ture of **5** was confirmed by elemental analysis, IR, MS and EPR spectroscopy.

Compound **5** was tested *in vivo* against lymphocytic leukemia P388 and lymphoid leukemia L1210 in mice CD2F1, according to methods described in the literature [7]. The results are presented in the Table in comparison with SLCNU and CCNU. Compound **5** prolonged the survival time of mice bearing leukemia L1210 by 831% at a dose of 60 mg/kg and leukemia P388 by 735% at a dose of 25 mg/kg. The LD_{50} for compound **5** was 72 mg/kg i. p. in mice; it showed greater cytotoxicity against leukemia L1210 and leukemia P388 than the clinically used drugs CCNU and SLCNU. Both of the compound **5** and SLCNU were less toxic than CCNU according to LD_{50} data.

In conclusion it can be pointed out that the hydrazone group in the spin labeled NU **5** influences its oncopharmacological properties.

Experimental

1. Starting compounds

4-Hydrazono-(2,2,6,6-tetramethylpiperidine-1-oxyl) (**1**) was prepared according to [8], N′-hydroxysuccinimide-N-(2-chloroethyl)-N-nitrosocarbamate (**3**) as described in [9] and 2-chloroethylnitrosocarbamoyl azide (**4**) was prepared according to [3].

2. 4-{N′-[N-(2-chloroethyl)-N-nitrosocarbamoyl]hydrazono}-2,2,6,6-tetramethylpiperidine-1-oxyl (**5**)

2.1. By N′-hydroxysuccinimide-N-(2-chloroethyl)-N-nitrosocarbamate (**3**)

(2,2,6,6-Tetramethylpiperidine-1-oxyl)-hydrazine 0.184 g (1 mM) was dissolved in DMF (2 ml at 0–5 °C). The mixture was stirred vigorously for 15 min and *N′*-hydroxysuccinimide-*N*-(2-chloroethyl)-*N*-nitrosocarbamate 0.23 g (1 mM) was added. After stirring of 3 h the mixture was poured into ice/H_2O (70 ml). The received yellow crude product was extracted with $(C_2H_5)_2O$ (3×10 ml). The combined extracts were successively washed with 10 citric acid (2×10 ml), 10% $NaHCO_3$ (2×10 ml) and a saturated solution of NaCl (2×10 ml). The organic layers were dried over anh. $MgSO_4$ and filtered. The solvent was evaporated under reduced pressure and the residue was crystallized twice from $(C_2H_5)_2O$/n-hexane as pale yellow crystals, yield 72%.

2.2. By 2-chloroethylnitrosocarbamoyl azide (**4**)

(2,2,6,6-Tetramethylpiperidine-1-oxyl)-hydrazine 0.184 g (1 mM) was dissolved in anh. pyridine (15 ml at 0 °C). A solution of 2-chloroethylnitrosocarbamoyl azide 0.178 g (1 mM) dissolved in 2 ml dry $(C_2H_5)_2O$ (3 ml) was added dropwise. The mixture was stirred at 0 °C for 3 h and was poured into iceH_2O. The organic layer was separated and the aqueous layer was extracted with $(C_2H_5)_2O$ (3×10 ml). All $(C_2H_5)_2O$-extracts were combined, washed with 2 N HCl (2×10 ml), 10% $NaHCO_3$ (2×10 ml) and a saturated solution of NaCl (2×10 ml), dried over anh. $MgSO_4$ and filtered. The solvent was evaporated under reduced pressure. The semisolid residue was crystallized twice from $(C_2H_5)_2OH$/n-hexane as pale yellow crystals, yield 58%.

M.p. 113–115 °C (dec.); Rf: 0.71 [$CHCl_3/CH_3OH$ (v/v 9 : 1)]; Ms m/z: 318 (M^+), 210 (M^+-108), 180 (M^+-138); IR (KBr): (3320, 1720, 1458, 1340 cm^{-1}); $C_{12}H_{21}ClN_5O_3$ (318.8); Calcd.: C 45.21, N 21.97, H 6.63; Found: C 44.5, N 21.5, H 6.4.

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Antimicrobial activity of some Nepalese medicinal plants

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Medicinal plants from Nepal are very popular as a source of pharmacologically active compounds. The local people of Nepal have rich folklore about the traditional use of different parts of various plants. The present paper describes the antimicrobial activity of a number of Nepalese medicinal plants used as remedies against various diseases.

Dried and powdered plant material (5 g each) was extracted successively with dichloromethane, methanol and 50% aqueous methanol in a soxhlet extraction apparatus. Evaporation of the solvent followed by drying in vacuo provided crude extracts. The plants and their parts used for extraction and the amount of extract obtained under different extraction conditions are summarized in Table 1. The extracts were screened for antimicrobial activity against Gram-positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus* and *Micrococcus flavus*), Gram-negative bacteria (*Proteus mirabilis*, *Serratia marcescens* and *Escherichia coli*) and the yeast species *Candida maltosa* using a modified disc diffusion method [1, 2].

Growth of the fungal species *C. maltosa* was only inhibited by the dichloromethane and methanol extracts of *Zanthozylum armatum* with MIC values of 500 and 1000 μg/disc, respectively.

Both extracts as well as the methanol extract of *Rhododendron anthopogon* showed weak activity against different gram-negative bacteria (MIC between 500 and 2000 μg/disc) while the aqueous methanol extracts of *Bergenia ligulata*, *Bombax cieba*, *Dipsacus mytis*, *Rh. anthopogon* and *Salvia coccinia* were slightly active against *P. mirabilis* only (MIC 2000 μg/disc).

Most of the extracts were active against the Gram-positive bacteria (see Table 2). The most pronounced activity was shown by the dichloromethane extract of *Maharanga bicolor* with a MIC value of 0.25 μg/disc for all the three Gram-positive bacteria included into the experiment. Generally, the dichloromethane extracts showed the highest activity followed by the methanol extracts while methanol-water extracts were less active with the exception of *B. ligulata* were the methanol extract was most active. The dichloromethane extract did not show any activity against *B. subtilis* and *M. flavus* and moderate activity against *S. aureus*. Divergent results were obtained with extracts of *Parnassia nubicola* with highest activity of dichloro-

Table 1: List of plants and yields (mg) obtained by extraction of 5 g of the dried plant material

Name of plants	Parts used	Dichloromethane extract	Methanol extract	50% Aqueous methanol extract
Adhatoda vasica Nees	Leaves & stem	167	1071	584
Allium wallichii Kunth	Whole plant	218	1101	662
Asparagus racemosus Willd.	Tubers	47	1400	375
Bergenia ligulata (Wall.) Engl.	Rhizomes	167	1285	234
Bombax ceiba L.	Flowers	87	370	514
Dypsacus mytis	Whole plant	80	583	237
Maharanga bicolor	Whole plant	475	415	436
Nigella sativa L.	Seeds	1387	538	212
Parnassia nubicola Wall. ex Arn.	Whole plant	985	608	140
Podophyllum hexandrum Royle	Roots	433	632	229
Rhododendron anthopogon	Leaves & stem	332	879	223
Salvia coccinea Buc'hoz ex Etl.	Whole plant	107	311	285
Zanthozylum armatum	Seeds	225	641	637

Table 2: Minimal inhibitory concentrations (µg/disc) of plant extracts against Gram-positive bacteria

Plant extracts	Organisms								
	B. subtilis			*S. aureus*			*M. flavus*		
	A	B	C	A	B	C	A	B	C
A. vasica	2000	>2000	>2000	1000	>2000	>2000	1000	>2000	2000
A. wallichi	1000	>2000	>2000	1000	>2000	>2000	1000	>2000	>2000
A. racemosus	2000	>2000	>2000	2000	>2000	>2000	>2000	>2000	>2000
B. ligulata	>2000	250	>2000	500	100	250	>2000	100	500
B. cieba	500	>2000	>2000	100	2000	1000	500	2000	>2000
D. mytis	500	>2000	>2000	1000	1000	2000	1000	2000	>2000
M. bicolor	0.25	>2000	>2000	0.25	1000	1000	0.25	2000	>2000
N. sativa	1000	>2000	>2000	1000	>2000	>2000	1000	>2000	>2000
P. nubicola	250	2000	250	250	1000	250	1000	2000	100
P. hexandrum	2000	1000	2000	1000	1000	2000	>2000	2000	50
R. anthopogon	250	>2000	2000	250	500	250	250	1000	250
S. coccinia	1000	>2000	>2000	100	1000	1000	500	1000	2000
Z. armatum	50	500	>2000	50	500	1000	50	100	2000

A: Dichloromethane extract, B: Methanol extract, C: Methanol-water (1 : 1) exctract

methane and methanol-water extracts and by extracts of *Podophyllum hexandrum* where only the water extract was active against *M. flavus*. The majority of extracts only showed weak or moderate activity (MIC 2000–500 µg/disc). Besides *M. bicolor* a pronounced activity was shown by extracts obtained from *B. ligulata*, *P. nubicola* and *Rh. anthopogon* with MIC values of 100 and 250 µg/disc.

The results obtained in the course of the present studies are in agreement with the traditional use of these plants. *M. bicolor*, *B. ligulata*, *P. nubicola* and *Rh. anthopogon* seem to be a source for new antibacterial drugs against Gram-positive bacteria. The bio-assay directed to an isolation of the active principle is under way in our laboratory.

Experimental

1. Plant materials

The plants were collected and identified in Nepal in summer 1996 and spring 1997. Voucher specimens are deposited at the Department of Plant Resources, Ministry of Forest and Soil Conservation, His Majesty's Government of Nepal.

2. Preparation of extracts

Dried and powdered plant material (5 g each) was extracted successively with dichloromethane, methanol and 50% aqueous methanol in a soxhlet extraction apparatus. Evaporation of the solvent followed by drying in vacuo provided crude extracts. Details are shown in Table 1.

3. Microorganisms

Cultures of *Bacillus subtilis* SBUG 14, *Staphylococcus aureus* SBUG 511, *Micrococcus flavus* SBUG 16 (Gram-positive), *Proteus mirabilis* SBUG 47, *Serratia marcescens* SBUG 9, *Escherichia coli* SBUG 13 (Gram-negative) and the fungal species *Candida maltosa* were prepared from stock cultures by streaking on nutrient agar for bacteria and malt agar for the yeast.

4. Antimicrobial assay

A modified disc diffusion method [1, 2] was used to determine the antimicrobial activity. Nutrient agar (Immunpräparate Berlin/Germany; composition: 6.75 parts of pancreatic peptone, 1.75 parts of protein hydrolysate, 1.5 parts of yeast extract, 5.0 parts of NaCl and 10.0 parts of agar) was used for bacteria, prepared by dissolving of 25 g/l in water. Malt agar was used for *C. maltosa*, prepared by mixing of 30 g/l of malt extract (Dr. Fränkle & Max Eck, Fellbach/Germany, composition: glucose 10–15%, maltose 40–50%, dextrine 10–15%, protein 5%) and 15 g/l of agar in water followed by adjustment of the pH to 5.5.

A sterile filter paper disc of 6 mm diameter impregnated with test extract was used for the assay. The paper disc was placed on the center of the agar plate seeded with the respective microorganisms. The plates were inverted and incubated overnight at 37 °C. In contrast, *M. flavus* was incubated at RT for 48 h and *C. maltosa* was incubated at 28 °C for 72 h. At the end of the incubation period the zones of inhibition around the paper disc were measured. Control experiments were performed using paper discs loaded with an equivalent volume of solvent only. The highest concentration of extract tested during the experiment was 2 mg/disc. The MIC corresponds to the lowest concentration of the test compound that produces a measurable zone of inhibition around the paper disc [1].

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Different kinetics of hydroquinone depletion in various medicinal plant tissue cultures producing arbutin

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Recently significant progress was made in production and accumulation of pharmaceutically important secondary metabolites by *in vitro* cultivated medicinal plant tissues. The biosynthetic capacity of such plant cell cultures can be utilised in biotransformation reactions with appropriate precursors added into the cultivation media. The glycoside arbutin is the main therapeutically active substance occurring in intact bearberry and other Ericaceae where it is biosynthesized from tyrosine. Arbutin is used as an antimicrobial agent [1, 2] in the treatment of urinary tract infections, it is also a powerful antitussive agent [3], it possesses an anti-inflammatory effect [4–6] and it is also known as effective inhibitor of the production of melanin [7]. This compound also reduces spore germination of decay fungi [8] and exhibits other biological effects. Its use as an indicator reagent in the assay of yeasts is well known. Isolation of arbutin from intact plant is rather time-consuming and economically inexpedient. Laboratory synthesis of arbutin is a multistep process. Hence it is obvious that the biotechnological production of arbutin and appropriate simple precursors in plant tissue cultures would be of practical interest to pharmaceutical chemists. We have found earlier [9] that bearberry plant tissues cultivated in conventional Murashige-Skoog (M-S) medium do not produce arbutin even when the M-S medium contained tyrosine or 4-hydroxybenzoic acid as precursors. However, the formation of arbutin in bearberry calluses cultivated in hydroquinone-treated M-S medium has been observed. Moreover, our previous studies [10] have also revealed that tissue cultures of other plants (that normally do not produce arbutin in intact state) are capable of glucosylating hydroquinone (HQ). This fact was also described for *Datura, Catharanthus* and *Rauwolfia* species [11–13]. The aim of this work was a comparative study of various plant tissue cultures *(Leuzea carthamoides, Bergenia crassifolia, Leonurus cardiaca, Rhodiola rosea, Datura meteloides)* with respect to the differences in the kinetics of HQ depletion leading to arbutin synthesis. For detailed long-term monitoring of the depletion of HQ during the biotransformation we employed an automated computer-aided FIA (flow-injection analysis) setup [14]. In the course of the experiment the following results were obtained.

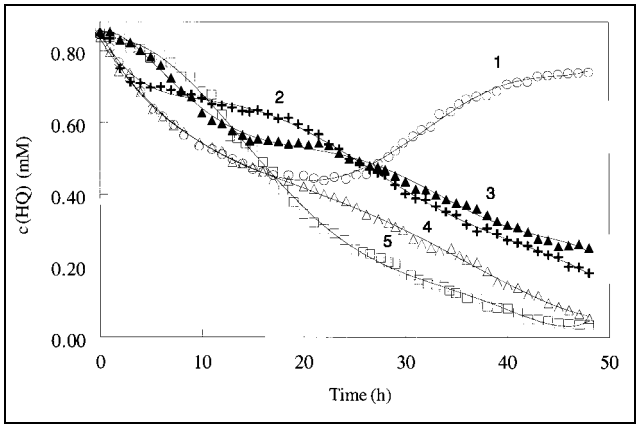

Fig. 1: Depletion of hydroquinone (HQ) from plant tissue cultivation media
Curves: 1: *Leuzea carthamoides*; 2: *Bergenia crassifolia*; 3: *Leonurus cardiaca*; 4: *Rhodiola rosea*; 5: *Datura meteloides*

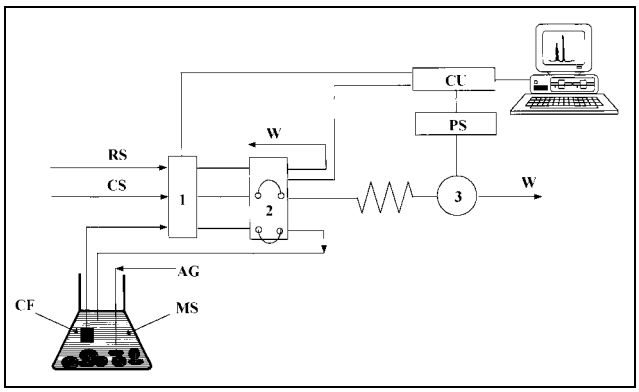

Fig. 2: Scheme of the automated flow-injection system for monitoring hydroquinone (HQ) concentration in plant tissue cultivation media
1: a 3-channel peristaltic pump; 2: dual sampling valve; 3: flow-through amperometric cell;
RS: reference sample (0.9 mM-HQ in pure plant cultivation medium); CS: carrier stream (0.1 M acetate buffer of pH 4.6); CU: control unit; PS: potentiostat; AG: inlet of agitation gas; MS: Murashige-Skoog cultivation medium (volume 300 ml); CF: polyamide cloth filter; W: waste

The course of time-dependent depletion of HQ is different in the various tissue cultures as depicted in Fig. 1. All the tissue cultures examined exhibit a relatively strong glycosylation activity during the initial 20 h of the experiment. After that time the *Leuzea* tissue culture shows the unique behaviour that the concentration of HQ in the cultivation medium gradually increases to approach its starting value in further 24 h, most probably due to the activation of hydrolytic enzymes. The tissue cultures of *Bergenia* and *Leonurus* biotransform HQ in two steps, namely during the initial 5 h (for *Bergenia*) or 10 h (for *Leonurus*) and then between the 20th and 48th hour; the period of "repose" corresponds to the night time. The highest depletion of HQ in the medium is attained with the tissue cultures of *Datura meteloides* and *Rhodiola rosea* where the conversion of HQ exceeds 90% after 48 h as confirmed by a HPLC determination of arbutin retained in the spent calluses [2]. The content of arbutin (calculated on dry mass of the culture) was 0.9%, 5.1%, 4.8%, 6.4% and 6.6% for *Leuzea*, *Bergenia*, *Leonurus*, *Rhodiola* and *Datura* which corresponds to 96.1 to 98.8% conversion of HQ to arbutin in the examined species.

It was further shown that the automated amperometric FIA system developed (Fig. 2) is suitable for a selective long-term monitoring of precursor changes in plant tissue cultivation media.