 (1989)
5 Sturm, C.: Dissertation, University of Regensburg 1999
6 Chapra, R. N.; Handa, K. I.; Kapur, L. D.: Indigenous Drugs of India 507 (1956)
7 Chapra, R. N.; Nayar, S. L.; Chopra, I. C.: Glossary of Indian Medicinal Plants 116 (1956)
8 Rahman, A. U.: J. Chem. Soc. Pak. **5**, 127 (1983)
9 Shaker, K. H.; Bernhardt, M.; Elgamal, M. H. A.; Seifert, K.: Phytochemistry **51**, 1049 (1999)
10 Shaker, K. H.; Bernhardt, M.; Elgamal, M. H. A.; Seifert; K.: Z. Naturforsch. **55c**, 520 (2000)
11 Miyase, T.; Shiokawa, K.-I.; Zhang, D. M.; Ueno, A.: Phytochemistry **41**, 1411 (1996)
12 Bernhardt, M.: Dissertation, University of Bayreuth 1999
13 Seto, H.; Otake, N.; Luo, S. Q.; Jin, H. F.: Agric. Biol. Chem. **50**, 939 (1986)
14 Lavaud, C.; Beauviere, S.; Massiot, G.; Le Men-Olivier, L.; Bourdy, G.: Phytochemistry **43**, 189 (1996)
15 Abe, F.; Yamauchi, T.; Shibuya, H.; Kitagawa, I.: Phytochemistry **42**, 809 (1996)
16 Hartwell, J. L.: Lloydia **33**, 288 (1970)
17 McCracken, D. S.; Schermeister, L. J.; Bhatti, W. H.: Lloydia **33**, 19 (1970)
18 Abdallah, R. M.; Ghazy, N. M.; El-Sebakhy, N. A.; Pirillo, A.; Verotta, L.: Pharmazie **48**, 452 (1993)
19 Verotta, L.; Orsini, F.; Tato, M.; El-Sebakhy, N. A.; Toaima; S. M.: Phytochemistry **49**, 845 (1998)
20 Wichtl, M.: Teedrogen und Phytopharmaka, 3. Auflage p. 609, Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart 1998
21 Seifert, K.; Preiss; A.; Johne, S.; Schmidt, J.; Lien, N. T.; Lavaud, C.; Massiot, G.: Phytochemistry **30**, 3395 (1991)

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Prof. Dr. K. Seifert
Universität Bayreuth
Lehrstuhl Organische Chemie I, NW II
D-95450 Bayreuth