Pharmacognosy Reviews [Phcog Rev.]
Vol 2, Issue 4, Jul-Dec, 2008
Page 206-218

PHCOG REV.
An official Publication of Phcog.Net

PHCOG REV.: Review Article
Anticancer compounds from in vitro cultures of rare medicinal plants

Iliana Ionkova

Faculty of Pharmacy, Medical University of Sofia, Dunav Str. 2, 1000 Sofia, Bulgaria
Correspondence:
e-mail: ionkova@pharmfac.acad.bg
Faculty of Pharmacy, Medical University of Sofia,
Dunav Str. 2, 1000 Sofia, Bulgaria

ABSTRACT
Malignant diseases are the second mortality cause within the human population. Due to their complex structure with several chiral centres important anticancer agents are still extracted from plants and not synthesized chemically on a commercial scale. In the coming decades, several new enabling technologies will be required to develop the next generation of advanced plant-based pharmaceuticals. This review summarizes our results concerning the production of certain anticancer compounds from tissue cultures of rare medicinal *Astragalus* and *Linum* plants. The various problems involved and the possible ways to overcome using biotechnological approaches are discussed on the basis of examples from our own research.
Keywords: plant anticancer compounds, *in vitro* production, saponins, lignans, *Astragalus*, *Linum*

INTRODUCTION

Plant-Made Pharmaceuticals is a category of therapeutic agents (pharmaceutical compounds) produced in live plants. The majority of high-value plant secondary metabolites which the pharmaceutical are still isolated from wild or cultivated plant species. However, many of these plants are difficult to cultivate or are becoming endangered due to over-harvesting (1). Furthermore, the chemical synthesis of plant-derived compounds is often not economically feasible due to their highly complex structures and the specific stereochemical requirements of the compounds. It has been estimated that about 40% of the pharmaceuticals used in the developed world was discovered from natural products, and about 25% from flowering plants (2). About 60% of antitumor and antiinfection drugs are of natural origin (3). Moreover, of the 252 drugs that are considered essential by the World Health Organisation, 11% are obtained exclusively from plants. On the other side it has been estimated that only about 10% of plant species have been tested for biological activity (4).

Cancer remains a significant unmet clinical need, causing millions deaths annually in the world. One out of every four deaths is from cancer. It is estimated that over 1.28 million people will develop cancer each year. Some of the most effective cancer treatments to date are natural products or compounds derived from plant products. Seven from the most consuming anticancer drugs are with plant origin: Etoposide, Teniposide, Taxol, Vinblastine, Vincristine, Topotecan, and Irinotecan. They are some of the most vigour products in cancer therapy and still derive from plants since the chemical synthesis of the chiral molecules is not economic (5, 8).

Market prices for the plant-derived anticancer drugs are quite high: 1 kg of vincristine (*Catharanthus* alkaloid) costs about US$20,000 and the annual world market is about US$5 million per year. Isolation of pharmaceuticals from plants is difficult due to their extremely low concentrations. The industry currently lacks sufficient methods for producing all of the desired plant-derived pharmaceutical molecules. Some substances can only be isolated from extremely rare plants. The biotechnological approach offers a quick and efficient method for producing these high-value medical compounds in cultivated cells. In the future, the new production method may also offer alternatives to other highly expensive drugs.

Biotechnological production in plant cell cultures is an attractive alternative but has so far had only limited commercial success (for example, paclitaxel or Taxol™), due to a lack of understanding of the complex multistep biosynthetic events leading to the desired end-product. Discoveries of cell cultures capable of producing specific medicinal compounds at a rate similar or superior to that of intact plants have accelerated in the last few years in Bulgaria.

Malignant diseases are the second mortality cause within the human population. Despite the serious progress in establishing and introduction of novel specifically targeted drugs the therapy of these diseases remains severe medical and social problem. Due to their complex structure with several chiral centres important anticancer agents are still extracted from plants and not synthesized chemically on a commercial scale. Nowadays, modern patients have been educated to receive highly defined medicines, which have been produced according to sophisticated techniques such as biotechnology. It could well be those very same patients who also turn toward traditional medicines. Europe is reported to have the highest turnover in herbal medicines with a retail sales volume of $ 6 billion per annum, compared to $2.1 billion in Japan, $2.3 billion in the rest of Asia, and around $1.5 billion in North America (5). In Bulgarien, an average of more of 6,000 tons of herbs are gathered annually, most of which represents natural plant resources. The increased interest in, and constantly expanding use of, the medicinal plants clearly raises questions about the alternative production of valuable

biologically active compounds. Although wild plants in many countries are presently under-utilised, the need for sustainable raw material for plant derived pharmaceuticals is projected to grow. The natural variation within plants is very wide and fairly untapped. In most countries, the reserves of these natural resources are highly depleted or the collecting of herbs from their natural habitats is unprofitable.

In the coming decades, several new enabling technologies will be required to develop the next generation of advanced plant-based pharmaceuticals. With modern biotechnology, it has become possible to use plant cells for the production of specific pharmaceuticals. Using the right culture medium and appropriate phytohormones it is possible to establish in vitro cultures of almost every plant species. Starting from callus tissue, cell suspension cultures can be established that can even be grown in large bioreactors. Moreover, the biotechnological production of these plant products is more environmentally friendly way than is currently occurring. Researchers at Ciba-Geigy AG, Basel, Switzerland, have produced the alkaloid scopolamine from cell cultures of *Hyoscyamus aegypticus* grown in air-lift bioreactors. In Germany, Alfermann and co-workers (6) of Boehringer Mannheim AG were able to grow cells of *Digitalis lanata* in 200-litre bioreactors and obtain 500 g of beta-methyl-digoxin in three months. The bioconversion rate of beta-methyl-digitoxin was very high, up to 93.5%. For instance, in France, Sanofi-Elf-Bio-industries have supported the research work on the production of bioconversion of ellipticine - an antitumour alkaloid - by cell cultures of *Ochrosia elliptica*, Apocynaceae (7). The production of anticancer compounds, such as the alkaloids vinblastine, vincristine, paclitaxel (Taxol), camptothecin, or the lignan podophyllotoxin, by plant in vitro cultures is reviewed (8). Taxanes can be produced in bioreactors using cell suspensions of various *Taxus* species with good yields; presently paclitaxel is produced on a commercial scale by Phyton Biotech (Germany). Paclitaxel can be produced in large-scale bioreactors (70,000 litres) with reasonable yields. Taxus is one of the few examples of large-scale production of a valuable secondary metabolite by undifferentiated cells. Such compounds are for example used for expensive special pharmaceuticals. These examples and others show that industrial production of plant cell biomass and secondary metabolites is possible with equipment and processes analogous to those used with micro-organisms.The biotechnological method will provide a more inexpensive and efficient method for producing anti-cancer drugs in the near future.

Research needs of production of anticancer compounds by plant in vitro cultures

Cancer comprises a large variety of malignant tumours that can affect nearly all organs of the body. Treatment includes surgery, radiotherapy and chemotherapy. Cancer cells usually divide much faster than 'normal' cells. Therefore, compounds that stop cell division (e.g. alkaloids, such as vinblastine, vincristine, paclitaxel, docetaxel, camptothecin, colchicine, demecolcine, or the lignan podophyllotoxin) or cytotoxic compounds that directly kill tumor cells (saponins), are the two most commonly employed means of chemotherapy with plant natural products. It was shown that certain triterpene saponins (avicins), are selectively toxic to tumor cells at very low doses -IC_{50}: 0.2 μg/mL for Jurkat cells (9). Many anticancer compounds are still derived from plants since the chemical synthesis of the chiral molecules is not economic. Often, the respective plants are grown in plantations as when they are harvested from nature, conservation problems may arise. Retail prices and demands are relatively high. Since the natural supply is limited, several research groups have explored the possibility of employing plant cell or organ *in vitro* cultures for the biotechnological production of these compounds as alternative. The development of paclitaxel (from *Taxus* species) as an effective drug for the treatment of breast and ovarian cancers illustrates how escalating demands necessitated the development of various methods of biomass production. An extensive program was initiated to develop alternative, renewable resources, scale-up production through tissue culture has been developed by the company, Phyton Inc.

If problems are encountered due to the scarcity of the wild plant or inability to adapt it to *in vivo* cultivation, alternative sources need to be sought. Other species of the same genus or closely related genera may be analyzed for drug content, and other biomass production techniques, such as plant tissue and cell culture can be investigated. Another potential route for bulk production of the active agent is total synthesis, but experience has shown that the complex structures of most bioactive natural products require the development of multi-step bench-scale syntheses which often are not readily adapted to economically feasible large-scale production.

The plant-specific secondary products were long considered as a major limitation for an extensive use of plant-made pharmaceuticals in human therapy. Our goal here is to emphasise all the progress recently made towards humanization of secondary metabolites in plant in vitro cultures, and to illustrate that plant typical anticancer compounds progressively emerge as additional advantages for using this promising expression system.

Production advantages provided by plant in vitro cultures

The two principal advantages of plant-based production systems are:

- Scalability: no other production system offers the potential scalability of plant products. High-value products could be produced in sufficient amounts in plant cell culture and will allow product manufacture on a massive scale that can match global demand.
- Adaptability: In the post-genomic era, it has become feasible to engineer plant cell and tissue cultures, not only to produce complex proteins but also to produce high-value secondary metabolites or entirely novel structures (such as new lead compounds for pharmaceutical industry) (90).

The additional major advantages of a cell culture system over the conventional cultivation of whole plants are: (a) Useful compounds can be produced under controlled conditions independent of climatic changes or soil conditions; (b) Cultured cells would be free of microbes and insects; (c) The

cells of any plants, tropical or alpine, could easily be multiplied to yield their specific metabolites; (d) Automated control of cell growth and rational regulation of metabolite processes would reduce of labour costs and improve productivity; (e) Organic substances are extractable from callus cultures. Production by cell cultures could be justified, for rare products that are costly and difficult to obtain through other means.

Characteristics of Target Botanicals

The target botanicals of this study (*Astragalus* and *Linum*) have been selected as possible candidates for in vitro cultivation for production of anticancer compounds, because each possesses the following characteristics:

- A long history of medical use for traditional treatments and/or modern treatments
- A defined market for raw material
- Diminishing natural supplies due to over harvesting
- Consumer and/or industrial interest

***Astragalus*:**

The medicinal use of *Astragalus* species dates back over 1000 years. *Radix Astragali* (*A.membranaceus, A.mongholicus)* is very old and well known drug in traditional Chinese medicine. The different species are used in traditional medicine in Bulgarien, Turkey, Romania, Serbian, Greece, Russia, China, Japan, Mongolia, Korea and other European and Asiatic countries (10). Some extracts or isolated compounds are clinically tested as well (11-13). Some medicines are introduced in clinical practice (14-16). In recent years, advances in research on the *Astragalus* species have been made, due to their anticancer constituents and medicinal application as an immunostimulant or as anticancer drugs. The *Astragalus* is also well-represented in the common flora, but such is not always the case. Some rare *Astragalus* plants are members of genera - e.g. *A.membranaceus, A.mongholicus* (China, Mongolia, Korea), *A. missouriensis* (New Mexico), *A. aitosensis, A. angustifolius* (Bulgarien) etc. Although many rare *Astragalus* plants are restricted in their distribution to specialized habitats, others can be found on a variety of soil types and in plant communities.

Currently, much of the pharmacological research on *Astragalus* is focused on its immune-stimulating and anticancer saponins, polysaccharides and flavonoids useful in treating immune deficiency conditions. *Astragalus* has demonstrated a wide range of potential therapeutic applications in immunodeficiency syndromes, as an adjunct cancer therapy, and for its adaptogenic effect on the heart and kidneys, described in many publication an summarized (16). *Astragalus* also stimulates the body's natural production of interferon (17).

Triterpenoid Saponins (Astragalosides): *Astragalus* root of many species contains a series of cycloartane triterpene glycosides denoted astragalosides (saponins), that are based on the aglycone cycloastragenol and contain from one to three sugars attached at the 3-, 6-, and 25-positions. (19-20). In the predominant astragalosides I-III, the 3-glucose is acetylated. Several saponins have also been reported that are based on the oleanene skeleton (17, 21).

Polysaccharides: The polysaccharides found in *Astragalus* have received a great deal of attention. They have been shown to play a role in immunomodulatory actions (18). New glucanes (Astraglucanes) and their mixture with other polysaccharides extracted from the roots and rhizomes of *A. membranaceus, A. mongholicus* and other species, endowed with immunomodulating properties, are disclosed. The polysaccharide fraction is highly branched, high molecular weight compound with 1,3 beta glucans. These new products find their application as an aid in radiation antineoplastic therapy, in chemotherapy and in the treatment and prevention of bacterial and viral infections.

Flavonoids: Epidemiologic data suggested that flavonoids consumption may protect against cancer induction in several human tissues. Chemoprevention has the potential to be a major component of colon, lung, prostate and bladder cancer control. The high intake of foods and beverages rich in polyphenols, especially in flavonoids, has been associated with decreased risk of neoplasm (22). Up to now 140 different flavonoids from 77 species *Astragalus* were isolated and identified (23). It is found, that *Astragalus* produced mainly derivatives of quercetin, kaempferol and isorhamnetin - ca. 90% in the genus in various quantity: *Astragalus membranaceus* 0.4% (24), 1.2% in *Astragalus angustifolius* (10) or 7.5% in *A. propingus* (25). New methods for isolation and identification of flavonoids are reviewed (26).

Mechanisms of Action: Research shows *Astragalus membranaceus* root stimulates the immune system in many ways. It increases the number of stem cells in bone marrow and lymph tissue and encourages their development into active immune cells. It appears to help trigger immune ceils from a "resting" state into heightened activity. It also enhances the body's production of immunoglobulin and stimulates macrophages. *Astragalus* can help activate T-cells and natural killer (NK) cells (27). Additionally, the flavonoids, saponins, and polysaccharides found in *Astragalus* root help minimize free radical damage to membranes. The flavonoids and saponins in *Astragalus* can significantly inhibit membrane lipid peroxidation, while the polysaccharides of Astragalus possess weaker protective activity (28).

Cancer Therapy: Different *Astragalus* species has been shown to increase resistance to the immunosuppressive effects of chemotherapy drugs, while stimulating macrophages to produce interleukin-6 and tumor necrosis factor (TNF). Human clinical trials demonstrated a substantial increase in survival rates when extracts from this plants is given to cancer patients receiving chemo- or radiotherapies. It has been also shown to increase IgA, IgC and interferon production in humans. The authors also suggest that by reducing the dosage required in treating cancer patients, the severe side effects of rIL-2 therapy (e.g., acute renal failure, capillary leakage syndrome, myocardial infarction, and fluid retention) might be reduced. These results were confirmed in another study where *Astragalus* potentiated the LAK cell-inducing activity of rIL-2 against an Hs294T melanoma cell line (29).

***Linum*:**

The lignans are a large group of natural substances, which occur in a range of plant species, but only in low concentrations. Their natural abundance however is scarce and their chemical synthesis is not yet economically feasible. Podophyllotoxin (PTOX) is a lignan, which is used as educt for the semisynthesis of etoposide and tenoposide. Both antineoplastic drugs are of importance for the therapie and today and in the future will be a high demand for the commercial drug and its precursors for the treatment of leukemia (30-31). For the production of podophyllotoxin different routes are known: extraction and isolation from *Podophyllum hexandrum, Linum* and other species, production in plant cell cultures (32) and Organic synthesis (31). Extraction and isolation of podophyllotoxin from Indian *Podophyllum hexandrum* species is not economic and characterised by a high price for the final product. Another problem is cultivation of the plant, why mostly the natural product is extracted from wild collected species. Main reason for this is the limited growth rate and not optimised culture conditions for accelerated growth. As a consequence from wild collection *P. hexandrum* is actually endangered in India, especially in the Himalaya region (31-32). Podophyllotoxin (PTOX) and related compounds are present in the plant families *Juniperaceae*, *Berberidaceae*, *Lamiaceae* and *Linaceae* (33).

Up to this moment aryltetralin lignans are actually derived exclusively from plant sources. Due to their restricted natural abundance and important pharmacological application, identification of new sources or rational in vitro synthesis is very important for the production of therapeutic candidates for cancer chemotherapy.

In the rich flora of Bulgarien the genus *Linum* is represented by 19 wild growing species, divided into 14 subspecies, 4 forms, 4 varietets. Of them 2 species are Balkan endemits, 1 – Balkan and Krim endemit, 2- restricted to Eastern and South-eastern Europe, 1 – to South-eastern Europe and 1 – to the Carpathians and Balkan peninsula (34).

A great number of aryltetralin lignans were identified from in vivo and in vitro species of the *Linum* genus in our research group (*L. tauricum* Willd. ssp. *bulgaricum* (Podp.) Petrova *L. tauricum* Willd. ssp. *tauricum L. tauricum* Willd. ssp. *serbicum* (Podp.) Petrova *L. tauricum* Willd. ssp. *linearifolium* (Lindem.) Petrova *L. elegans* Sprun. ex Boiss. *L. flavum* L. ssp. *sparsiflorum* (Stoj.) Petrova *L.capitatum* Kit. ex Schult. var. *laxiflorum* (Stoj.) Petrova, *L. cariense* Boiss, *L. altaicum* Ledeb., *L. austriacum ver euxinum* Juz., *L. lewissi* Pursh., *L. campanulatum* L., *L. setaceum* Brot., *L. africanum, L. strictum* L., *L. leonii* F. W. Schulz., *L. narbonense* L.). Lignans in different samples of *Linum* species, mainly occurring in Bulgaria, were analysed by HPLC-ESI/MS and HPLC-UV/DAD. The ESI/MS fragmentation pathways recently established for aryltetralin lignans are now extended to ester and glycoside derivatives. In total, ca 40 different lignans, mainly of the aryltetralin type, were identified (49). 6-Methoxypodophyllotoxin and its glucoside were present as major constituents in all samples. Differences between the investigated taxa were observed especially with respect to the accumulation of 6-deoxy-7-hydroxy-aryltetralins such as podophyllotoxin and of 6-hydroxy-7-deoxy-aryltetralin lignans of the peltatin type (35). Studies of extracts of in vitro cultures of *L. narbonese* and *L. leonii* have shown cytotoxic activity, due to the presence of arylnaphtalene lignan Justicidin B (36).

The determination of the biosynthesis of various valuable aryltetralin derivatives gives the grounds to conduct precise biotechnological, phytochemical and pharmacological investigations, including elucidation the neu structures of aryltetralin lignans in *Linum* species (35, 49, 73).

Development of *in vitro* cultutres

Plants have always been a suitable source for the production of pharmaceuticals. However, the quality and quantity of active substances from wild collected and field grown plants is often fluctuating and heterogeneous depending on environmental conditions. Infestation, diseases and the application of pesticides additionally decrease the quality of the plant material. *In vitro* culture of plants can overcome these problems, since the environmental conditions that affect plant metabolism can be strictly controlled. Working with plant cells drastically reduces the preparation time, handling and storage costs associated with the traditional whole plant approaches (37). The strong and growing demand in today's marketplace for natural, renewable products has refocused attention on in vitro plant materials as potential factories for secondary phytochemical products, and has paved the way for new research exploring secondary product expression in vitro. However, it is not only commercial significance that drives the research initiatives. There is a series of distinct advantages to producing a valuable secondary product in plant cell culture, rather than in vivo in the whole crop plant. These include the following:

- Production can be more reliable, simpler, and more predictable
- Isolation of the phytochemical can be rapid and efficient, as compared to extraction from complex whole plants
- Compounds produced in vitro can directly parallel compounds in the whole plant
- Interfering compounds that occur in the field-grown plant can be avoided in cell cultures
- Cell cultures can yield a source of defined standard phytochemicals in large volumes

Due to their complex structures saponins, polysaccharides and flavonoids are still most efficiently produced by the plants. Within the *Fabaceae,* production of these compounds has been reported for several genera (10). However there are several problems connected with this production method (16). Variable quantities and qualities of the plant material, plants that need to grow several years before they are ready for harvesting (*Astragalus* roots*)* and over collecting of endangered species (*A.membranaceus*, *A.mongholicus, A. missouriensis, A. angustifolius, A. thracicus, A. aitosensis* etc.) are just a few of the problems connected with the production of these natural products. Therefore, cultured cells rather than plants are as a possible alternative production method.

The studies on culture cells of *Astragalus* began with the report on callus cultures of *A. hamosus* and *A. boeticus* (38). In order to achieve an industrial production one has to obtain a stable high-producing cell line of the *Astragalus* plant. For this - two approaches are being used - screening and selection for high producing cell lines and optimization of growth and production medium and conditions (39-40).

Our research was focused on the use of plant tissue cultures of *Astragalus* plants for improvement of natural compound production (41-42). Now we have cell lines of various plant species of interest in our laboratory. In a screening program a large number of cell lines from different species of *Astragalus* are set up (43), derived preferably from high-producing plants. Since the root is mostly the site of saponin and polysaccharide biosynthesis, in vitro root cultures from different *Astragalus* species have been able to produce large amounts of secondary metabolites (16). Summary of studies on in vitro cultures of *Astragalus* is given in Table 1.

Although undifferentiated suspension cultures are very "convenient" for the development of secondary metabolites production, with their high growth rates and many possibilities to enhance it, mentioned above, there is one very significant drawback. Although the plant cell is considered to be totipotent, the biosynthesis of many second metabolites requires a certain level of differentiation of the tissues. Experiments have proven that although not present in an undifferentiated culture, with the inducement of somatic embryoids, roots or shoots, these compounds are detectable in the plant tissue again (44). The maintenance of a culture in a certain differentiated state is obtained through the phytohormone regime. A certain type of "hairy roots" culture can be induced by means of transformation with a specific soil *Agrobacterium rhizogenes*, and can be further maintained without phytohormones in the medium (45).

Four different bacterial strains were used in our experiments with *Astragalus* plants - TR 105, R 1601, ATCC 15834, and LBA 9402. The phenotypic response results from the insertion into the plant genom of T-DNA (transfer DNA) was different. In some of the more recalcitrant *Astragalus* species, successful transformation was achieved with a variety of *Agrobacterium rhizogenes* strains showing different host specificites. Howewer, different strains of bacteria showed various abilities to induce hairy roots on the leaf explants on the same *Astragalus* species. The difference in virulence could be explained by the plasmids harboured by bacterial strains (46).

In order to improve the frequency of transformation, two methods were attempted. It has been demonstrated that *Agrobacteria* are attracted to host across chemical gradients of phenolic compounds released by injured plant cells. One specific highly active compound in this respect has been identified as acetosyringone. Acetosyringone have been reported to increase *Agrobacterium* - mediated transformation frequencies in a number of plants (47). In some of the more recalcitrant species, successful transformation was achieved by including 10 ¤M acetylsyringon in the medium in which the bacteria are suspended. The addition to the cocultivation medium of acetylsyringone changed the frequency of transformation and resulted in a substantial increase in the number of transformation events. This compound, produced during the wounding response of plants, activates the *vir* genes of *Agrobacterium*, aiding plasmid T-DNA transfer (48). The number of individual roots formed has been used as a measure of virulence. The results show that acetylsyringone can be used to increase *Agrobacterium*-mediated transformation frequencies in *Astragalus* species as well.

The hairy root cultures (HR) from different *Astragalus* species grown rapidly in simple media without phytochormones (43) were stable in their growth rate, polysaccharide and saponine production over a period of more than 10 years in cultures (49). The hairy root clones, transformed with different bacterium, were cultured individually. Agrobacterium strain had a some effect on growth and content of biologically active compounds. The clones infected by ATCC 15834 and LBA 9402 grew the fastest and the biomass increased 2-3 fold more than the other clones after 28 days of cultures. Moreover, different *Agrobacterium* strains have an effect on total saponin content in older hairy roots. Maximum saponin content was found in HR of *A. mongholicus*, transformed with LBA 9402 bacteria (49).

Saponins: The results of Tappe et al. (50) demonstrate that the saponin production by the hairy root cultures is typical of those found in many species of *Astragalus* (43, 49). The transformed roots produce cicloartane saponins which do not differ significantly from those produced by the parent plants. Analysis of saponins of *A. mongholicus*-HR and *A. membranaceus* was performed after lyophilization, extraction and column chromatography, as described earlier (10, 16). In order to confirm the structure of saponins, the NI-FAB, MS/MS, EI-MS, ^{1}H- and ^{13}C-NMR (1D and 2D) were made (16, 50). A. *mongholicus* HR produces cycloastragenol-saponins: astragalosid I, astragalosid II and astragalosid III (42, 50). On the basis of spectral analysis and chemical investigations including MS, ^{13}C-NMR, and ^{1}H- NMR, the structure of aglicons was assignated as 9,19-cyclolanostane (cycloastragenol), one artifact, - lanost-9(11)-ene (astragenol), which was secondary formed during acid hydrolysis from cycloastragenol (molecular ion at m/z 490, base pea at m/z 143) and soyasapogenol B with molecular ion at m/z 458, base peak at m/z 234 (10, 16). Another work concerning cycloartane triterpeneglycosides from the hairy root cultures of *A. membranaceus* has been reported by Hirotani et al. (51). Agroastragalosid II, a new astragaloside was isolated from this hairy roots line. Its structure was established as 3-O-¤-(2'-O-acetyl)-D-xylopyranosyl-6-O-¤-D-glucopyranosyl-(24S)-3¤,6¤,16¤,24,25-pentahydroxy-9,19-cyclolanostane on the basis of various spectroscopic data.

HR cultures of *Astragalus* spp. released part of the saponin product (about 16-20% of the total saponin) into the extracellular medium. This is essential, in order to establish continuous production of saponins, because permit recycling of the biomass (e.g. via immobilization) and would help reduce production costs. The most apparent effect in saponin production was seen when sucrose levels of the medium were

modified. A medium (MS) containing 2% sucrose increased overall saponin yield, but the growth was very low. The optimal medium for both yield and growth was supplemented with 4% sucrose. The saponin content was not impaired significantly at high levels of nitrogen. These results suggest that biosynthetic regulation is affected by altered cell organization in roots, as might be expected. HR cultures of *Astragalus* spp. can therefore be an interesting system by which fast growth of the biomass as well as relatively high saponin production can be achieved, facilitating further studies on tritrpene saponin biosynthesis.

Because phytosterols (campesterol, stigmasterol and ¤-sitosterol) are present in hairy roots of *Astragalus* species (10), the effect on the total saponin production of these substances were examined. Since the biosynthetic route of saponins and that of phytosterols are branched at 2,3-epoxyscvalen (42, 49) it may be possible to increase the production of saponins by end-product inhibition. In the experiments, contents of saponins after the addition of 10 mg/50ml of ¤-sitosterol in MS liquid medium without ammonium nitrate were examined at different stages of growth. The saponine content (total saponins) in the hairy roots induced by LBA 9402 reached 5.25 % of dry wt at the 28 day of cultivation. When ¤ -sitosterol was added in the culture media of this hairy roots, astragaloside production was remarkably increased to 7.13 % of dry wt and led to an increase of 36% of the total saponin content in comparison with the control. From these data, ¤-sitosterol seem to behave as inhibitor in biosynthetic route when amount added is relatively large. These results prove that the hairy root cultures of *Astragalus mongholicus* can be a valuable alternative approach for the over-production of cycloartane saponins compared with the whole plant. Using a selected high productive clone, inducing by *Agrobacterium rhizogenes* LBA 9402, optimized culture medium (MS without ammonium nitrate) and end-product inhibition, a relatively high saponin production can be achieved.

Polysaccharides: Dedifferentiated callus and suspension yield very few polysaccharides (16, 52). However, HR of *A. gummifer, A. mongholicus, A. membranaceus, A. brachycera, A. canadensisis, A. falcatus, A. oxyglotis* and *A. sulcatus* produce significant amounts of polysaccharides, which are secreted into the liquid growth media. They account for the viscosity of the media. The presence of macromolecules in the culture media has been described by a number of investigators (16, 53); experiments with different *Astragalus* species showed similar results. *A.mongholicus* secreted water-soluble polysaccharides 8.5 wt%, *A. gummifer* 19.5 wt%, and *A. membranaceus* 7.3 wt%. These polysaccharides can be isolated from the medium by simple ethanol precipitation (16, 49). Monosaccharide composition of the macromolecules in various cultures and parent plants have been imvestigated. Differences in the sugar composition of polysaccharides between HR and intact plants have been found. Furthermore, the amounts of each neutral monosaccharide produced in the various HR cultures were not uniform, and composition varied widely. The polysaccharides of the mother plant contained more compounds different to the mucilage of HR. We suggest that this difference can be attributed to the different stages of development in the transformed roots.

Flavonoids: The use of flavonoids for prevention and cure of human diseases is already widespread. Epidemiologic data suggested that flavonoids consumption may protect against cancer induction in several human tissues. Chemoprevention has the potential to be a major component of colon, lung, prostate and bladder cancer control (54). A number of investigators have reported that flavonoids inhibit the tumour growth by interfering with some phases of the cell cycle. Quercetin shows anti-proliferative effects against various cancer cell lines. These aspects made flavonoids an interesting object for industrial production.

Flavonoid produced from cell cultures of *A. missouriensis, A. hamosus, A. edulis* as well as optimization of cultures and production conditions, has been achieved (16). Flavonoids produced in uniform plant-cell culture systems offer a novel vehicle for in-depth investigation of these compounds individually. Cell-culture-derived flavonoids can be more easily separated in an intact polymeric form than flavonoids within complex plant tissues. In *A. missouriensis* cell cultures the main aglycon identified was Quercetin in both free and bound forms (as glycosides). Isoquercitrine (quercetin-3-O-glucoside) and Quercitrine (quercetin-3-O-rhamnoside) were the main flavonoid glycosides in all tested cell lines. Rutin (quercetin-3-O-rutinoside), Hyperoside (quercetin-3-O-galactoside), Scopoletin and Phenolcarbonic acids - p-coumaric and chlorogenic have been also detected. In order to increase flavonoid production *in vitro* the effect of plant growth regulators (auxins and cytokinins) and sucrose concentration were optimized (55).

Phytosterols: As for cancer protection, it has been estimated that diets rich in phytochemicals can significantly reduce cancer risk by as much as 20%. Phytosterols are specific phytochemicals that resemble cholesterol in structure but are found exclusively in plants. Phytosterols are absorbed from the diet in small but significant amounts. Epidemiological data suggest that the phytosterol content of the diet is associated with a reduction in common cancers including cancers of the colon, breast, and prostate (56). Hairy root lines from *Astragalus* species are rich of sterols, identified by GC-MS (Incos 50, Finigan MAT, 10 mA/sec.) as β- sitosterol, Stigmasterol and Campesterol (49).

Bioreactor strategie for Transformed roots of Astragalus: The special morphological characteristics of hairy roots cultures call for the introduction of bioreactors which provide a suitable environment for growth and production of secondary metabolites. Transformed roots have low oxygen demand, unlike suspension cultures where change in oxygen level can significantly affect growth and productivity. A rapidly growing root lines of *A. membranaceus/A. mongholicus* were selected for cultivation in air lift bioreactor, because they have been considered to be sensitive in mechanically agitation. The biomass concentration showed a high final density above 17 g dry roots pro liter medium for 35 days of culturing. Part of the saponins and polysaccharides were released in growth

medium. The roots retained high internal product levels and remained viatble. The results provides an efficient means for cycloartan saponin production on large scale, irrespective of geographic locations and climatic conditions (16, 49).

The above mentioned examples clearly indicate that present investigations provides a high astragaloside-yielding line of *A. membranaceus*/*A. mongholicus* that can synthesize and accumulate astragalosides (both in content and quality) that are produced in field grown roots of this plant species. The main advantages are:

- The hairy roots line is capable of producing astragalosides (within 30-40 days) in yield more and quality comparable to that of 3-5 years old roots of field-grown plants.
- It has resulted in the generation of a viable alternative source (roots line) for the commercial production of astragalosides.
- The investigations assumes significance considering the worldwide demand for *Astragalus* saponins and the problems associated with *Astragalus* cultivation on account of its prolonged seed dormancy and long gestation period from planting to harvest (3-5 years).
- The cultural procedure and conditions used are fully defined and reproducible.

Lignans - promising anticancer agents: Antitumor activity will undoubtedly continue to be the most clinically relevant property of lignans. The production of anticancer compounds, such as lignan podophyllotoxin, by plant in vitro cultures from different plant species is reviewed (33). Cell cultures of different Linum species of section Syllinum are shown to produce considerable amounts of lignans, mainly MPTOX. Although the both PTOX and MPTOX have comparable cytotoxic activity, due to the different substitution in position 6, MPTOX is not used for the production of anticancer drugs (57). Results, summarized in Table 2, show that a broad range of experiments have been carried out, resulting in enhancement of lignan production of *in vitro* cultures from *Linum* species. Since PTOX is the preferred precursor for the semi-synthesis of anti-cancer drugs like etoposide and etopophos®, the accumulation of predominantly PTOX is especially interesting. *L. linearifolium* is now beside *L. album and L. persicum* the third *Linum* species of section Syllinum with PTOX (ca 0.8% DW) as the main lignan (58).

As a new biotechnological alternative is our success in stereoselective hydroxylation of deoxypodophyllotoxin by recombinant CYP3A4 in *Escherichia coli* to epipodophyllotoxin (59), the direct precursor for the semi-synthesis of anti-cancer drugs etoposide (VP-16) and teniposide (VM-26).

Detection of Potential Anticancer Agents from in vitro plant cultures

Mechanism-based approach

The tremendous biodiversity within the plants is based upon a large pool of genetic information which encode an enormous diversity of chemicals. The enabling plant cell culture technology accesses this chemical diversity and further amplifies it by culture manipulation, yielding a variety of chemicals with pharmaceutical potential. Rapid discovery of lead compounds with high activity is essential for the successful development of novel drug candidates. During the last decade, natural product drug discovery research has evolved into a mechanism-based approach (60) as advances in selection of specific products (61). An important feature of mechanism of some antitumor agents is that they have ability to interaction with DNA. Application of a this approach, employing DNA for screening and bioassay-guided fractionation of higher plants and cell culture extracts led to the found of natural product with potential anticancer activity. Application of HPLC system for the detection of compounds capable of binding to DNA can be used for early drug discovery research. The results of Gupta et al (61) and Ionkova and Alfermann (62) show that, in general, there is a corelation between the DNA-intercalation and tumor inhibition or cytotoxicity. The use of mechanism-based screens has the advantage of speed and simplicity, and we have applied this approach in our work. As a continuation of our phytochemical and biological studies on potential anti-tumor chemoprevantive agents, we carried out primary screening of many natural products and plant extracts (62).

The purpose of this investigation was to evaluate several plant species and cell cultures for a potential antitumor agents. In addition, the test-system was optimised in an attempt to gain more obective information on the possible antitumor substances present in the plant extracts evaluated. Extracts of various plant species was tested for the presence of DNA binding compounds. The results of a primary screening test on the DNA-intercalation effects of over 100 plant extracts and isolated compounds from plants and plant cell cultures were described (62). Of the ca 100 tested plant extracts - 23% proved active in DNA intercalation. Methanol and butanol extracts of *Astragalus angustifolius, Astragalus aitosensis, Astragalus thracicus* showed significant DNA intercalation effect (over 50%) (Table 3).

Calf thymus DNA and pUC19 (ATCC 37254) E.coli plasmid was evaluated in order to optimize the test system. For routine screening procedures, there are some advantages to using pUC19, since is technically easier to handle in comparison with high-molecular weight DNA. A good correlation was obtained between the response observed with calf thymus DNA and pUC19 plasmid. The results, obtained with pUC19 were more clear. The time for washed of the column was reduced to 15 min. These minor modification gave better reproducibility in DNA determination and solutions produced are less viscous (62).

Extracts of callus and transformed roots from different plant species (Table 4) was tested for their DNA-interaction possibilities. As a result the DNA - intercalation effect, was clearly seen in many extracts from callus culture of *Astragalus* in the HPLC - detection system. The majority of the *Astragalus* extracts are rich of saponins. We have found that there was correlation in DNA - interaction and the hemolytic effect in some *Astragalus* plant extracts (Table 5). This results are supported by published from Bader et al. (63), for accordance of cytotoxicity against tumor cells with the hemolytic effect of saponins.

PHCOG REV.
An official Publication of Phcog.Net

Table 1. Production of secondary metabolites from in vitro cultures of Astragalus species

Astragalus spp.	Explant	Culture system	Compounds isolated	Ref.
A. hamosus	Leaves, stem, root	Callus, suspension hairy roots	Saponins, Soyasapogenol B, β-sitosterol, Astragalin, Rutin, Isorhamnetin-3-O-glycoside phenol acids	38, 40
A. boeticus	Leaves, stem, root	Callus, suspension hairy roots	Saponins, Soyasapogenol B, β-sitosterol,Flavonoids, Phenol acids	16, 38
A. missouriensis	Leaves, stem, root	Callus, suspension hairy roots	Isoquercitrin, Quercitrin, Rutin, Hyperoside, Phenol acids, Saponins	16, 38
A. edulis	Leaves, root	Callus	Quercetin, Kempferol. Isorhamnetin, Saponins	16, 38
A. gummifer	Leaves, stem, root	Callus	Polysaccharides	53
		Root cultures	Polysaccharides	53
		hairy roots	Polysaccharides	53, 16, 52
A. membranaceus	Leaves, stem, root	hairy roots	Polysaccharides	16, 52, 67, 51
		Air lift fermenter	Cycloastragenol, Astragenol, Soyasapogenol B, β-sitosterol, Stigmasterol, Campesterol, Astragalosides I-III, new astragaloside	
A. mongholicus	Leaves, stem, root	hairy roots	Astragalosides I-III, β- sitosterol, Stigmasterol, Campesterol, Polysaccharides	16, 52, 67, 68
A. glycypyllos	Leaves, stem	hairy roots	Cycloastragenol, Astragenol, Soyasapogenol B, Cycloartane saponins	16, 49, 67
A. englerianus	Leaves, stem	hairy roots	Cycloartane saponins	16, 67
A. monspessulanus	Leaves, stem	hairy roots	Cycloartane saponins	16, 67
A.brachycera	Leaves, stem	hairy roots	Cycloartane saponins Polysaccharides, Sterols	16, 69
A.canadensis	Leaves, root	hairy roots	Cycloartane saponins Cycloastragenol, Astragenol, Polysaccharides	16, 49, 69
A.falcatus	Leaves, root	hairy roots	Cycloartane saponins Polysaccharides	16, 49, 69
A. oxyglotis	Leaves, stem	hairy roots	Cycloartane saponins Polysaccharides	16, 69
A. sulcatus	Leaves, stem	hairy roots	Cycloartane saponins Polysaccharides, Sterols, Swensonine	16, 69

Table 2. Production of secondary metabolites from in vitro cultures of Linum species

Species	In vitro culture	Lignans synthesized	Ref.
Linum album	Suspension	PTOX, 6MPTOX, DPTOX, Pinoresinol, Matairesinol, Lariciresinol, β-peltatin, α-peltatin	72
Linum altaicum	Cell cultures	Justicidin B Isojusticidin B	73
Linum austriacum	Callus, Suspension, Root, Hairy root	Justicidin B, Isojusticidin B	74
Linum austriacum ssp. *euxinum*	Cell cultures	Justicidin B, Iisojusticidin B	73
Linum africanum	Callus, Suspension	PTOX, DPTOX	75
Linum campanulatum	Callus, Suspension	Justicidin B	76, 77
Linum cariense	Suspension	6MPTOX 5'-demethoxy-6-methoxypodophyllotoxin, and the corresponding 8'-epimers 6-methoxypicropodophyllin, 5'-demethoxy-6-methoxypicropodophyllin	73
Linum elegans	Callus, Suspension	6MPTOX	71
Linum flavum	Root	6MPTOX	78, 79
Linum flavum	Suspension	6MPTOX	80, 81
Linum flavum	Suspension, Root like tissue	6MPTOX, 5'-demethoxy-6-methoxy-PTOX	82, 83
Linum flavum	Hairy roots	6MPTOX	84
Linum flavum	Hairy roots	Coniferin	85
Linum leonii	Callus,	Justicidin B	36, 76

Linum leonii	Hairy roots	Justicidin B	66
Linum lewisii	Cell cultures	Justicidin B, Isojusticidin B	73
Linum linearifolium	Callus, Suspension	PTOX, 6MPTOX	49
Linum mucronatum ssp. *armenum*	Shoot, Suspension	6MPTOX, PTOX	86
Linum narbonese	Callus	Justicidin B	36,76
Linum nodiflorum	Suspension	6MPTOX	87
Linum nodiflorum	Suspension	6-MPTOX, DPTOX, PTOX	88
Linum persicum	Callus, Suspension	PTOX, 6MPTOX, α- and β-peltatin	89
Linum tauricum	Callus, Suspension	6MPTOX	70
	Shoots, Hairy roots	4'-demethyl-6MPTOX	

Table 3. Evaluation for DNA-interaction activity of extracts from Astragalus species.

Plant species	*In vitro* cultures	Extract	DNA-interaction (%)
Astragalus aitosensis	roots	BuOH	0%
Astragalus aitosensis	above-ground	BuOH	57%
Astragalus angustifolius	roots	BuOH	0%
Astragalus angustifolius	above-ground	BuOH	90%
Astragalus asper	leaf/flower	MeOH	0%
Astragalus brachicera	above-ground	MeOH	68%
Astragalus cicer	seeds	MeOH	90%
Astragalus contortuplicatus	seeds	MeOH	90%
Astragalus cymbicarpus	seeds	MeOH	63%
Astragalus echinatus	seeds	MeOH	0%
Astragalus englerianus	above-ground	MeOH	0%
Astragalus glycyphyllos	above-ground	MeOH	85%
Astragalus lusitanicus	above-ground	MeOH	58%
Astragalus monspessulanus	twig/leaf	MeOH	56%
Astragalus onobrichis	above-ground	MeOH	0%
Astragalus pugionifer	roots	MeOH	0%
Astragalus sesameus	seeds	MeOH	70%
Astragalus spruneri	twig-leaf	MeOH	63%
Astragalus tracicus	roots	BuOH	90%
Astragalus tracicus	above-ground	BuOH	0%

Table 4. Evaluation for DNA-interaction activity of extracts from in vitro cultures.

Plant species	*In vitro* cultures	Extract	DNA-interaction (%)
Albizia julibrissin	callus	MeOH	48%
Althaea officinalis	HR	MeOH	0%
Astragalus brachicera	HR	BuOH	43%
Astragalus glycyphyllos	HR	MeOH	41%
Astragalus gummifer	HR	BuOH	0%
Astragalus membranaceus	HR	BuOH	54%
Astragalus missouriensis	callus	MeOH	90%
Catharanthus roseus	callus	MeOH	0%
Glycyrrhiza echinata	callus	MeOH	0%
Glycyrrhiza glabra	callus	MeOH	0%
Linum africanum	callus	MeOH	28%
Verbascum pseudonobile	callus	MeOH	0%
Astragalus mongholicus	HR	BuOH	51%
Hyoscyamus reticulatus	HR	$CHCl_3$	0%
Datura innoxia	HR	$CHCl_3$	0%
Hypericum revolutum	callus	MeOH	0%
Hypericum innodorum	callus	MeOH	0%
Hypericum olimpicum	callus	MeOH	0%

Table 5. Correlation in DNA-interaction and hemolytic activity

Plant species	Plant part	Hemolytic Index	DNA-interaction (%)
Astragalus aitosensis	roots	<1000	0%
	above-ground	5000	57%
Astragalus angustifolius	roots	<1000	0%
	above-ground	7000	90%
Astragalus thracicus	roots	>7000	90%
	above-ground	1000	0%

This method do not necessarily establish that the compound under investigation has antitumor properties, but it is indicative of the DNA-toxic nature of the material under investigation. Most, but not all compounds exhibiting DNA-intercalation activity well be found to elicit in vivo antitumor activity. Therefore the final selection of an antitumor agent for clinical use should be made only after extensive *in vivo* pharmacological test. Application of the intercalation test is repaid and economic for the prescreen and bioassay-guided fractionation of crud extracts from higher plants and *in vitro* - cell cultures.

Cytotoxic activity on human tumor cell lines

To evaluate new molecules using cell culture studies and to select the molecules with the best chance of treating cancer in vitro cytotoxic activity on human tumor cell lines were investigated. The antiproliferative action of the extracts was tested against panel malignant cell lines (the chronic myeloid leukemia – derived cell lines K-562 and LAMA-84, the Hodgkin lymphoma-derived HD-MY-Z and the human urinary bladder carcinoma-derived EJ cells) with etoposide as a positive control. The leukemic cells were supplied from the German Collection of Microorganisms and Cell Cultures (DSMZ GmbH, Braunschweig, Germany), whereas the human urinary bladder carcinoma-derived cell line EJ was obtained from the American Type Culture collection (Rockville, MD, USA). The cells were maintained as suspension type culture (leukemias), semiadherent culture (HD-MY-Z) or monolayer culture (EJ) in a controlled environment. The MTT-dye reduction assay results in dose-response curves that allow estimating the IC_{50} values for the different compounds. The *Linum* - extracts showed a moderate cytotoxicity to all tested cell lines (36, 58, 64, 65).

Conclusion

Plant cell and organ cultures constitute a promising future for the production of numerous valuable anticancer compounds, although efforts in this field have so far had limited commercial success. Empirical approaches have long been employed for the development and optimization of plant cell-based bioprocesses, focusing on input (cell line, medium, culture parameters, bioreactors, process operations, etc.) and output factors (cell growth, nutrient uptake, productivity, yield, etc.). In this context, we have developed conventional cell and hairy root cultures of many rare medicinal plant species, mainly spread in Bulgarien and Balkans, for the optimization of the production of anticancer compounds. Recently, the interest of international pharmaceutical industries has been directed more and more to biotechnology. We believe that cell cultures of *Astragalus* and *Linum* plants as source of biologically active anticancer compounds can play a role in this respect.

Abbreviations. - PTOX – podophyllotoxin; 6MPTOX – 6-methoxypodophyllotoxin; DPTOX – deoxypodophyllotoxin; 4'DM-6MPTOX -4'-demethyl-6-MPTOX, HR -hairy root cultures.

Acknowledgements

Financial support from Ministry of Education and Science, Sofia, Bulgaria (grant I. Ionkova) is acknowledged.

References

1. I. Raskin. Plants and pharmaceuticals in the 21st century. In: Proceedings of the 10th IAPTC&B Congress - Plant Biotechnology 2002 and Beyond. I. Vassil (ed.) Kluwer Academic Publishers, Dordrecht, Boston, London pp. 83-95 (2002).
2. P.J.Houghton. The A B C of modern pharmacognosy. In Proceedings of the International Conference on Medicinal and Aromatic Plants part II Eds.E Szoke et al., Acta Hort. 597: 151-158 (2002).
3. Yue-Zhong Shu. Recent Natural Products Based Drug Development: A Pharmaceutical Industry Perspetive. *J. Nat. Prod.* 61: 1053-1071 (1998).
4. R.Verpoorte. Pharmacognosy in the New Millennium: Leadfinding and Biotechnology. *Journal of Pharmacy and Pharmacology* **52:** 253-262 (2000).
5. J. Dechamp. Herbal medicinal products and patents need in Europe. *Drug Information Journal* **33**: 309-313 (1999).
6. W. Alfermann, H. Spieler, E. Reinhard, Biotranformation of cardiac glycosides by Digitalis cell cultures in air lift reactors. In: Neumann, K.H.; Ban, W.; Reinhard, E. (eds.). Primary and secondary metabolisomf plant cell cultures, Berlin, Springer-Verlag, 316-224 (1985)
7. A. Sasson. Production of useful biochemicals by higher-plant cell cultures: biotechnological and economic aspects. *Options Méditerranéennes* - Série Séminaires **14**: 59-74 (1991)
8. M. Wink, W. Alfermann, R. Franke, B. Wetterauer, M. Distl, J. Windhövel, O. Krohn, E. Fuss, H. Garden, A. Mohagheghzadeh, E. Wildi and P. Ripplinger. Sustainable bioproduction of phytochemicals by plant *in vitro* cultures: anticancer agents. *Plant Genetic Resources*, **3**: 90-100 (2005)
9. V. Haridas, C. Arntzen, J. Gutterman. Avicins, a family of triterpenoid saponins from *Acacia victoriae* (Bentham), inhibit activation of nuclear factor-κB by inhibiting both its nuclear localization and ability to bind DNA. *PNAS*, 98: 11557-11562 (2001).

10. I. Ionkova. Phytochemical investigation of saponins, sapogenins and flavonoids species of *Fabaceae* family. Ph.D. Dissertation, Medical Academy, Bulgaria, Fac. Pharm, Sofia (1983).
11. N. Stojanov. Astraglus glycyphyllos. In: Ularova K (ed) Our medicinal plants, Nauka and Iskustvo, part I, Sofia, pp 165-166 (1972).
12. M. Alania. Cycloartanic triterpenoids of plants. *Rast.Resur.* **4:** 510-523 (1985).
13. J. Zhon. Chinese medicinal herbs in the treatment of viral hepatitis. In: Chang et al. (eds) Advances in Chinese medicinal materials research. World Sci, Singapore, pp. 215-219(1985).
14. I. Isaev, Gorovitz A, Abubakirov H Progress in chemistry of cycloartanes. *Chim Prirod Soedin*, **2:** 156-157 (1989).
15. I. Gubanev, Krilova I, Tichonova V. *Astragalus* In: Agrar plants in USSR, Misl, Moscow, pp193-202 (1976).
16. Ionkova I. In vitro cultures and formation of secondary metabolites in Astragalus, In: Biotechnology in Agriculture and Foresty, Medicinal and Aromatic Plants, VIII, (ed. Y.P.S. Bajaj), Springer Verlag, Berlin, Heidelberg, 97-138 (1995).
17. Pistelli L. Secondary metabolites of genus Astragalus: Structure and biological activity, In: *Studies in Natural Product Chemistry* (ed. Atta Rhaman), Vol. 27: 443-545 (2002).
18. McKenna DJ, Hughes K, Jones K. Astragalus. *Int J Integr Med*, **4**: 40-46 (2002).
19. He Z, Findlay J. Constituents of Astragalus membranaceus. *J Nat Prod,* **54**: 810 (1991).
20. H. Budzikiewicz, P.Kormali, I.Ionkova. The stereochemistry of Astragalus saponins: A new sapogenine from Astragalus aitosensis Ivanisch., In: Essays on Science, H.Said (ed.), Hamdard Fondation Pakistan, Karachi, 75-83 (1992).
21. S. Nikolov, I.Ionkova, D.Panova, H.Budzikiewicz, E.Schroder. Soyasapogenol B from Astragalus angustifolius lam. *Comptes rendus de l/Academie Bulgare des Science*,38(7), 875-879 (1985).
22. S.Thomasset, Berry, D. Garcea, G. Marczylo, T. Steward, W. Gescher, A. Dietary polyphenolic phytochemicals - promising cancer chemopreventive agents in humans? A review of their clinical properties. *Int. J. Cancer*, **120**: 451-458 (2007).
23. Krasteva I., Benbasad N., S. Nikolov (2000), Pharmacia, Sofia, 47, (3-4): 20-25
24. Lin LZ, He XG, Lindenmaier M. Liquid chromatography-electrospray ionization mass spectrometry study of the flavonoids of the roots of Astragalus mongholicus and A. membranaceus. *J Chromatography A*, **876:** 87-95 (2000).
25. A. Rizk. Astragalus In: The phytochemistry of the flora of Qatar (Rizk A ed), A Copyright, Qatar, pp 246-254 (1986).
26. Rijk E., P. Out, Wilfried M., A. Niessen, F. Ariese, Cees G., U. Brinkman. Review: Analytical separation and detection methods for flavonoids, *Journal of Chromatography A*, Vol. 1112, (1-2): 31-63 (2006).
27. Yang YZ, Jin PY, Guo Q, et al. Effect of *Astragalus membranaceus* on natural killer cell activity and induction of alpha- and gamma-interferon in patients with Coxsackie B viral myocarditis. *Chin Med J* (Engl) **103**:304-307 (1990).
28. Toda S, Shirataki Y. Inhibitory effects of Astragali radix, a crude drug in Oriental medicines, on lipid peroxidation and protein oxidative modification by copper. *J. Ethnopharmacol* **68**: 331-333 (1999).
29. Wang Y, Qian XJ, Hadley HR, Lau BH. Phytochemicals potentiate interleukin-2 generated lymphokine-activated killer cell cytotoxicity against murine renal cell carcinoma. *Mol Biother*, **4:**143-146 (1992).
30. Damayanthi Y, Lown JW. Podophyllotoxins: current status and recent developments. *Curr Med Chem.*, **5:** 205-252(1998)
31. You, Y. Podophyllotoxin derivatives: current synthetic approaches for new anticancer agents. *Curr Pharm Des.* 11(13):1695-717 (2005).
32. Farkya,2004 Farkya S, Bisaria VS, Srivastava AK. Biotechnological aspects of the production of the anticancer drug podophyllotoxin. *Appl Microbiol Biotechnol*., **65:** 504-519 (2004).
33. I. Ionkova. Biotechnological Approaches for the Production of lignans, *Pharmacognosy Reviews,* Vol.1, Issue 1, 57-68 (2007)
34. N. Andreev. Flora of The Peoples Republic of Bulgaria, Sofia, Bulgarian Academy of Sciences, VII, 82-84 (1979).
35. N. Vasilev, R. Ebel, R. Edrada, W. Alfermann, E. Fuss, I. Ionkova, A. Petrova, and T. Schmidt. Metabolic Profiling of Lignan Variability in Linum species of Section Syllinum native to Bulgaria. *Planta Medica*, **74:** 273-280 (2008).
36. N. Vasilev and I. Ionkova Cytotoxic activity of extracts from *Linum* cell cultures. *Fitoterapia* **76:** 50-53(2005).
37. A. Gerth, D. Schmidt, D. Wilken. The production of plant secondary metabolites using bioreactors. *Acta Horticulturae* 764 Internat. Symposium on Plant Biotechnology: From Bench to Commercialization. (2006).
38. I. Ionkova, W.Alfermann. Transformation of Astragalus species by Agrobacterium rhizogenes and their saponin production. *Planta Medica*, **56:** 634-635 (1990).
39. I. Ionkova, W.Alfermann. Production of flavonoids in callus and suspension cultures from Fagopyrum tataricum, *Pharmacy*, Sofia, XLI, **5-6:** 21-26 (1991).
40. I. Ionkova. Production of saponins by konventional and transformed cultures of Astragalus hamosus (Fabaceae), *Problems of Pharmacy and Pharmacology*, Sofia, vol.V, 31-37 (1991).
41. I. Ionkova, T. Kartnig. Densitometric determination of cycloartane triterpene saponins from the transformed root cultures of Astragalus mongholics Bge., *Die Pharmazie*, vol. 52 (3): 218-220 (1997).
42. I. Ionkova, T. Kartnig, W. Alfermann. Cycloartane saponin production in hairy root cultures of Astragalus mongholicus Bge., *Phytochemistry*, vol. 45, N 8, 1597-1600 (1997).
43. I. Ionkova Genetic transformation in Astragalus spp., In: Biotechnology in Agriculture and Forestry vol. 45, Transgenic Medicinal Plants (ed. YPS Bajaj), Springer Verlag, Berlin, Heidelberg, 55-72 (1999).
44. J. V. Shanks and J. Morgan, Plant hairy root culture, *Curr. Opin. Biotechnol.* **10:** 151-155 (1999).
45. A. Giri and M. Narasu, Transgenic hairy roots: recent trends and applications, *Biotechnol. Adv.* **18**: 1-22 (2000).
46. M. Cardarelli, Spanol L, De Paolis A, Mauro ML, Nitali G, Constantino P. Identification of the genetic locus responsible for non-polar root induction by *Agrobacterium rhizogenes* 1855. *Plant Mol Biol* **5:** 385-391(1985).

47. L. Vanhala, Hiltunen R, Oksman-Caldentey MK. Virulence of different *Agrobacterium* strains on hairy root formation of *Hyoscyamus muticus, Plant Cell Report*, **14:** 236-240 (1995).
48. MJC. Rhodes, Robins JR, Hamill JD, Paar AJ, Walton NJ. Secondary product formation using Agrobacterium rhizogenes-transformed "hairy root" cultures. Newsletter **53:** 2-15 (1987).
49. I. Ionkova. Pharmaceutically significant biologically active compounds from sources with optimized phytochemical potential. Thesis of Dr. of Science, Sofia, (2008).
50. R. Tape, H. Budzikiewicz, I. Ionkova, W. Alfermann. Triterpene glycosides from transformed root cultures of Astragalus mongholicus Bge, *Spectroscopy* (IOS Press, USA), 12 (1):1-8 (1994).
51. M. Hirotani, Zhou Y, Rui H, Furuya T. Cycloartane triterpene glycosides from the hairy root cultures of *Astragalus membranaceus , Phytochemistry*, vol.37(5): 1403-1407(1994).
52. I. Ionkova, Z, Hu, W. Alfermann. Polysaccharide Production by Hairy Root Cultures of Some Higher Medicinal Plants, *Planta Medica*, **59(7):** A 658 (1993)
53. T. Isa. Mucilage production by hairy root cultures of *Astragalus gummifer.* In: Plant Cell Culture in Japan. Komamine A et al. (eds), CMC Co., Ltd.Copyright, Japan, pp 99-103 (1991).
54. S.Thomasset, Berry, D. Garcea, G. Marczylo, T. Steward, W. Gescher, A. Dietary polyphenolic phytochemicals - promising cancer chemopreventive agents in humans? A review of their clinical properties. *Int. J. Cancer*, **120**: 451-458 (2007).
55. I. Ionkova. Optimization of flavonoid production in cell cultures of *Astragalus missouriensis* Nutt. (Fabaceae) *Pharmacognosy Magazine,*submitted, (2008).
56. P. Bradford , A. Awad, Phytosterols as anticancer compounds Molecular Nutrition & Food, 51: 161 - 170 (2007).
57. S. Kuhlmann, K. Kranz, B. Lücking, A.W. Alfermann and M. Petersen. Aspects of cytotoxic lignan biosynthesis in suspension cultures of *Linum nodiflorum, Phytochem. Rev.* **1:** 37-43 (2002).
58. I. Ionkova, I. Antonova, G. Momekov, E. Fuss. Cytotoxic activity of extracts from Bulgarian Linum species, *Pharmacognosy Magazine Vol.3, Issu e* **11**: 192-195 (2007).
59. N. Vasilev, M. Julsing, A. Koulman, C. Clarkson, H. Woenderbag, I. Ionkova, R. Boss, J. Jaroszewski, O. Kaiser, Bioconversion of deoxypodophyllotoxin into epipodophyllotoxin in E. coli using human cytochrome P450 3A4, *J. of Biotechnology*, **126**: 383-393(2006)
60. J. Pezzuto, Che C., McPherson D. DNA as an affinity probe useful in the detection and isolation of natural products, *J.Nat.Prod.*, 54: 1522 (1991)
61. M.Gupta, Monge A., Karikas G. Screening of Panamanian plants for brine shrimp toxicity, grown gall tumor inhibitor, cytotoxicity and DNA interaction, *Int. J.of Pharmacognosy*, 34: 1-19 (1996).
62. I.Ionkova, A.W.Alfermann. Use of DNA for detection and isolation of Potential Anticancer Agents From Plants, *Pharmacy*, Sofia, **1-2**:10-16 (2000).
63. G. Bader, Plohmann B, Hiller K, Franz. Cytotoxicity of triterpenoid saponins, part 1: Activities ageinst tumor cells in vitro and hemolytical index. Pharmazie, 51, **6:** 414-417 (1996).
64. I. Ionkova, Vasilev N., Konstantinov S., Ninov St., Tzvetanova V. Callus and suspension cultures in vitro of *Linum* species and their pharmacological activities, *Pharmacia* L(1): 14-18 (2003).
65. N. Vasilev, Momekov G., Zaharieva M., Konstantinov S., Bremner P., Heinrich M., Ionkova I. Cytotoxic activity of a podophyllotoxin-like lignan from *Linum tauricum* Willd, *Neoplasma*, 52, **5:** 425-429 (2005).
66. N. Vasilev, Elfahmi, R. Boss, O. Kaiser, G. Momekov, S. Konstantinov, I. Ionkova Production of Justicidine B, a Cytotoxic Arylnaphthalene Lignan from Genetically Transformed Root Cultures of *Linum leonii*, *JNP*, **69:** 1014-1017 (2006).
67. Ionkova I. Utilisation of transformed root cultures of Astragalus sp. in saponin production, Dutch-German, Workshop of Plant Cell and Tissue Culture, 3-6 Oct., Kerkrade, The Netherlands 2 (1995).
68. I. Ionkova, H. Budzikiewicz, A.W.Alfermann. Cycloartane-Derived Saponin from Transformed Root Cultures of Astragalus mongholocus, *Planta Medica*, 59(7): A66 (1993).
69. I. Ionkova, A.W. Alfermann. Genetic transformation in medicinal plants & the power of Agrobacterium-mediated transformation for producrion of biologically active compounds, Proceedings of the Conference on Medicinal and Aromatic Plants, May 29 – Juni 3, Yugoslavia, 197-202 (2000).
70. I. Ionkova, E. Fuss. Influence of different strains of *Agrobacterium rhizogenes* on induction of hairy roots and lignan production in *Linum tauricum ssp. tauricum, Pharmacognosy Magazine,* accepted (2009).
71. I. Ionkova, I. Antonova, G. Momekov. Ariltetralin lignan from in vitro cultures of *Linum elegans* and their cytotoxic activity, *Pharmacy,* Sofia, submitted.(2009).
72. T. Smollny, H. Wichers, S. Kalenberg, A. Shasavary, M. Petersen and A.W. Alfermann, Accumulation of podophyllotoxin and related lignans in cell suspension cultures of *Linum album*, *Phytochemistry* **48:** 975-979 (1998).
73. Konuklugil B., I. Ionkova, N. Vasilev, T. Schmidt, J. Windhövec, E. Fuss, and A. Alfermann, Lignans from Linum species of sections Sylinum and Linum, *Nat. Prod. Research* **21**: 1-6(2007).
74. A. Mohagheghzadeh, T. J. Schmidt and A. W. Alfermann, Arylnaphtalene lignans from *in vitro* cultures of *Linum austriacum* L., *Nat. Prod.* **65:** 69-71(2002).
75. I. Ionkova, V. Tzvetavova, A. W. Alfermann, Ariltetralin lignans from callus and suspension cultures of *Linum bienne* Miller and *Linum africanum* L. in vitro, Proceedings of the II Pharmaceutical Congress "Neu Perspectives in Controlled Release" April 28-29 CD, Panhellenic Pharmaceutical Association - Amphitrion Athens 2001, pp. 154-156.
76. N. Vasilev, P. Nedialkov, I. I. Ionkova and St. Ninov HPTLC densitometric determination of Justicidin B in *Linum* cultures in vitro, *Pharmazie* **59:** 528-529 (2004).
77. N. Vasilev, I. Ionkova, Lignan production by cell cultures of Linum setaceum and Linum campanulatum, *Pharm. Biol.,* **43:** 1-3(2005).
78. J. Berlin, V. Wray, C. Mollenschott and F. Sasse, Formation of β-peltatin-A methyl ether and coniferin by root cultures of *Linum flavum, J. Nat. Prod.* **49:** 435-439(1986).

79. J. Berlin, N. Bedorf, C. Mollenschott, V. Wray, F. Sasse and G. Höfle, On the podophyllotoxins of root cultures of *Linum flavum, Planta Med.* **54:** 204-206 (1988).
80. W. Van Uden, N. Pras, S. Battermann. J. F. Visser and T. M. Malingré, The accumulation and isolation of coniferin from a high producing cell suspension of *Linum flavum* L., *Planta* **183:** 25-30(1990).
81. W. Van Uden, N. Pras, E. M. Vossebeld, J. N. M. Mol and T. M. Malingré, Production of in cell suspension cultures of *Linum flavum* L., *Plant Cell Tiss. Org. Cult.* **20:** 81-87(1990).
82. H. J. Wichers, M. P. Harkes and R. J. Arroo, Occurrence of 5-methoxypodophyllotoxin in plants, cell cultures and regenerated plants of *Linum flavum, Plant Cell Tiss. Org. Cult.* **23:** 93-100 (1990).
83. H. J. Wichers, G. G. Versluis-De Haan, J. W. Marsman and M. P. Harkes, Podophyllotoxin related lignans in plants and cell cultures of *Linum flavum, Phytochemistry* **30:** 3601-3604 (1991).
84. A. Oostdam, J. N. M. Mol and L .H. W. van der Plas, Establishment of hairy roots cultures of *Linum flavum* producing the lignan 5-methoxypodophyllotoxin, *Plant Cell Rep.* **12:** 474-477 (1993).
85. Han-wei Lin, K. H. Kwok and P.M. Doran, Development of *Linum flavum* hairy root cultures for the production of coniferin, *Biotech. Lett.* **25:**521-525 (2003).
86. B. Konuklugil, T. J. Schmidt and A. W. Alfermann, Accumulation of lignans in suspension cultures of *Linum mucronatum* ssp. *armenum* (Bordz. and Davis), Z. *Naturforsch* **56c:** 1164-1165 (2001).
87. K. Kranz and M. Petersen, β-Peltatin 6-0-methyltransferase from suspension cultures of *Linum nodiflorum, Phytochemistry* **64:** 453-458 (2003).
88. S. Kuhlmann, K. Kranz, B. Lücking, A.W. Alfermann and M. Petersen, Aspects of cytotoxic lignan biosynthesis in suspension cultures of *Linum nodiflorum, Phytochem. Rev.* **1:** 37-43 (2002).
89. A. Mohagheghzadeh, S. Hemmati, I. Mehregan and A.W. Alfermann, *Linum persicum:* Lignans and placement in Linaceae, *Phytochem. Rev.* **2:** 363-369 (2003).
90. Stakeholders proposal for a Strategic Research Agenda to 2025, including Draft Action Plan 2010 - 3.2.1. WP 2-2. Natural plant pharmaceutical products. (http://europa.eu.int/comm/research/biosociety/library/glossaryfind_en.cfm) (2005).