Cytotoxic and Antitumor Activities of Ethanolic Extracts of Salt Marsh Plants from the Lower Saxonian Wadden Sea, Southern North Sea

Thomas F. Lellau* and Gerd Liebezeit*

*Forschungszentrum Terramare, Wilhelmshaven, Germany; FB Chemie, Carl von Ossietzky-Universität Oldenburg, Germany

Abstract

Twenty-eight plant species belonging to fifteen families were subjected to a screening for cytotoxic and antitumor activities with three test organisms in six bioassays. Freshly hatched larvae and four week old adults of Artemia salina LEACH are used for the detection of acute toxic activity after 6 h and chronic toxicity after 24 h. Adult Daphnia magna STRAUS were used to establish toxic activities of the extract after 48 h. Finally the potato disc assay with Agrobacterium tumefaciens was used to detect antitumor compounds. Of the screened plants, Limonium vulgare MILL., Artemisia maritima L. and Salicornia europaea L. showed significant cytotoxic activity against Artemia salina LEACH and Daphnia magna STRAUS. Extracts of these plants also showed anti-neoplastic activities in the potato disc assay. The extracts of Ononis spinosa L., Trifolium fragiferum L. and Trifolium repens L. showed tumor growth inhibiting activities.

Keywords: Salt marsh plants, biological screening, cytotoxic activity, antitumor activity, Artemia assay, Daphnia assay, potato disc assay.

Introduction

The search for anticancer agents from plants dates back to 1947, when the cytotoxic properties of podophyllotoxin from Podophyllum peltatum (Berberidaceae) were detected (Kelly & Hartwell, 1954). The discovery of the antileukemic properties of vinblastine and vincristine from Catharanthus roseus (Apocynaceae) soon followed (Noble et al., 1958) and gave the impulse for wider ranging investigations of plant extracts and plant-derived compounds for possible anticancer activity. In addition to the two plants mentioned above, Taxus brevifolia (Taxaceae), Ochrosia elliptica (Apocynaceae) and Camptotheca acuminata (Nyssaceae) are among the species that so far have provided clinically useful drugs. The former one is the source of the diterpene taxol (Suffness, 1993), the second is one of the sources of the pyridocarbazole alkaloid ellipticine, while the last contains the pyrrolo[3,4-b]quinoline alkaloid camptothecin (Hamburger et al., 1991). Potential anticancer principles from plants and other natural sources have been reviewed by Frei et al. (1967), Strauch and Hiller (1974), Cordell and Farnsworth (1976), Suffness and Dourou (1979) and Kraus (1990).

As part of a phytochemical and biological screening programme, we are presently investigating the activities of ethanolic extracts of plants characteristic for the salt marshes of the German North Sea coast. This flora has so far not been subjected to such a screening programme. Salt marshes may be considered promising in this respect because of the highly variable environmental conditions. Species here do not just interact with each other as elsewhere but have to deal with, for example, salinities ranging from fresh water conditions after heavy rains to hypersaline conditions due to evaporation. These seasonal and daily changes of environmental conditions may act in a way like a natural combinatorial chemistry. The organisms might react with the production of a different spectrum of secondary metabolites than the same plants under more even environmental conditions (Verpoorte, 1998).

Accepted: September 10, 2002
Address correspondence to: Prof. Dr. Gerd Liebezeit, Forschungszentrum Terramare, Schleusenstraße 1, 26382 Wilhelmshaven, Germany. Fax: +49 4421 944 199; E-mail: Gerd.Liebezeit@terramare.de
One of the simplest biological responses to monitor is lethality. An animal that has been extensively used for this purpose is the brine shrimp, *Artemia salina* LEACH (Sam, 1993). This animal has been used since 1956 (Michael et al., 1956) in general screening for bioactive substances in plant extracts (Meyer et al., 1982) and even proposed as a standard test (van Hacke & Personne, 1982).

Another organism used in toxicity testing is *Daphnia magna* STRAUS. First mentioned by Flücker & Flück (1949) it is still in use today for the detection of toxic compounds (Sandbacka et al., 2000). It has proven to be a sensitive (Adema, 1978) and simple laboratory model for predictive toxicity studies (De Waart et al., 1972). One of the reasons why *D. magna* is a commonly used test animal in toxicology is that it can be easily cultured in the laboratory (Ten Berge, 1978). Furthermore, short-term toxicity tests are reasonably reproducible (Canton & Adema, 1978).

A totally different test that has proven useful in monitoring the inhibition of crown-gall tumour is the potato disc assay (Ferrigni et al., 1982). Crown gall is a neoplastic disease of plants induced by specific strains of the Gram-negative bacterium *Agrobacterium tumefaciens* (Braun & Stonier, 1958), first reported by Smith and Townsend (1907) and Jensen (1910, 1918). This malignancy, normally affecting dicotyledonous plants, is induced by inoculation of a wound site with *A. tumefaciens* followed by the transfer of a large plasmid from *A. tumefaciens* to the plant (Watson et al., 1975). The genetic information (T-DNA) of the large plasmid transforms normal plant cells into autonomous tumour cells (Chilton et al., 1980). Once the tumour induction has taken place, the autonomous proliferation of the tumour cells becomes entirely independent of the bacteria (Zaenen et al., 1974).

Galsky et al. (1980) showed that inhibition of crown gall tumour initiation on potato discs showed good agreement with compounds and plant extracts known to be active in the 3PS (*in vivo*, mouse leukaemia) antitumor assay. In addition to the inhibition of tumour initiation also the inhibition of the growth of the tumours agrees well with 3PS activity (Galsky et al., 1981).

The combination of *A. salina* and *D. magna* tests for cytoxicity and the potato disk assay for the investigation of antineoplastic activities appears to be a promising and easy to perform method for investigation of plant extracts. The assays are cheap, quick and yield good results in a first testing of plant material but further investigations have to follow.

In the present communication, results are presented from such a screening of 28 plant species from salt marshes from the Lower Saxonian Wadden Sea.

**Material and methods**

**Plant material**

Plant species were chosen according their abundance in the salt marshes sampled (von Glahn et al., 1989). Of the 82 species found here only the 28 most prominent species were used. Sampling locations and dates of collection, identification and preparation of plant extracts have been described in detail by Lellau and Liebezeit (2001).

**Screening for cytotoxic activity with *Artemia salina* LEACH**

Eggs of *A. salina* were bought at a local aquarium store (Novotemia Salinenkrebs-Eier, JBL GmbH). A spoonful of the eggs was placed in a 1L flask filled half with artificial seawater medium (ASW, Sam, 1993). The culture medium was aerated with a moderate flow of air to ensure mixing. The organisms were cultivated at room temperature and normal day length in the laboratory. The first seven days the larvae were fed with Liquizell (Dohse Aquaristik) and then with Mikrozell (Dohse Aquaristik).

For convenience, only seven extracts together with a negative and a positive control were analysed at one time. All tests of extracts and controls were carried out in triplicate. The negative control was carried out with 200μL EtOH instead of the extract and the positive control with 200μL HgCl₂ solution (1% w/v).

For the bioassay, larvae about 36h old were used. Each extract was tested separately. In a vial, 4.3 mL ASW were placed together with 200μL of the ethanolic extract to be tested. Then, 500μL of the larvae suspension with about 20–80 larvae were pipetted to the test solution. To determine the acute toxicity, the mortality of the test organisms was measured after 6h by counting dead and living larvae. The chronic toxicity was determined after 24h exposure.

The screening procedure with adult *A. salina* was the same except that only 10 organisms of similar size were used.

**Screening for cytotoxic activity with *Daphnia magna* STRAUS**

Adult *D. magna* was obtained at a local aquarium store. Mikrozell (Dohse Aquaristik) was used as food (Kersting, 1978).

The screening with *D. magna* was performed as described above for adult *A. salina*. Toxicity was measured after a 48h exposure time (de Waart et al., 1972).

The average death rate (AD) and relative toxicity (RT) of each extract and blank were calculated based on de Waart et al. (1972).

**Screening with the potato disc assay**

*A. tumefaciens* was obtained from the DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany (http://www.dsmz.de). The initial cultivation of the bacteria was performed in nutrient medium (peptone 0.5%, meat extract 0.3%, pH = 7.0) according to DSMZ.
Fresh, disease-free potatoes were obtained from a local market. Tubers of moderate size were surface sterilised by immersion in sodium hypochlorite (Chlorox) for 20 min. Ends were removed and the potatoes were soaked for an additional 10 min in Chlorox. A core of the tissue was extracted from each tuber with a surface-sterilised 1 cm cork borer. Pieces of 2 cm were removed from each end and discarded. The remainder of the cylinder was cut into 0.5 cm discs with a surface-sterilised scalpel. The discs were then transferred to agar plates (1.5 g of agar dissolved in 100 mL double distilled water (DDW), autoclaved for 20 min at 121 °C, 20 mL poured into each Petri dish). Each plate contained 6 discs and 3 Petri dishes were used for each extract.

Of each extract 500 μL were added to 1.5 mL DDW and mixed with 2 mL of a broth culture of A. tumefaciens (a 48 h culture containing 5 \times 10^9 cell mL^{-1}). Of this suspension, 50 μL were spread over the surfaces of 3 potato discs for inoculation. Controls were prepared with 500 μL ethanol instead of the extract. The remaining 3 discs per plate were inoculated with a suspension prepared with 500 μL DDW instead of extract solution. After 1 week, the latter were inoculated with 20 μL of extract.

The discs inoculated immediately with plant extracts tested the inhibition of tumour induction (tumour induction inhibition test, TIIT), the other three tumour growth inhibition (tumour growth inhibition test, TGIT).

The plates were incubated at room temperature for 21 days. Tumours were counted with the aid of a dissecting scope, after staining with LUGOL's solution. The tumour cells lack starch. For the TIIT the average number of tumours per sample was calculated following McLaughlin et al. (1991). The average number of tumours in the controls was 22.

The TGIT investigates the modification of tumour growth after tumours have been allowed to grow for 7 days. The TGIT lacks quantitative criteria and results are given as (−) for no inhibition, (±) for ambiguous inhibition, and (+) for inhibition of tumour growth after visual comparison with the control.

Results

Cytotoxic activity

All results are summarised in Table 1. Figure 1 shows the effect of the samples tested for toxic activities on A. salina larvae. The maximum toxicity (>50%) was observed with Ononis spinosa L. (Fabaceae), Salicornia europaea L. (Chenopodiaceae), Limonium vulgare Mill. (Plumbaginaceae) and Artemisia maritima L. (Asteraceae). A toxicity of about 20% was found with Centaurea pulchella (Sw.) DRUCE. (Gentianaceae) and Spergularia media L. (Caryophyllaceae). In every case the chronic toxicity is higher than the acute toxicity. All other species investigated showed effects <20% (Fig. 1).

Figure 2 shows the effect of the samples tested for toxic activities on A. salina adults. The maximum toxicity (>50%) was observed with L. vulgaris, A. maritima, O. spinosa and S. europaea. A toxicity of about 20% was reached by C. pulchellum only. As for A. salina larvae, chronic toxicities were higher than acute toxicities.

Figure 3 shows the effect of the samples tested for toxic activities on adult D. magna. Again the maximum toxicities were observed with A. maritima, O. spinosa, S. europaea and L. vulgaris although relative toxicities reach only about 40%. A toxicity between 20 and 30% was found with C. pulchellum and S. media.

Inhibition of tumour induction (TIIT)

The results are given in Table 1. Figure 4 shows the effect of the samples tested for inhibition of tumour induction on potato discs. The maximum activity in the potato disc assay is shown by the extracts of O. spinosa, L. vulgaris and A. maritima (in decreasing order). A lower, but still recognisable activity is shown by S. europaea, C. pulchellum, S. media, Puccinellia maritima (Huds.) PARL. (Poaceae), Plantago maritima L. (Plantaginaceae), Odontites litoralis Fr. (Serephulariaceae) and Aster tripolium L. (Asteraceae).

Inhibition of tumour growth (TGIT)

The results of the TGIT are difficult to quantify. It was only possible to compare the size of the tumours at the end of the investigation time visually on a disc of 1 cm diameter.

The only obvious growth inhibition was recognised with the extracts of the three species or the Fabaceae, Trifolium repens L., T. fragiferum L. and O. spinosa.

Discussion

The ethanolic extracts of 28 plant species were tested for their cytotoxic and antitumour effects. The results shown in Table 1 indicate that the effectiveness varied from no effect to promising in the assays employed.

High relative toxicities with both A. salina and D. magna are shown by A. maritima, L. vulgaris, O. spinosa and S. europaea (Table 1, Figs. 1–3). In addition C. pulchellum and S. media show slightly increased relative toxicities. In every case the chronic toxicity is higher than the acute toxicity as was to be expected. The values reached by extracts of the other species tested are considered to be non-significant. This is due to the observation that especially for the adult test organisms an apparent promotion of survival was observed in some cases (Figs. 1–3). Whether this is related to the inherent variability of biological tests or the actual presence of compounds reducing ethanol effects cannot be ascertained with the present data. Therefore, relative toxicities <10% are considered not to be significant.

The highest antitumor activities (>70%) in the TIIT were observed with O. spinosa, L. vulgaris and A. maritima. Moderate effects (50–70%) were found with S. europaea and C.
<table>
<thead>
<tr>
<th>species</th>
<th>Artemia salina LEACH</th>
<th></th>
<th>Daphnia magna STRAUS</th>
<th>inhibition of tumor initiation</th>
<th>inhibition of tumor growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>larvae acute [%]</td>
<td>larvae chronic [%]</td>
<td>adult acute [%]</td>
<td>adult chronic [%]</td>
<td></td>
</tr>
<tr>
<td>Aciaceae (Umbelliferae)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apium graveolens L.</td>
<td>14</td>
<td>3</td>
<td>17</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>Oenanthe lachenalii C. C. Gmel.</td>
<td>6</td>
<td>8</td>
<td>7</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Asteraceae (Compositae)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Artemisia maritima L.</td>
<td>53</td>
<td>56</td>
<td>58</td>
<td>68</td>
<td>48</td>
</tr>
<tr>
<td>Aster tripolium L.</td>
<td>1</td>
<td>0</td>
<td>-8</td>
<td>-4</td>
<td>-4</td>
</tr>
<tr>
<td>Leontodon autumnalis L.</td>
<td>1</td>
<td>-1</td>
<td>0</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>Brassicaceae (Cruciferae)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cochlearia anglica L.</td>
<td>6</td>
<td>16</td>
<td>7</td>
<td>-4</td>
<td>3</td>
</tr>
<tr>
<td>Caryophyllaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stellaria media L.</td>
<td>17</td>
<td>23</td>
<td>-7</td>
<td>7</td>
<td>24</td>
</tr>
<tr>
<td>Chenopodiaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Halimione portulacoides (L.) Aellen</td>
<td>4</td>
<td>1</td>
<td>7</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Salicornia europaea L.</td>
<td>52</td>
<td>63</td>
<td>47</td>
<td>57</td>
<td>41</td>
</tr>
<tr>
<td>Suaeda maritima (L.) Dum.</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cyperaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carex distans L.</td>
<td>2</td>
<td>-1</td>
<td>8</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Carex extensa Good.</td>
<td>2</td>
<td>-1</td>
<td>8</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Fabaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ononis spinosa L.</td>
<td>49</td>
<td>68</td>
<td>53</td>
<td>63</td>
<td>45</td>
</tr>
<tr>
<td>Trifolium fragiferum L.</td>
<td>13</td>
<td>16</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Trifolium repens L.</td>
<td>5</td>
<td>14</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Gentianaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Centaurium pulchellum (Sw.) Druce.</td>
<td>22</td>
<td>23</td>
<td>20</td>
<td>23</td>
<td>28</td>
</tr>
<tr>
<td>Juncaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Juncus gerardii Lois.</td>
<td>6</td>
<td>17</td>
<td>13</td>
<td>-4</td>
<td>7</td>
</tr>
<tr>
<td>Juncus maritimus Lam.</td>
<td>6</td>
<td>15</td>
<td>7</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Juncaginaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglochin maritima L.</td>
<td>6</td>
<td>6</td>
<td>7</td>
<td>7</td>
<td>-4</td>
</tr>
<tr>
<td>Plantaginaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plantago maritima L.</td>
<td>14</td>
<td>18</td>
<td>13</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>Plumbaginaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Armeria maritima (Mill.) Willd.</td>
<td>6</td>
<td>3</td>
<td>8</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Limonium vulgare Mill.</td>
<td>49</td>
<td>60</td>
<td>67</td>
<td>70</td>
<td>38</td>
</tr>
<tr>
<td>Poaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agrostis maritima Lam.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-4</td>
<td>0</td>
</tr>
<tr>
<td>Festuca villosoa Schweigg.</td>
<td>3</td>
<td>2</td>
<td>13</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Puccinellia maritima (Huds.) Parl.</td>
<td>4</td>
<td>6</td>
<td>13</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Sporina maritima (Curt.) Fern.</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Primulaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glaux maritima L.</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Scrophulariaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Odontites litoralis Fr.</td>
<td>13</td>
<td>19</td>
<td>13</td>
<td>7</td>
<td>7</td>
</tr>
</tbody>
</table>

no inhibition –
doubtful inhibition ±
inhibition +
Figure 1. Relative and chronic toxicities of plant extracts to *Artemia salina* larvae.

Figure 2. Relative and chronic toxicities of plant extracts to *Artemia salina* adults.
Figure 3. Relative and chronic toxicities of plant extracts to *Daphnia magna* adults.

Figure 4. Inhibition of tumor growth (potato disc assay) by plant extracts.
The results of the TGIT are totally different from the results of the TIIT although the TGIT method is a variation of the TIIT. The results obtained by visual comparison with the control suggest that only the three Fabaceae species tested show tumour growth inhibiting activities.

The results obtained in the present study are in agreement with literature data at least as far as antitumor activities are concerned. Tests with *A. salina* and *D. magna* have, to our knowledge, not been described in the literature.

The use of *A. maritima* L. against tumours has already been documented in the review by Hartwell (1967) who collected information on over 3000 plants used by several indigenous people for cancer treatment. Other species of the genus *Artemisia* exhibit similar activities (Hartwell, 1968). *L. vulgare* has been employed as a medicinal plant for cancer treatment in Israel (Silva & Abraham, 1981).

Of the Fabaceae *O. spinosa* is known as a medicinal plant used against cancerous ulcers (Hartwell, 1970a). For *O. matrix* L. from Amman, Jordan, a similar inhibition has been reported (Oran, 1999) as obtained here for *O. spinosa* probably indicating genus specific antitumor activity.

Several *Trifolium* species among them *T. repens* have also been employed in cancer treatment (Hartwell, 1970a). In the present study the species of the genus *Trifolium* showed some activities only in the tumour growth inhibition test.

The use of *S. europaea* for cancer treatment has also been reported (Hartwell, 1968) as the use of *Centaurium* species for this purpose (Hartwell, 1969a).

Despite the reported use of species of the genus *Juncus* as anti-tumour medicinal plants (Hartwell, 1969b) the tested Juncaceae *J. gerardii* Lois. and *J. maritimus* lamin. did not show any cytotoxic or antineoplastic activities.

For *Plantago* species a wide range of anticancer uses has been reported (Hartwell, 1970b). The tested *P. maritima* L. was not among them and in the present investigation it did not show any activity.

**Conclusions**

All results together suggest that of the 28 salt marsh species investigated, *Limonium vulgare*, *Artemisia maritima*, *Salicornia europaea* and *Ononis spinosa* are promising candidates for new anticancer compounds and merit further investigation.

**References**


Jensen CO (1918): Undersøgelser vedrørende nogle svulstlig-
nede dannelser hos planter. *Kgl. Veterinaer-Landbo-
hyskoles Aarskrift* 2: 91–143.


