Investigations on Wild *Allium* Species. Part I: Cysteine Sulfoxides of Flowers. Part 2: Anticancer Activity of Bulb Extracts

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Eingereicht am **…….....**

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Dedicated to my wife and life partner Sevda, our little hearts-children Said and Esma, my beloved parents and my proud brother Orkhan, to the supporting parents-in-law and brother-in-law Pervin.

If learning the truth is the scientist's goal… then he must make himself the enemy of all that he reads

Ibn al-Haytham (Alhazen)

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A.	*Allium*
LF	Lachrymatory factor
HPLC	High performance liquid chromatography
HR-MS	High resolution mass spectrometry
1H NMR, ^{1}H-NMR	Proton nuclear magnetic resonance
IPK Gatersleben	The Leibniz Institute of Plant Genetics and Crop Plant Research at Gatersleben (Germany)
Tax / Acc No	Taxonimic identifier/ Accession number
PBS	Phosphate-buffered saline
IC50, IC_{50}	Concentration of an inhibitor where the population of cells reduced by 50%
CSO	Cysteine sulfoxide
HO-1	Heme oxygenase enzyme
Nrf2	The nuclear factor erythroid 2 (NFE2)-related factor 2
DMSO	Dimethyl sulfoxide
MeOH	Methanol
H_2O	Water
PDA	Photodiode array detector
UV	Ultraviolet
NaCl	Sodium chloride
N_2	Nitrogen gas
ESI	Electrospray ionisation

m/z	Mass to charge ratio
V	Volt
TOF	Time of flight
l/min	Liter per minute
ml/min	Milliliter per minute
EtOAc	Ethyl acetate
SD; STD	Standard deviation
p	Statistics value probability of error
J	Coupling constant
MS	Mass spectrometry
GSH	Glutathione
PNO	2-(methyldithio)pyridine-N-oxide
MTPNO	2-[(methylthiomethyl)dithio]pyridine-N-oxide
DPT	2,2′-dithio-bis-pyridine-N-oxide; dipyrithione
RSD	Relative standard deviation
OPA	ortho-Phthalaldehyde
µm	Micrometer
XTT	(2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide)
PCR	Polymerase chain reaction
RPMI 1640 medium	Roswell Park Memorial Institute 1640 Medium
h	Hour

µg/ml	Microgram per milliliter
DNAse I	Deoxyribonuclease I
mRNA	Messenger Ribonucleic acid
ΔCt	Delta cycle threshold
CDKN1a	Cyclin Dependent Kinase Inhibitor 1A
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
T24	Human bladder carcinoma cell line
UMUC3	Human bladder carcinoma cell line
HFF-1	Healthy human fibroplast cell line
Sub-G1	Cells with deficit in DNA content
G1-S	Stage in the cell cycle between G1 and S phases
G2-M	Stage in the cell cycle between G2 and mitosis phases
MMTSI	Dimethyl disulfide sulfoxide
MMTSO	Dimethyl disulfide sulfone

Summary

Since ancient times, onions, garlic and some other species of the genus *Allium* L. (onions) have been used as phyto-pharmaceutics, seasonings, and vegetables. Most prominent are common onion (*A. cepa* L.) and garlic (*A. sativum* L.). The medicinal benefits of these two species were intensely investigated during the last decades and lipid lowering, antibiotic, anti-atherosclerotic and anti-diabetic effects were described. In addition, a number of ethnic studies proved their canceroprotective effect. The health benefits of *Allium* vegetables are mainly related to sulfur containing compounds as well as saponins. The so-called cysteine sulfoxides of these plants are believed to be mainly responsible for these health benefits. These compounds are converted to thiosulfinates like allicin, when plant material is disrupted. This reaction is catalyzed by the action of the enzyme alliinase.

Flowers of *Allium* species of subgenus *Melanocrommyum*, most of them for the first time, were analyzed for their cysteine sulfoxide(CSO) content. While all of the flowers carried methiin, only a few of flowers carried any of the following CSOs: isoalliin, alliin, marasmin, S-(2-pyrrolyl)-cysteine sulfoxide, S-(2-pyridyl)-cysteine N-oxide and butiin. Total CSO content of analyzed flowers varied from 0.12% to 7.05%, relatively to fresh material weight.

Bulbs of several *Allium* species were extracted using ethyl acetate and screened for their cytotoxic activity against bladder cancer cell lines (T24 and UMUC3). Four most cytotoxic extracts were investigated further for their mechanism of action using flow cytometry assay, real-time PCR and Western blot analysis. Same four extracts were also investigated for their bioactive compounds. It was found that *A.aflatunense*, *A. stipitatum*, *A.rosenorum* and *A. pallens* acted as cancer cell killers. Mechanisms of action of the extracts were apoptosis, antioxidant response and cell cycle arrest. Bioactive compounds of *A. stipitatum* were found to be 2-(methyldithio)pyridine-N-oxide and as 2-[(methylthiomethyl)dithio]pyridine-N-oxide which were responsible for cell cycle arrest and inducting antioxidant response via Nrf2/HO-1 system. Tentatively identified bioactive compounds in *A.aflatunense* are 2,2′-dithio-bis-pyridine-N-oxide and 2-[(methylthiomethyl)dithio]pyridine-N-oxide.

Zusammenfassung

Zwiebeln, Knoblauch und einige andere Spezies der Gattung *Allium* L. werden seit Jahrtausenden als Gewürz, Gemüse oder Phytopharmaka verwendet. Die bekanntesten Arten sind Knoblauch (*Allium sativum* L.) und die Küchenzwiebel (*Allium cepa* L.). Die medizinische Wirkung dieser zwei Spezies wurden während des letzten Jahrhunderts intensiv erforscht; es zeigte sich, dass sie ein sehr breites Wirkspektrum besitzen. Zu ihren pharmazeutisch und medizinischen Eigenschaften werden antibiotische, lipidsenkende, anti-arthreosclerotische und eine anti-diabetische Wirkung gezählt. Auch ein krebsvorbeugender Effekt wurde in einer Vielzahl ethnischer Studien bestätigt. Diese gesundheitsfördernden Eigenschaften der *Allium*-Spezies sind in erster Linie auf schwefelhaltigen Verbindungen und Saponine zurückzuführen. Man geht davon aus, dass hauptsächlich Cysteinsulfoxide (CSO) verantwortlich für die pharmazeutische Wirkung sind. Die sekundären Stoffwechselprodukte aus der Gruppe der Thiosulfinate wie Allicin bilden sich bei Verletzung der Pflanzenzellen. Es handelt sich dabei um eine von Allinase-katalysierte Enzymreaktion. Hauptsächlich Blüten der Gattung *Allium* Subgattung *Melanocrommyum* wurden auf ihren Gehalt an Cysteinsulfoxiden (CSO) untersucht.

Während alle der untersuchten Blüten Methiin enthielten, fanden sich in wenigen Spezies weitere CSO: Isoalliin, Alliin, Marasmin, *S*-(2-Pyrrolyl)-cysteinsulfoxid, *S*-(2-Pyridyl)-cystein N-oxid und Butiin. Der Gesamtgehalt an CSO schwankte stark und lag zwischen 0,12% und 7,05%, relativ zum Gewicht der frischen Probe.

Zwiebeln verschiedener *Allium*-Spezies wurden mit Ethylacetat extrahiert und hinsichtlich ihrer zytotoxischen Aktivität gegen Blasenkrebszellen (T24 und UMUC3) untersucht. Die vier Extrakte höchster Aktivität wurden hinsichtlich ihres Wirkmechanismus erforscht; es wurden dabei real-time PCR, Western Blot und Durchflusszytometrie-Analysen verwendet. Die Zusammensetzung dieser vier Extrakte hinsichtlich ihrer bioaktiven Substanzen wurde aufgeklärt. Hierbei zeigte sich, dass *A. aflatunense*, *A. stipitatum*, *A. rosenorum* and *A. pallens* alle in der Lage sind Krebszellen zu töten. Die Wirkung beruht auf Apoptose, „antioxidant response“ und

Zellzyklus-Arrest der betroffenen Zellen. Die bioaktiven Substanzen aus *A. stipitatum* sind 2-(Methyldithio)pyridin-N-oxid und 2-[(Methylthiomethyl)dithio]pyridin-N-oxid. Diese Verbindungen waren sowohl für den Zellzyklus-Arrest als auch für die Induktion des „antioxidant response“ via Nrf2/HO-1 System verantwortlich. Die vorläufig identifizierten bioaktiven Verbindungen in *A. aflatunense* sind 2,2′-Dithio-bis-pyridin-N-oxid sowie 2-[(Methylthiomethyl)dithio]pyridin-N-oxid.

1. INTRODUCTION

1.1. The genus Allium

Humanity has extensively utilized main representatives of the genus, garlic (*A. sativum*), common onion (*A. cepa*) and leek (*A. porrum*) since the dawn of its history. Their importance to the human race can be understood from the fact, that garlic and onions are mentioned in the Talmud, Bible and Koran.

The species-rich gender *Allium* has a main centre of distribution reaching from Southwest Asia to the high mountains of Central Asia. In this area, the local population utilizes several wild species as medical plant [1,2]. Another location of distribution of the species is North America. The genus Allium has around 750 species. Although the plants are adapted to various climatic growth conditions, majority of the species prefer arid and moderately humid climates and open, sunny areas.

First documented reference to the medical utilization of garlic and onion can be found in Ebers Papyrus (1550 BC) found in Egypt. The Ebers Papyrus is an ancient medical document, which also describes several formulations. These formulas include garlic and onions as a treatment for headaches, heart problems, tumors, worms and bites. Ancient Egyptians should have had much interest in garlic, leek and onions since they can be found depicted on funeral plaques as well as onion bulbs found placed in body cavities of mummies and garlic was used in embalming process. Findings of well-preserved garlic in Tutankhamun's tomb and in tombs of other Egyptian ancient rulers, stress the importance of this plant in the ancient culture. Eventually, Greeks and Romans have known and used these plants as food and medication [3,4].

A botanist of 19th century, Eduard Regel, is among the ones who greatly and specifically contributed to botanical research of the genus *Allium*. Because of his explorations in Asia, Regel authored two monographs, which feature more than 250 species, large number of which were described for the first time [5].

1.2. General chemistry and sulfur compounds of Allium

Besides the water, which may account between 65-95% of the weight of the plant, *Allium* species have flavonoids, fructans, minerals and vitamins [6–9]. Two canthin-6-one alkaloids were also reported to be found in *A. neapolitanum.* They exhibited some antibacterial activity against fast-growing *Mycobacterium* species and multidrug-resistant and methicillin-resistant (MRSA) strains of *Staphylococcus aureus* [10].

Cysteine sulfoxides(CSOs) found in *Allium* species are of main research interest as they serve as precursors of organic sulfur compounds (OSCs) such as thiosulfinates and sulfines [11]. A glutamyl derivative of cysteine sulfoxide, γ-glutamyl-(S_S,R_C)-marasmin, was reported to be found in a mushroom *Marasmius alliaceus* from genus *Marasmius* [12]. First CSO to be found was alliin from garlic [13]. Eventually methiin, propiin, and isoalliin were discovered [14,15]. By derivatising CSOs with ethyl chloroformate and analyzing with GC it was possible to observe a new CSO, ethiin. It was reported to be present in small amounts in several common species such as elephant garlic, shallot, leek, chive and wild garlic [16].

Cysteine sulfoxides examined in this investigation are (+)-S-methyl-L-cysteine sulfoxide (methiin), (+)-S-ethyl-L-cysteine sulfoxide (ethiin), (+)-S-propyl-L-cysteine sulfoxide (propiin), (+)-S-(2-propenyl)-L-cysteine sulfoxide (alliin), (+)-S-(1-propenyl)-L-cysteine sulfoxide (isoalliin), (+)-S-butyl-L-cysteine sulfoxide (butiin), (+)-S-butenyl-L-cysteine sulfoxide (homoisoalliin), (+)-S-methylthiomethyl-L-cysteine sulfoxide (marasmin), S-(2-pyrrolyl)cysteine sulfoxide, S-(2-pyridyl)cysteine N-oxide (Figure 1.1). Additionally, essential amino acids, alanine, serine, valine, threonine, leucine/isoleucine, asparagine, aspartic acid, glutamine, glutamic acid, histidine, phenylalanine, arginine, tyrosine, tryptophane, were also evaluated qualitatively and quantitatively.

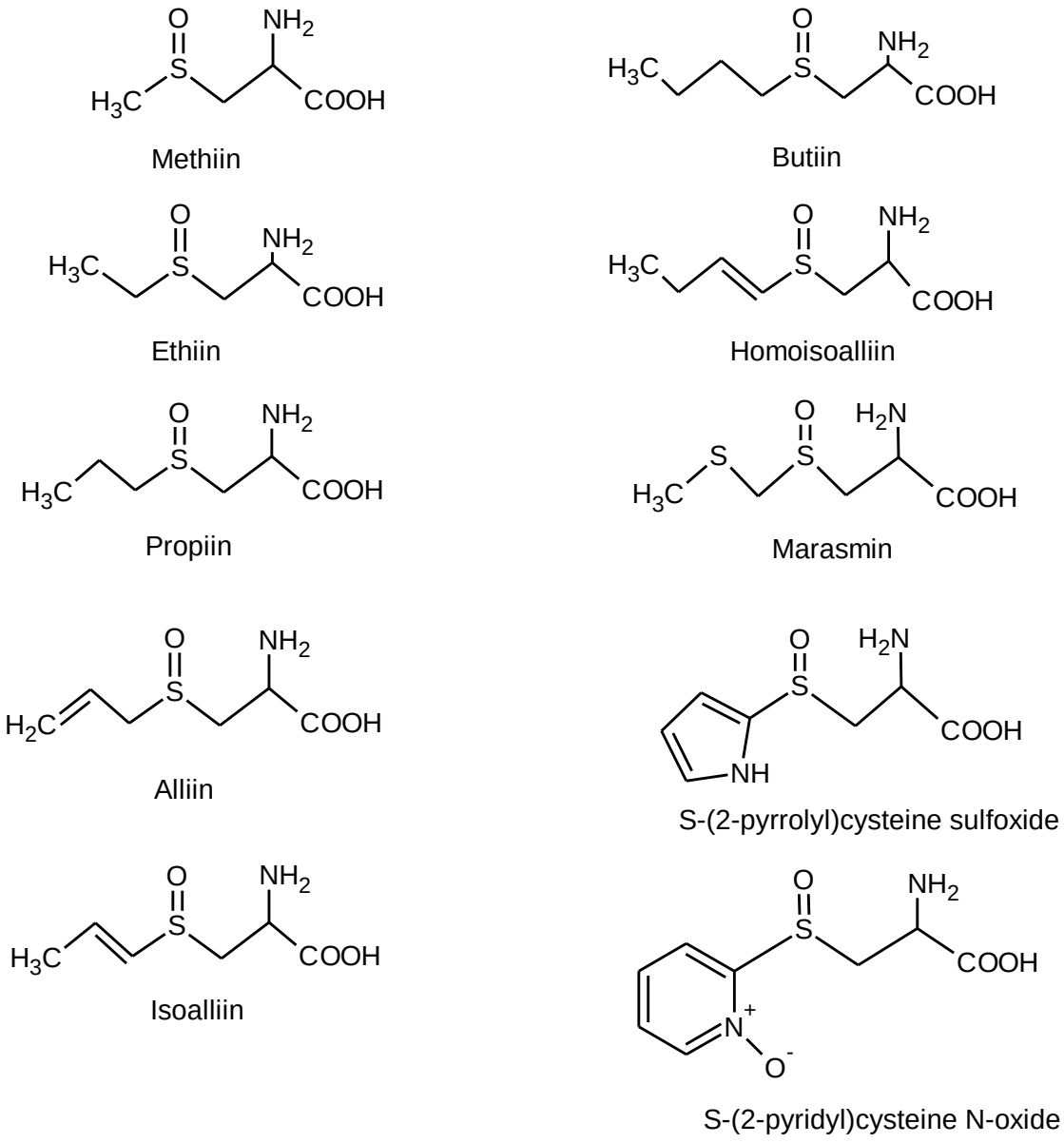

Figure 1.1. Cysteine sulfoxides examined in this study.

Thiosulfinates like allicin, and sulfines are biosynthesized from cysteine sulfoxides when the plant cell is disrupted. This reaction is catalyzed by the action of the enzyme alliinase. The activity of the alliinase enzyme may be specific to species and CSO and stereoselective [12,17]. One of distinctive properties between garlic (*A. sativum*) and onion (*A. cepa*) is that garlic possesses alliin, while onion has isoalliin (Figure 1.1). This

difference results in synthesis of allicin from alliin catalyzed by the alliinase enzyme (Figure 1.2). Allicin has been shown to be physiologically active in microbial, plant and mammalian cells, by inhibiting proliferation of cancer cells, kill outright bacteria, fungi, and lower cholesterol and blood pressure [18]. Yellowish oily allicin with characteristic garlic smell is highly unstable and decomposes to yield more stable, colorless and odorless liquid-ajoene [19,20]. Ajoene, in turn, found to have some biological activities, such as inhibiting prostaglandin synthesis by a mechanism common to non-steroidal anti-inflammatory drugs [21], antitumor [22–24], antifungal [25] and antithrombotic [26].

In addition to alliinase enzyme, in onion, a LF synthase enzyme, steps in to the reaction after alliinase and converts (E)-1-propenesulfenic acid into (Z)-propanethial S-oxide, the lacrimatory factor of onion [14,27–29]. Other structures such as cepaenes, *cis*- and *trans*- zwibelanes also reported to be found as products of alliinase reaction in onion (Figure 1.3)

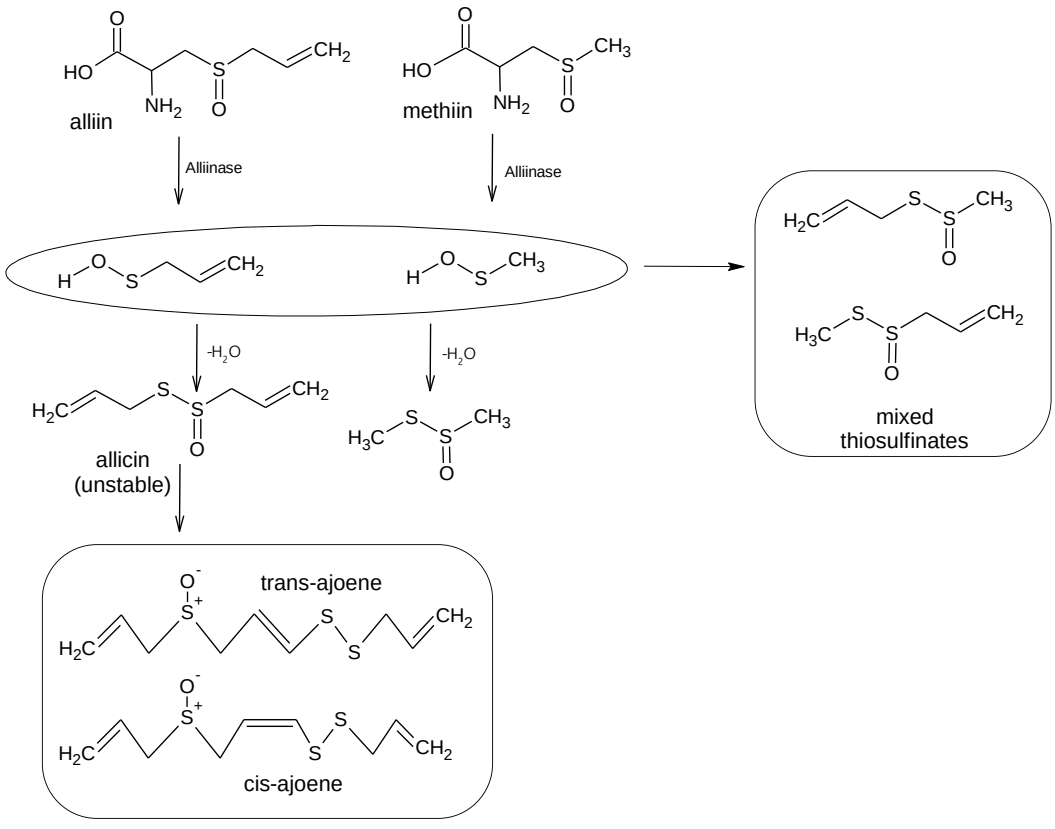

Figure 1.2. Biosynthetic pathway of thiosulfinates in garlic

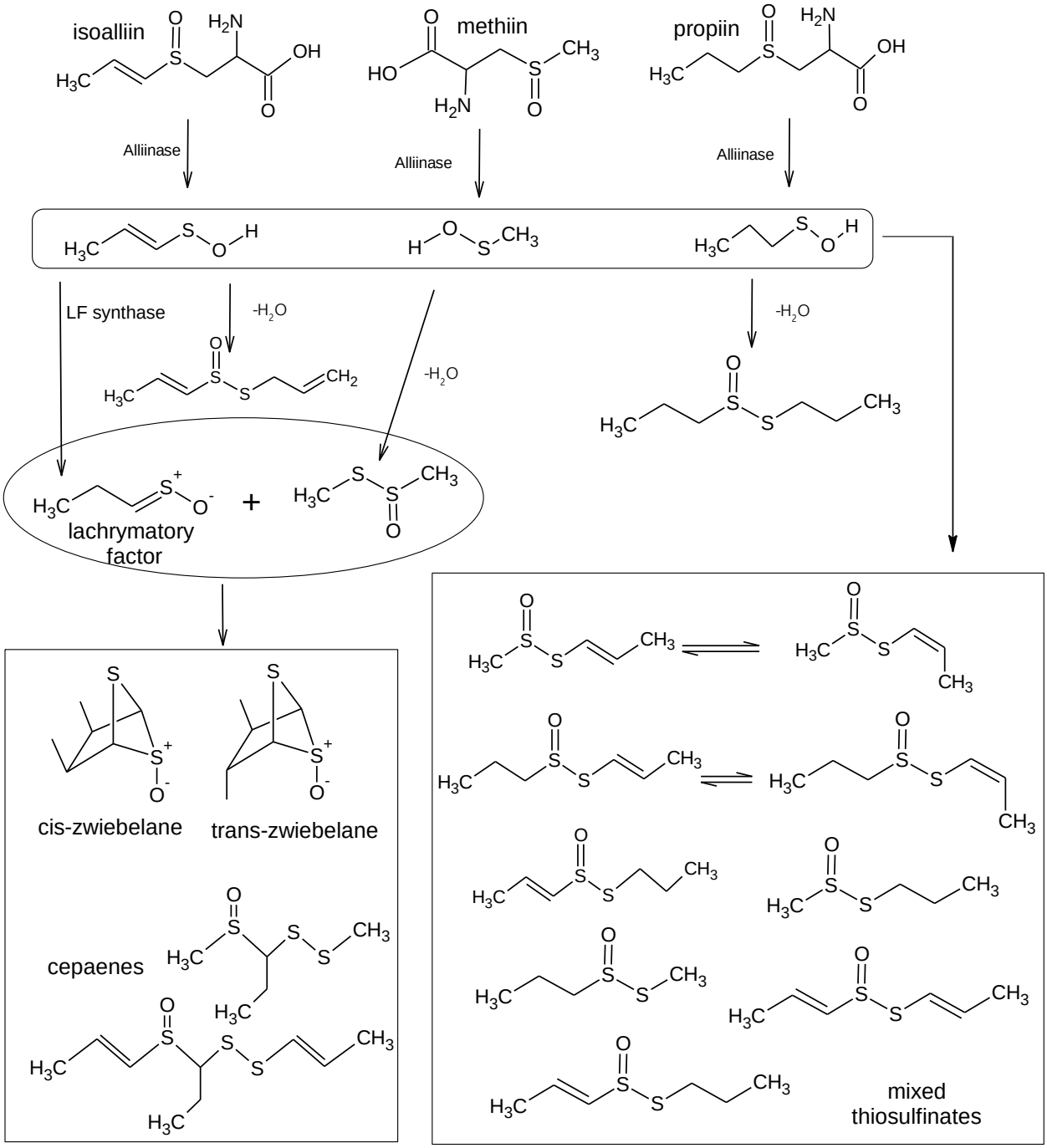

Figure 1.3. Biosynthetic pathway of thiosulfinates in onion

More recently aromatic sulfur compounds were reported to be present in subgenus *Melanocrommyum* (Figure 1.4) [30–33]. Compound 2,2'-epidithio-3,3'-dipyrrole is found

to be responsible for color discoloration specific to species of the subgenus, when the tissue is disrupted. Compounds 2-(methyldithio)pyridine-N-oxide (**1**), 2-[(methylthiomethyl)dithio]pyridine-N-oxide (**2**), 2,2′-dithio-bis-pyridine-N-oxide (dipyrithione) (**3**), 2-(methyldithio)pyridine (**4**), 2-(methyldithio)pyrimidine (**5**), 2-(methyldithio)quinoline (**6**), 2-(methyldithio)benzene (**7**), 2-(methyldithio) thiophene (**8**) were reported to be isolated from *A. stipitatum*.

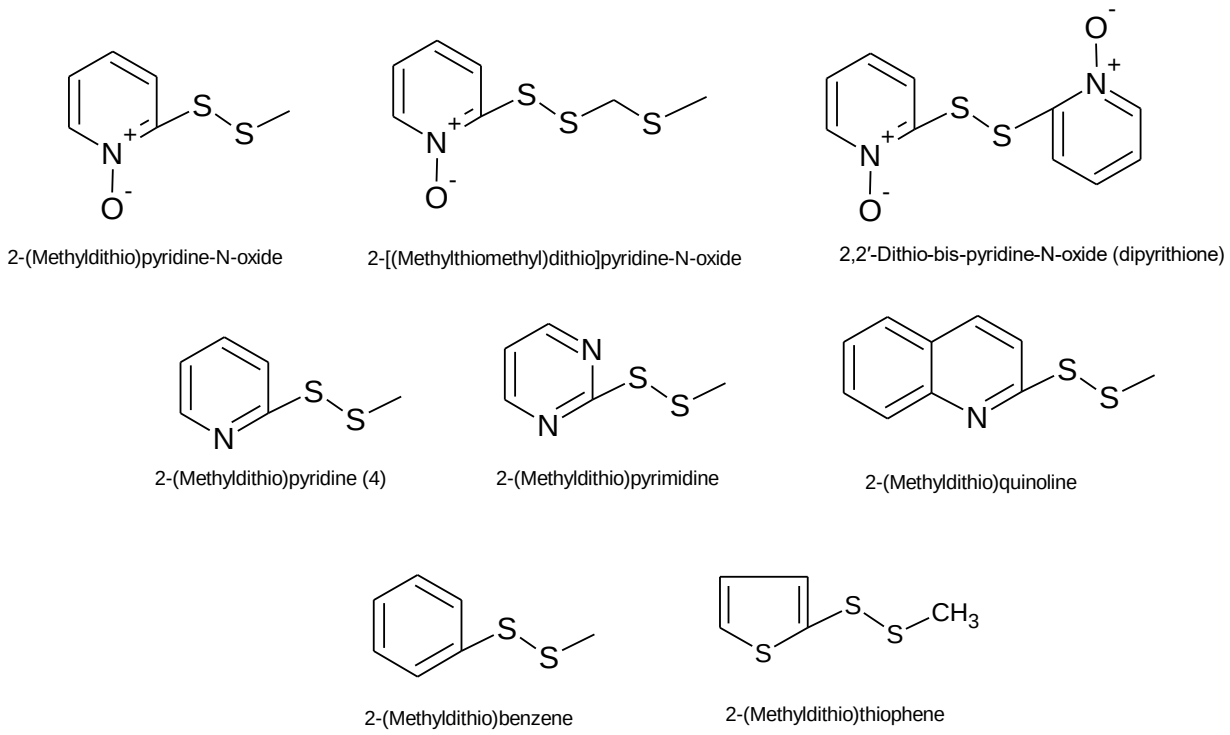

Figure 1.4. Structures of aromatic sulfur compounds isolated from *A. stipitatum* [31].

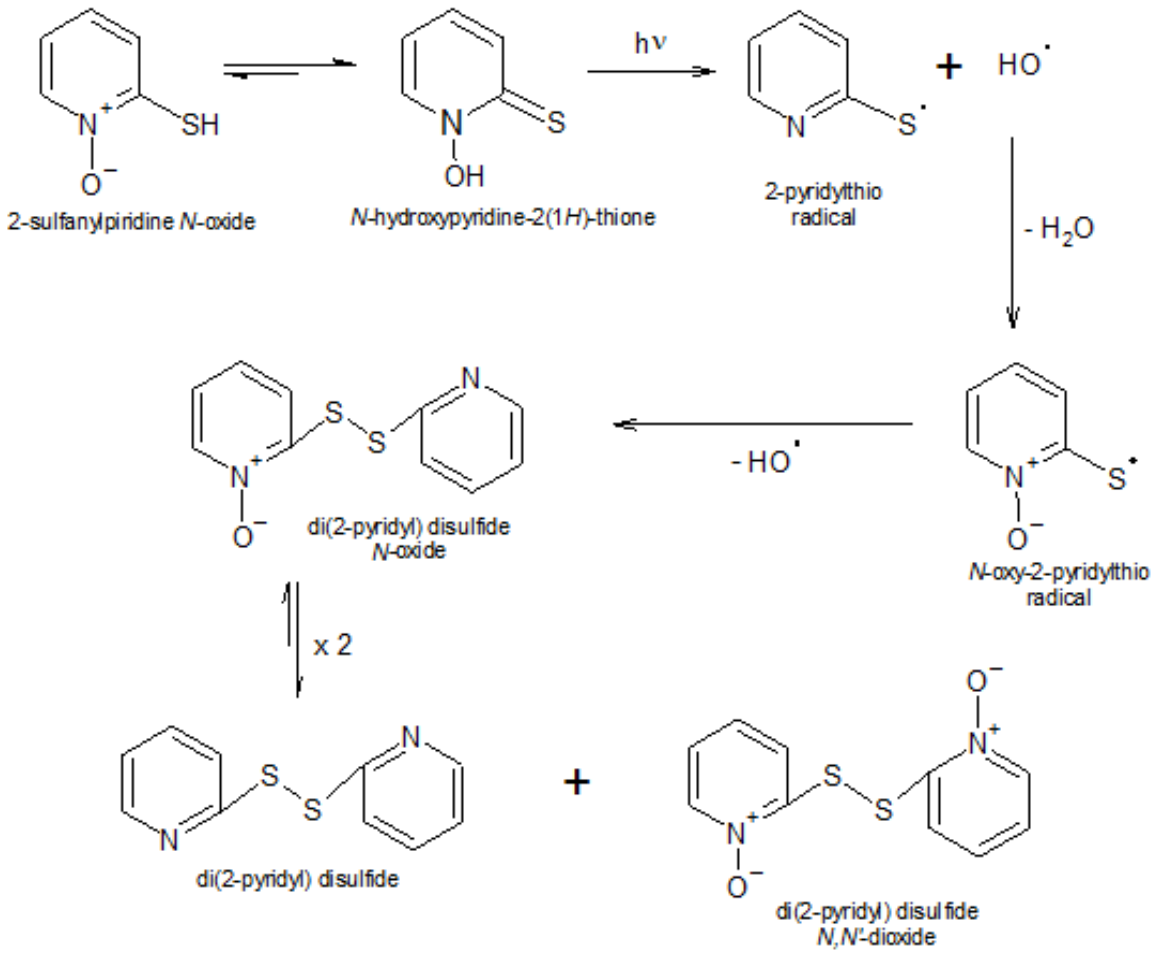

Figure 1.5.Biosynthesis of pyrithione and other pyridyl containing compounds from (R)-S-(2-pyridyl)cysteine N-oxide [33].

Kubec et al, proposed formation of pyridyl-containing compounds from pyrithione and formation of the latter from naturally occurring precursor (R)-S-(2-pyridyl)cysteine N-oxide (Figure 1.5) [33].

Authors of one recent study researched changes in metabolite profile of sprouted garlic (*A. sativum*) and their antioxidant effect [34] . They found that ethanolic extract of garlic sprouted for 5 days enhanced DPPH radical scavenging ability and suppressed the generation of peroxy radical more than raw garlic and garlic extracts sprouted for shorter times. In addition, sprouted garlic extract prevented glutamate-induced cytotoxicity in the HT22 mouse hippocampal neuronal cell line while raw garlic extract

had limited protective effect. Extract of garlic sprouted for 4-5 days also significantly suppressed reactive oxygen generation mediated by glutamate treatment in HT22 cells. As there was only slight increase in total phenolic content in garlic during sprouting and no significant difference when garlic was sprouted for different periods, authors cannot provide clear reason for enhanced antioxidant effect of the extract sprouted for longer period. Metabolites isoguanosine (or 1-aminoinosine), *γ*-glutamyl-S-trans-1- propenyl cysteine, glucoside, 8,11,12-trihydroxy-9-octade-cenoic acid, and spirostane-3,6-diol, 6-O-D-glucopyranoside increased during sprouting period, therefore authors suggest to further investigate and define their antioxidative potential.

1.3. Role of arginine in Alliums and plants in general

Considering widespread use of *Allium* in human diet, it may be reasonable to analyze amino acid content of bulbs as humans cannot synthesize them in their organism and dependent on external sources. One of the early studies analyzed relative approximate amino acid amounts in bulbs of four cultivars of common onion (*A. cepa*) using densitometry and colorimetry [35]. In all four cultivars arginine (Figure 1.6) found to be the most abundant amino acid with amounts ranging between 16-25 mg/100 g onion on fresh weight basis.

Another study evaluated amino acid composition of seeds from 200 angiospermous plant species with the purpose to estimate the nutritional quality of a seed protein [36]. Among 12, 18 and 23 members of *Asteraceae*, *Brassicaceae* and *Fabaceae*, respectively, glutamic acid (Figure 1.6) was in the highest amounts, ranging between 15.2-20.5 grams per 16 grams of nitrogen and amount of arginine was second highest, ranging between 7.1-9.1 grams per 16 grams of nitrogen, across all three families. Glutamic acid and arginine amounts of seeds of *Allium porrum* (*Amaryllidaceae*) reflected same pattern having glutamic acid in highest, 20.6 grams per 16 grams of nitrogen, and arginine second highest amounts with 10.8 grams per 16 grams of nitrogen, among evaluated amino acids of the *A. porrum* seeds.

A multiyear study conducted by Schuphan and Schwerdtfeger [37], showed a significant change in amounts of arginine in bulbs of *A. cepa* over vegetative cycle. They reported that during growth of *A. cepa*, arginine content increased by 29%, glutamic acid/glutamine (Figure 1.6) by 7%, whilst most of the contents of the other amino acids decreased. Sprouted bulbs contained 43% of that of non-sprouted ones. The inner scales surrounding the buds contained the highest concentration of arginine. The outer fleshy scales contained 27%, the buds 35% less arginine than the inner scales. They also stated that, arginine, in addition to glutamic acid appears to play a special role providing evidence that *A. cepa* belongs to a rather small number of plants using arginine as a pool for nitrogen.

Arginine was found to be predominant free amino acid in cotyledons of seeds of peas (*Pisum sativum* L. cv Marzia) [38,39] and *Glycine max* (L.) Merrill.

Seeds of loblolly pine (*Pinus taeda* L.) were studied for the major storage proteins and arginine was the most abundant amino acid among the principal storage proteins of the megagametophyte and was a major component of the free amino acid pools in both the seedling and the megagametophyte [40].

Underground storage forms of ericaceous shrubs *Vaccinium vitis-idaea* and *V. myrtillus*, the fern *Gymnocarpium dryopteris*, the grass *Deschampsia flexuosa*, and the herbs *Epilobium angustifolium*, *Maianthemum bifolium*, *Solidago virgaurea*, *Geranium sylvaticum* and *Trientalis europaea* growing in boreal forest were studied for their storage forms of nitrogen [41]. Authors reported that, in *D. flexuosa* and *S. virgurea*, asparagine (Figure 1.6) and arginine together were the major forms of stored nitrogen, in *G. dryopteris* glutamine was major free amino acid and nitrogen storage form, and in all other species arginine dominated the pool of free amino acids and was the major form of stored nitrogen.

In taproots of the forb *Rumex acetosa*, free amino acids (with arginine and glutamine being major ones) and proteins were identified to be main storage compounds [42]. During early vegetative growth, the taproots were found to be main source of

remobilized nitrogen, which also supports an idea of arginine and glutamine being main nitrogen sources.

Amino acids (mainly arginine, glutamine and asparagine) are considered as source of nitrogen in trees as well. Arginine, containing four moles of nitrogen per mole, and the amides glutamine and asparagine, containing two moles of nitrogen per mole, are of particular significance as soluble storage amino compounds [43].

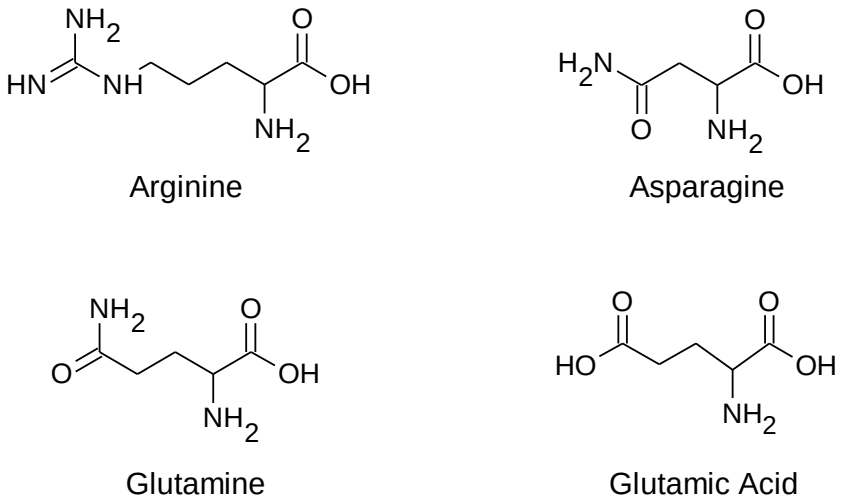

Figure 1.6. Chemical structures of arginine, asparagine, glutamine and glutamic acid

Arginine and its metabolism are of central importance in plant biology and Slocum (2005) reviewed the genes involved in arginine biosynthesis in *Arabidopsis thaliana* [44]. In plants and other organisms arginine biosynthesis involves two steps: synthesis of ornithine from glutamate either in a cyclic or a linear pathway followed by the synthesis of arginine from ornithine (Figure 1.7) [44,45].

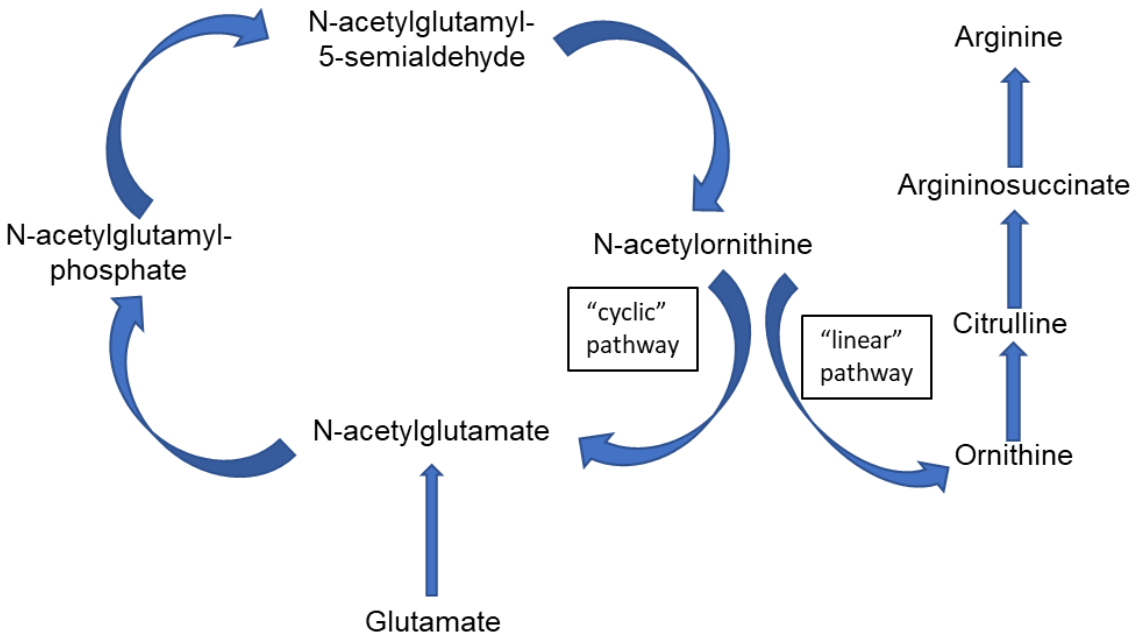

Figure 1.7. Arginine biosynthesis in plastids [44,45]

1.4. Alliums and their anticancer activity

First experimental evidence of anticancer activity of *Allium sativum* was reported by Weisberger and Pensky (1958) [46]. In this study, authors prepared dimethyl, diethyl, dipropyl, and dibutyl thiosulfinic esters and tested them for their anticancer activity *in vitro* and *in vivo*. The tumors studied included the ascitic and solid forms of Sarcoma 180 in CFW Swiss mice and the Murphy-Sturm lymphosarcoma in Wistar rats. Tumor cell growth was inhibited both *in vitro* and *in vivo.*

One of the first epidemiological studies on *Allium* and cancer relationship was conducted in China in 1989. The authors of the study suggest that if taken for long period, beginning in childhood, *Allium* consumption may prevent development of stomach cancer [47], which is in correlation with the results of relatively recent study (2005) on frequency of onion intake and stomach cancer in Qingdao and Shanghai

populations [48]. Another, population-based, case–control study of the risk of prostate cancer in Chinese men, identified a reduced risk associated with consumption of *Allium* vegetables, especially garlic and scallions [49]. Again, a study in China suggests that a decreased risk of esophageal and stomach cancer is associated with increased consumption of *Allium* vegetables (garlic, onion, Welsh onion and Chinese chives) [50]. A hospital-based case-control study of esophageal cancer carried out in the Heilongjiang Province, China, points out to a significant inverse dose-risk trend for combined consumption of vegetables and fruits, including garlic and onion [51]. Results of a US study suggest that increasing vegetable and fruit consumption (including onions and garlic) may impart some protection against developing pancreatic cancer [52]. In Europe, multiannual independent breast cancer studies conducted in France and Switzerland, suggest that a diet rich in fiber or garlic and onions may lower the risk of breast cancer and could represent effective preventive measures [53,54]. A series of case-control studies were conducted in Italy between 1991 and 2004, in order to determine canceroprotective effect of garlic and onion consumption [55]. Authors found a protective role of a moderate frequency of onion consumption against colorectal, laryngeal, and ovarian cancers. The inverse relation was even more evident for high frequency of use, when it was also significant for oral cavity and esophageal cancers, but not for prostate, breast, or renal cell cancers.

Various *in vitro* studies have shown anti-tumor properties of garlic and its constituents. Diallyl trisulfides have shown high efficacy in prostate cancer cells by induction of apoptosis and cell cycle arrest [56,57], in gastric cancer cells [58], in liver tumor via G_2/M arrest [59], in breast cancer cells by inhibiting anchorage-dependent growth of cells [60], in colon tumor cells by leading to apoptosis and inhibition of tubulin polymerization[61]. Diallyl disulfides were effective against breast cancer cells by inhibiting anchorage-dependent growth of cells [60], human leukemia cells by inhibiting NAT activity [62], human colon adenocarcinoma via G2/M cell cycle arrest and inhibition of p34 [63]. Diallyl sulfides fought human leukemia cells by inhibiting NAT activity and modulation of MDR [62,64]. Ajoene, more stable compound than allicin, found to be

active against leukemia cells by arresting G2/M phase and inducing apoptosis *via* stimulation of peroxide production and activation of nuclear factor kB [22,23].

In an animal study, Lamm and Riggs [65] evaluated intralesional and oral aged garlic extract treatment of transitional cell carcinoma in the murine model. The results show that intralesional garlic extract was highly effective in the treatment of subcutaneously transplanted MBT2 bladder cancer, although repeated intralesional garlic injection was toxic, resulting in the death of as many as 42% of treated animals. As a conclusion, authors suggest that, garlic stimulates the proliferation of macrophages and lymphocytes and protects against the suppression of immunity by chemotherapy and ultraviolet radiation and may be seriously considered in clinical trials for the prevention and treatment of bladder cancer.

1.4.1. Effect of sulfides from Allium spp on Nrf2/HO-1 antioxidant system in cancer cells

Nuclear factor erythroid 2-related factor (Nrf2) is a member of cap'n'collar (CNC) family proteins are transcription factors. CNC family is required for metabolic detoxification of xenobiotics, and play a pivotal role in the cellular response to oxidative or electrophilic stresses [66]. Cellular redox balance and protective antioxidant and phase II detoxification responses in mammals is regulated by the Nrf2 transcription factor [67,68]. Oxidative stress is defined as an imbalance between production of free radicals and reactive metabolites, so-called oxidants, and their elimination by protective mechanisms, referred to as antioxidative systems [69]. Since, the production of O_2^- in cancer cells generally is more active than in normal cells, cancer cells are under intrinsic oxidative stress, therefore more vulnerable to damage by reactive oxygen species (ROS)-generating agents [70]. The overproduction of ROS can result in detrimental cellular damage including lipid peroxidation, DNA adduct formation, protein oxidation and enzyme inactivation, which can all ultimately lead to cell death.
Heme oxygenase-1 (HO-1) is one of the genes regulated through Nrf2 [71]. HO-1 is a cytoprotective enzyme, which can be stimulated by number of stimuli like heme, nitric

oxide, heavy metals, growth factor, cytokines, modified lipids and others. Its activation eventually leads to generation of iron ions, biliverdin and CO. Along with generating potent antioxidants, other products of HO-1 activity regulate important biological processes including inflammation, apoptosis, cell proliferation, fibrosis, and angiogenesis [66,67,72] .

Studies, which investigated ability of sulfides from *Alliums* to cause oxidative damage to cells through generating reactive oxygen species, reported elevated levels of HO-1 and Nrf2 proteins in the cells, thus confirming oxidative damage caused by the sulfides [73,74].

1.4.2. Sulfides from Allium spp as inducers of cell cycle inhibitor CDKN1a (p21) protein

In human cancer cells, scheduled cell cycle activity is often hijacked and by affecting the expression of cell cycle regulatory proteins, causing overexpression of cyclins and loss of expression of cyclin-dependent kinase (cdk) inhibitors [75]. This provides a target for drug discovery against cancer cells. Several anticancer drugs, such as Doxorubicin, are known for their ability to interfere with cell cycle. A protein, p21, was discovered to be present in normal cells, while to be absent in many transformed cells and it was suggested to be a universal inhibitor of cyclin kinases [76].

In 2004, Druesne et al in their investigation showed that, 200 µM of diallyl disulfide (DADS), one of main compounds of garlic, has inhibited proliferation of Caco-2 and HT-29 colon tumor cells and [77]. The inhibition of the proliferation was associated with increase in $p21^{waf1/cip1}$ expression after 6 and 24h incubations. There was also significant accumulation of the cells in G2 phase of cell cycle.

Another study also reported increase in $p21^{waf1/cip1}$ levels to 116% and 124% in prostate cancer cells, PC-3, as a result of effect of DADS in concentrations of 25 µM and 40 µM respectively, after 24 h of treatment [78].

An aromatic sulfur compound, dipyrithione, resulted in apoptosis of HeLa cells in a dose and time dependent manner and IC50 was calculated as 2.3 µg/ml after 36 h [79].

Authors claim that overexpression of p21 $^{waf1/cip1}$ also triggered apoptosis associated with cytochrome c release and caspase-3 activation. Cytochrome c plays an important role in the mitochondrial apoptosis pathway, and when released from mitochondria activates caspase-3, an important downstream effector in the apoptosis pathway.

1.5. Objectives of this investigation

Current work consists of two parts that were explained in more details below. First part deals with investigation of cysteine sulfoxides and essential amino acids in flowers of *Allium* species. Contrary to bulbs, the flowers of *Allium* species are underinvestigated for their sulfur chemistry and probably for their chemistry overall; therefore, they may be good point of research interest. It was decided to screen flowers of *Allium* species for their cysteine sulfoxide and essential amino acid content.
There many publications investigating *Allium* species and several small volatile sulfur compounds for their anticancer activities are present. Unfortunately, species of interest are mainly limited to a few onion varieties and garlic. This fact also leads to the idea of necessity of thorough investigation of other *Allium* species for their anticancer activities.

1.5.1. Screening flowers of Allium species for cysteine sulfoxide and amino acids

One of the scopes of this investigation is to screen for cysteine sulfoxide and amino acid content of the flowers of *Allium* spp. The species were originally collected from Middle East and Central Asia and maintained at a local *Allium* collection in IPK, Germany. Flowers of several species were collected from wild in Afghanistan, Tajikistan or Kyrgyzstan. Extensive consumption of the *Allium* species by the local people of the mentioned areas leads to the idea of possible undiscovered valuable pharmacological effects.

While there is considerable amount of information on cysteine sulfoxide content of bulbs of the species, the information about the content in flowers is rather scarce or missing at

all. Variations in cysteine sulfoxide compositions lead to the biosynthesis of various volatile sulfur compounds catalyzed by the enzyme alliinase.

Qualitative and quantitative analysis of the cysteine sulfoxide and amino acid content was performed using HPLC and HPLC/MS methods. Calibration curve was plotted using measurements of known concentrations of alliin dilutions. Amounts of cysteine sulfoxides and amino acids were calculated using equation of the calibration curve.

1.5.2. Anticancer effect of bulbs of Allium species

Some studies showed that species of subgenera *Melanocrommyum* and *Nectaroscordum* may contain different bioactive OSCs such as dipyrithione and several related sulfur-containing pyridine *N*-oxides. Considering that genus *Allium* consists of more than 750 species and mainly a few varieties of onion and garlic are mainly investigated, it is possible to see that the majority of these species is not yet investigated for its antiproliferative and/or cytotoxic activity against cancer cells. In this study, we also screened ethyl acetate (EtOAc) extracts of bulbs (52), flowers (2), stalk (1), mushroom (*Marasmius alliaceus*) and black paste-like viscous extract of *A. carolianum* (obtained by Prof. Dr. M. Keusgen from local people in Tajikistan in June 2015) for their antiproliferative activity against human bladder cancer cell lines T24 and UMUC3. Pure compounds 2,2′-dipyridyl disulfide and dipyrithione were also tested as they have been previously reported to be present in *A. stipitatum*. The four most cytotoxic extracts were tested for their mechanism of action by measuring the change in protein levels in cancer cell lines. Furthermore, they were subjected to bioactivity-guided fractionation by using preparative HPLC technique. Cytotoxicity of isolated fractions was confirmed, and structures of compounds were elucidated using HR-MS and ^{1}H NMR techniques.

Due to overlap of active *Allium* extracts and fractions from *Allium* extracts, same chromatograms and spectrograms present in this thesis and in doctorate thesis of Sevda Jivishova with title “Nematicidal, Phytotoxic and Brine Shrimp Lethality Activity of

Some *Allium* Species and Their Bioactive Sulfur Compounds". The list of same chromatograms is as follows: "Preparative HPLC Chromatogram of 7002 *A. stipitatum* EtOAc bulb extract", "HPLC Chromatogram of Aldrithiol-2® (2,2′-dipyridyl disulfide) standard", "HPLC Chromatogram of dipyrithione (2,2'-dithiobis(pyridine-N-oxide)) standard", "NMR Chromatogram of fraction 3 from 7002 *A. stipitatum* EtOAc bulb extract-(2-(methyldithio)pyridine N-oxide)", "NMR Chromatogram of fraction 4 from 7002 *A. stipitatum* EtOAc bulb extract-(2-[(methylthio)methyldithio]pyridine N-oxide)". At the time of writing, the thesis of Sevda Jivishova is not published, therefore reference above is provided. Any other identical chromatographic and spectroscopic data found in this thesis and the thesis of Sevda Jivishova is requested to be considered as a result of mutual work and agreement and not as a plagiarism.

2. MATERIALS AND METHODS

2.1. Plant materials

Plant material was mainly collected from the living plant collection at IPK in Gatersleben, Germany. Bulbs of *Allium stipitatum* (7002) were collected from wild in Afghanistan in 2013 for cytotoxicity screening and in 2014 for bioactivity guided fractionation. Dried *A. stipitatum* (Iran) bulb slices were bought from local grocery shop in Iran. Samples of *Marasmius alliaceus* were collected from suburban areas of Marburg. Samples of *Tulbaghia violacea* were collected from living plant collection at Botanical Garden of University of Marburg. The lists of tested species for CSO analysis and cytotoxicity are shown in Table 2.1 and Table 2.2, respectively.

Table 2.1. List of *Allium* species, flowers of which were analyzed for CSO and amino acid content.

Tax/Acc. Nr.	Plant Name	Weight (mg)
4293	*A. rosenorum* R.M. Fritsch	310.0
1653	*A. hollandicum* R.M. Fritsch	787.3
4283	*A. giganteum* Regel	600.0
1222	*A. jesdianum* Boiss. & Buhse subsp. *angustitepalum* (Wendelbo) F.O. Khass. & R.M. Fritsch	724.3
4287	*A. suworowii* Regel	490.0
1198	*A. keusgenii* R.M. Fritsch	467.7
4296	*A. suworowii* Regel	610.0
4282	*A. protensum* Wendelbo	340.0
4285	*A. caspium* (Pall.) M. Bieb.	610.0
4280	*A. gypsaceum* Popov & Vved.	510.0
1148	*A. moderense* R.M. Fritsch	858.0
1207	*A. derderianum* Regel	754.3
5451	*A. nevskianum* Vved. ex Wendelbo	616.0
4291	*A. protensum* Wendelbo	320.0
1082	*A. jesdianum* Boiss. & Buhse subsp. *Angustitepalum* (Wendelbo) F.O. Khass. & R.M. Fritsch	555.0
2530	*A. rosenorum* R.M. Fritsch	1067.0
1177	*A. stipitatum* Regel	1883.5
3953	*A. jesdianum* Boiss. & Buhse subsp. *angustitepalum*	911.0

	(Wendelbo) F.O. Khass. & R.M. Fritsch	
2802	*A. hollandicum* R.M. Fritsch	921.0
1083	*A. jesdianum* Boiss. & Buhse subsp. *angustitepalum* (Wendelbo) F.O. Khass. & R.M. Fritsch	1072.0
2415	*A. macleanii* Baker	973.0
2800	*A. hollandicum* R.M. Fritsch	892.0
3951	*A. jesdianum* Boiss. & Buhse subsp. *angustitepalum* (Wendelbo) F.O. Khass. & R.M. Fritsch	1494.0
1886	*A. rosenorum* R.M. Fritsch	892.0
6275	*A. trautvetterianum* Regel	452.0

Table 2.2. List of *Allium* species tested for cytotoxicity against cancer cells. Bulbs of the plants were used, unless another part specifically mentioned.

TAX/ACC	Plant Name	Source/Year	Subgenus
0985	*A. paniculatum* L. *subsp. fuscum* (Waldst. & Kit.) Arcang.	IPK, Gatersleben, May, 2013	subg. Allium sect. Codonoprasum Rchb. in Mossl.
3183	*A. strictum* Schrad.	IPK, Gatersleben, May,2013	subg. Reticulatobulbosa (Kamelin) N. Friesen sect. Reticulatobulbosa Kamelin
1482	*A. tuberosum* Rottler ex Spreng.	IPK, Gatersleben, May,2013	subg. Butomissa (Salisb.) N. Friesen sect. Butomissa (Salisb.) Kamelin
3548	*A. senescens* L.	IPK, Gatersleben, May, 2013	subg. Rhizirideum (G. Don ex Koch) Wendelbo s. strictiss. sect. Rhizirideum G. Don ex Koch s. str
5410	*A. ramosum* L.	IPK, Gatersleben, May, 2013	subg. Butomissa (Salisb.) N. Friesen sect. Butomissa (Salisb.) Kamelin
1017	*A. atropurpureum* Waldst. & Kit.	IPK, Gatersleben, May, 2013	subg. Melanocrommyum (Webb et Berthel.) Rouy sect. Melanocrommyum Webb et Berthel. s. str.

			Allium multibulbosum alliance
5011	*A. fedschenkoanum* Regel	Kyrgyzstan, 2013	subg. Polyprason Radic sect. Falcatifolia N. Friesen
7057	*A. oreoprasum* Schrenk	Kyrgyzstan, 2013	subg. Butomissa (Salisb.) N. Friesen sect. Austromontana N. Friesen
7051	*A. oreoprasum* Schrenk	Kyrgyzstan, 2013	subg. Butomissa (Salisb.) N. Friesen sect. Austromontana N. Friesen
5022	*A. oreoprasum Schrenk*	Kyrgyzstan, 2013	subg. Butomissa (Salisb.) N. Friesen sect. Austromontana N. Friesen
7053	*A. talassicum* Regel	Kyrgyzstan, 2013	subg. Polyprason Radic sect. Oreiprason F. Herm
6178	*A. pskemense* B. Fedtsch.	IPK, Gatersleben, May, 2013	subg. Cepa (Mill.) Radic sect. Cepa (Mill.) Prokh. subsect. Cepa (Mill.) Stearn
3513	*A. angulosum* L.	IPK, Gatersleben, May, 2013	subg. Rhizirideum (G. Don ex Koch) Wendelbo s. strictiss. sect. Rhizirideum G. Don ex Koch s. str.
3558	*A. victorialis* L. *subsp. victorialis*	IPK, Gatersleben, May, 2013	subg. Anguinum (G. Don ex Koch) N. Friesen sect. Anguinum G. Don ex Koch
5475	*A. stipitatum* Regel	IPK, Gatersleben, May,2013	subg. Melanocrommyum (Webb et Berthel.) Rouy sect. Procerallium R.M. Fritsch subsect. Elatae R.M. Fritsch

5738	*A. stellerianum* Willd.	IPK, Gatersleben, May, 2013	subg. Rhizirideum (G. Don ex Koch) Wendelbo s. strictiss. sect. Rhizirideum G. Don ex Koch s. str.
1642	*A. spirale* Willd.	IPK, Gatersleben, May,2013	subg. Rhizirideum (G. Don ex Koch) Wendelbo s. strictiss. sect. Rhizirideum G. Don ex Koch s. str.
3740	*A. denudatum* F. Delaroche	IPK, Gatersleben, May, 2013	subg. Rhizirideum (G. Don ex Koch) Wendelbo s. strictiss. sect. Rhizirideum G. Don ex Koch s. str.
0192	*A. schoenoprasum* L.	IPK, Gatersleben, May, 2013	subg. Cepa (Mill.) Radic sect. Schoenoprasum Dumort.
10001	*A. schoenoprasum* L.	Finland, August, 2014	subg. Cepa (Mill.) Radic sect. Schoenoprasum Dumort.
7002	*A. stipitatum* Regel	Afghanistan, 2013	subg. Melanocrommyum (Webb et Berthel.) Rouy sect. Procerallium R.M. Fritsch subsect. Elatae R.M. Fritsch
1732	*A. rupestre* Steven	IPK, Gatersleben, May, 2013	subg. Allium sect. Codonoprasum Rchb. in Mossl.
3193	*A. rubens* Schrad. ex Willd.	IPK, Gatersleben, May, 2013	subg. Rhizirideum (G. Don ex Koch) Wendelbo s. strictiss. sect. Rhizirideum G. Don ex Koch s. str.
5309	*A. rotundum* L. *subsp. rotundum*	IPK, Gatersleben, May, 2013	subg. Allium sect. Allium
5316	*A. pictistamineum* O. Schwarz	IPK, Gatersleben, May,2013	subg. Allium sect. Codonoprasum Rchb. in Mossl.

0068	*A. cernuum* Roth	IPK, Gatersleben, May,2013	subg. Amerallium Traub sect. Lophioprason Traub subsect. Cernua Ownbey et Traub
2800	*A. hollandicum* R.M. Fritsch	IPK, Gatersleben, May, 2013	subg. Melanocrommyum (Webb et Berthel.) Rouy sect. Procerallium R.M. Fritsch subsect. Costatae R.M. Fritsch
5193	*A. cornutum* Clementi	IPK, Gatersleben, May, 2013	subg. Cepa (Mill.) Radic sect. Cepa (Mill.) Prokh. subsect. Cepa (Mill.) Stearn
3187	*A. obliquum* L.	IPK, Gatersleben, May, 2013	subg. Polyprason Radic sect. Petroprason F. Herm.
2672	*A. maximowiczii* Regel	IPK, Gatersleben, May, 2013	subg. Cepa (Mill.) Radic sect. Schoenoprasum Dumort.
1513	*A. lusitanicum* Lam.	IPK, Gatersleben, May, 2013	subg. Rhizirideum (G. Don ex Koch) Wendelbo s. strictiss. sect. Rhizirideum G. Don ex Koch s. str.
3200	*A. altaicum* Pall.	IPK, Gatersleben, May, 2013	subg. Cepa (Mill.) Radic sect. Cepa (Mill.) Prokh. subsect. Phyllodolon (Salisb.) Kamelin
3208	*A. nutans* L.	IPK, Gatersleben, May, 2013	subg. Rhizirideum (G. Don ex Koch) Wendelbo s. strictiss. sect. Rhizirideum G. Don ex Koch s. str.
0126	*A. rotundum* L. *subsp. rotundum*	IPK, Gatersleben, May, 2013	subg. Allium sect. Allium
5263	*A. stipitatum* Regel	IPK, Gatersleben, April, 2014	subg. Melanocrommyum (Webb et Berthel.) Rouy sect.

			Procerallium R.M. Fritsch subsect. Elatae R.M. Fritsch
2390	*A. pallens* L. subsp*. pallens*	IPK, Gatersleben, May, 2013	subg. Allium sect. Codonoprasum Rchb. in Mossl.
5301	*A. rupestre* Steven	IPK, Gatersleben, May, 2013	subg. Allium sect. Codonoprasum Rchb. in Mossl.
2584	*A. douglasii* Hook*.*	IPK, Gatersleben, May, 2013	subg. Amerallium Traub sect. Lophioprason Traub subsect. Falcifolia Traub
0469	*A. flavum* L. *var.ssp. flavum*	IPK, Gatersleben, May, 2013	subg. Allium sect. Codonoprasum Rchb. in Mossl.
2023	*A. canadense* L. *var. canadense*	IPK, Gatersleben, May, 2013	subg. Amerallium Traub subsect. Canadensia Ownbey et Traub
2256	*A. rosenorum* R.M.Fritsch	IPK, Gatersleben, May, 2013	subg. Melanocrommyum (Webb et Berthel.) Rouy sect. Procerallium R.M. Fritsch subsect. Costatae R.M. Fritsch
2218	*A. macleanii* Baker (Flower)	IPK, Gatersleben, May, 2013	subg. Melanocrommyum (Webb et Berthel.) Rouy sect. Compactoprason R.M. Fritsch subsect. Erectopetala F.O. Khass
5451-IPK2013	*A. nevskianum* Vved. ex Wendelbo (Flower)	IPK, Gatersleben, May, 2013	subg. Melanocrommyum (Webb et Berthel.) Rouy sect. Kaloprason K. Koch subsect. Ligulifolia R.M. Fritsch

0779-IPK2013	*A. karataviense* Regel	IPK, Gatersleben, May, 2013	subg. Melanocrommyum (Webb et Berthel.) Rouy sect. Miniprason R.M. Fritsch
0882-IPK2013	*A. oreophilum* C.A. Mey.	IPK, Gatersleben, May, 2013	subg. Porphyroprason (Ekberg) R.M. Fritsch sect. Porphyroprason Ekberg
5372-IPK2013	*A. sphaerocephalon* L. subsp. *sphaerocephalon*	IPK, Gatersleben, May, 2013	subg. Allium sect. Allium
IRAN	*A. stipitatum* Regel	IRAN, Commercial, 2011	subg. Melanocrommyum (Webb et Berthel.) Rouy sect. Procerallium R.M. Fritsch subsect. Elatae R.M. Fritsch
2996	*A. platyspathum* Schrenk *subsp. amblyophyllum* (Kar. & Kir.) N. Friesen	IPK, Gatersleben, May, 2013	subg. Polyprason Radic sect. Falcatifolia N. Friesen
1178	*A. aflatunense* B. Fedt.	IPK, Gatersleben, May, 2013	subg. Melanocrommyum (Webb et Berthel.) Rouy sect. Acmopetala R.M. Fritsch subsect. Acmopetala R.M. Fritsch
TGT-2014	*A. sativum* L.	Commercial, Tegut,Germany, 2014	subg. Allium sect. Allium
	Marasmius alliaceus Jacq.	Marburg, 2014	genus *Marasmius*
	Tulbaghia violacea Harv. (Stalks)	U. of Marburg, Bot. Garden, April, 2015	*Tulbaghia* L.
	Tulbaghia violacea Harv. (Bulbs)	U. of Marburg, Bot. Garden, April, 2015	*Tulbaghia* L.
7107	*A. mirum* Wendelbo	Afghanistan, 2015	subg. Melanocrommyum (Webb et Berthel.) Rouy sect. Thaumasioprason Wendelbo

6275	*A. trautvetterianum* Regel	Tajikistan, 2015	subg. Melanocrommyum (Webb et Berthel.) Rouy sect. Compactoprason R.M. Fritsch subsect. Erectopetala F.O. Khass.
6268	*A. carolinianum* DC.	Tajikistan, 2015	subg. Polyprason Radic sect. Falcatifolia N. Friesen
Taj-2015	Black paste-like extract from *Allium carolianum*	Tajikistan, 2015	subg. Melanocrommyum (Webb et Berthel.) Rouy sect. Compactoprason R.M. Fritsch subsect. Erectopetala F.O. Khass.

2.1.1. Preparation of ethyl acetate extracts of plant material

Depending on availability of plant material, 5-10 g of bulb or flower were cut into small pieces and ground in mortar with sea sand using pestle. About 10-20 mL of PBS (pH 7.4) were added to the ground plant material in mortar to provide favorable environment for alliinase reaction and ensure completion of biosynthesis of volatile sulfur compounds. After 1 hour of incubation at room temperature, the mixture was transferred into Erlenmeyer flask and was subjected for further extraction with ethyl acetate. For this, 200 ml of ethyl acetate were added into the flask and carefully and slowly swirled by hand, to avoid formation of emulsion. Swirling process was performed for 15 minutes. After 15 minutes, the ethyl acetate phase containing extracted compounds was carefully transferred into a collection flask. The plant material was subjected to extraction with ethyl acetate 3 times more in the same manner, but using only 100 ml of ethyl acetate each time. In total 500 ml of ethyl acetate was used to extract sulfur compounds from the plant material. Each time organic phases were collected and dried over anhydrous magnesium sulfate. Organic solvent containing *Allium* extract was evaporated using rotary evaporator at about 27 °C until preferably oily residue left. The

oily residue was transferred equally into 5 or 6 vials and carefully evaporated under N_2 gas. The vials were weighed and then stored at -20 °C until further utilization. These extracts were used in XTT cell proliferation assay and real-time PCR.

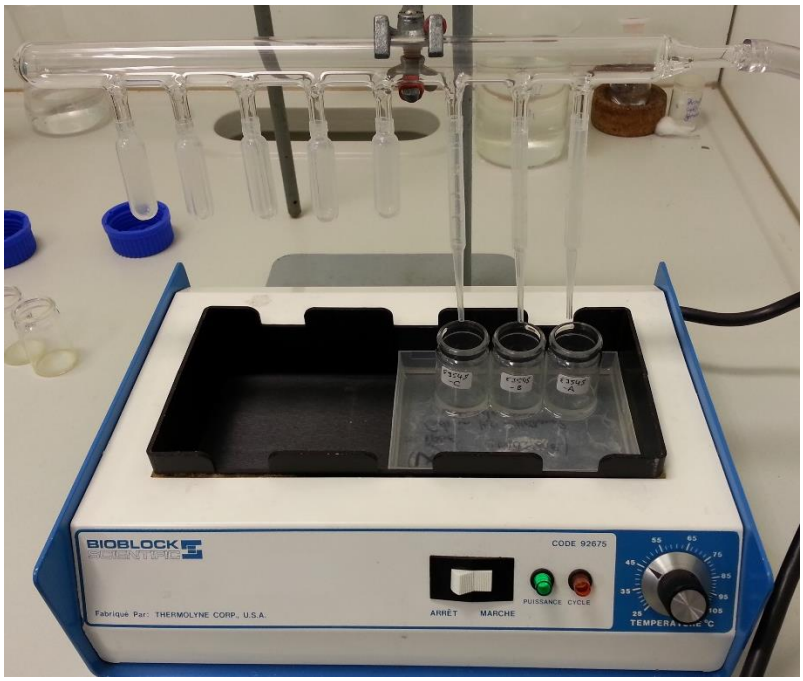

Figure 2.1. Preparation of *Allium* extracts for quantitative and qualitative analysis. Evaporating EtOAc from the extracts using nitrogen gas.

Between 500-1000 mg of *Allium* flowers were weighed. The flowers were roughly cut and heated in 10 mL of methanol under reflux. After 10 minutes the sample was cooled and homogenized in mortar. The material was completely trasnferred back into the flask by rinsing the mortar with methanol. Then, 10 ml of distilled water was added in the flask and heated for next 10 minutes under reflux. The sample was cooled, filtered to get rid of plant material and carefully evaporated under reduced pressure at 35 °C. The samples were weighed and then stored at -20 °C until further utilization.

2.2. Reagents, equipment and devices

Reagents, equipment and devices used in this investigation are listed in Table 2.3.

Table 2.3. Reagents, equipment and devices used in this investigation.

Reagent	Source
Ethyl acetate, ≥99.5%	Merck, Darmstadt
di-Sodium hydrogen phosphate dihydrate, > 98% USP	Merck, Darmstadt
Propidium iodide solution (1.0 mg/ml in water), ≥94%	Sigma-Aldrich, Seelze/Deisenhofen
2-Methyl-2-propanthiol	Merck, Darmstadt
Ammonium acetate > 96%, pure	Carl Roth GmbH + Co. KG, Karlsruhe
Anhydrous MgSO4	Merck, Darmstadt
Phosphate buffer saline solution (PBS, pH 7.4)	Merck, Darmstadt
di-Sodium hydrogen phosphate monohydrate	Merck, Darmstadt
Methanol	Merck, Darmstadt
Aldrithiol-2	Sigma-Aldrich, Seelze/Deisenhofen
Acetonitrile LiChrosolv®	Merck, Darmstadt
Methanol HPLC-grade	Merck, Darmstadt
Methanol HPLC-MS grade	Merck, Darmstadt
Iodacetamide	Fluka (Sigma-Aldrich Fine Chemicals); Seelze/Deisenhofen
o-Phthalaldehyde [for HPLC Labeling]	TCI Deutschland GmbH, Eschborn
Sodium tetraborate decahydrate (Na2B4O7*10H2O)	Merck, Darmstadt

Roswell Park Memorial Institute (RPMI) 1640 Medium	Sigma Chemical Company, St. Louis. MO, USA
Fetal bovine serum	Gibco BRL, Grand Island, NY, USA
TriFast reagent	PEQLAB-Life Science, Erlangen,
SYBR green	ThermoScientific, UK
IQ5 Real-Time PCR	Biorad, Germany
HO-1, Nrf2, CDKN1a, ß-actin primer sets	Biomers, Germany
Pierce BCA Protein Assay	Thermo Scientific, Rockford, USA
Primary antibody for Hemeoxygenase (HO-1)	Cell Signaling, Leiden, Netherlands
Primary antibody for β-actin (monoclonal mouse)	Abcam, Cambridge, UK
Anti-rabbit immunoglobulin	Thermo Scientific, Rockford, USA
Chemiluminescent luciferase kit	Thermo Scientific, Rockford, USA
2,2′-dipyridyl disulfide	Sigma-Aldrich, Seelze/Deisenhofen
Dipyrithione	Sigma-Aldrich, Seelze/Deisenhofen
XTT cytotoxicity proliferation assay kit	AMS Biotech, UK
Equipment and Devices	**Source**
VP 250/2 Nucleodur 100-5 C18 EC HPLC Column	Macherey-Nagel, Düren
VP 250/4 Nucleodur 100-5 C18 EC HPLC Column	Macherey-Nagel, Düren
VP 250/16 Nucleodur 100-5 C18 EC HPLC Column	Macherey-Nagel, Düren
Merck Hitachi 7000 HPLC-System	Merck KGaA, Darmstadt,
Waters HPLC-System (600E System Controller, 991 Waters PDA)	Waters, Milford, USA
JEOL-ECA 500 NMR-Spektrometer	Jeol, Tokyo, Japan
Emax Microplate reader	Molecular Devices, Sunnyvale, CA, USA

Attune Acoustic Focusing Cytometer	Thermo Fisher Scientific, Waltham, MA USA
Electroblot	Millipore, Bedford, USA
Fluorchem IS-8900 imager system	Alpha Innotech, San Leandro, USA

2.2.1. Preparation of borate buffer pH 9.5

Firstly, 4.77 g of sodium tetraborate decahydrate ($Na_2B_4O_7*10H_2O$) were dissolved in 100 ml of distilled water. The pH of the solution was adjusted to 9.5 using 1M sodium hydroxide solution. The resulting solution was transferred into 250 mL volumetric flask and filled with distilled water to the graduation mark.

2.2.2. Preparation of derivatisation agent

Due to toxicity and volatility of the reagent, it was prepared under the hood. 140 mg of ortho-phthalaldehyde was completely dissolved in 5 ml of methanol. 200 µl of 2-methyl-2-propanethiol were added. Finally, 50 ml of borate buffer (pH 9.5) were added slowly and swirling after each addition. The reagent was kept for 12 hours in the dark before utilization. To refresh the reagent after 48 hours, 100 µl of 2-methyl-2-propanethiol were added.

2.2.3. Preparation of 1M iodoacetamide solution

Exactly 185 mg of iodoacetamide were dissolved completely in 1 ml of methanol. The reagent was stored at -20 °C when not used. At first signs of yellowing, the reagent was discarded, and new reagent was prepared.

2.2.4. Preparation of phosphate buffer (pH 6.5) for HPLC

Full amount of disodium hydrogen phosphate and sodium dihydrogen phosphate dihydrate were completely dissolved in about 1500 ml of distilled water using magnetic

stirrer. When needed the acidity of resulting solution was adjusted to pH of 6.5 with 1 M NaOH. The solution was transferred into a 2 L volumetric flask and filled with distilled water to the graduation mark.

2.2.5. Preparation of 50 mM ammonium acetate buffer (pH 6.5) for HPLC-MS

3.84 g of ammonium acetate were dissolved completely in 350 mg of distilled water. When needed the pH was adjusted to 6.5 using acetic acid [c(CH_3COOH)=0.2 mol/L]. The solution was transferred to volumetric flask and filled with distilled water to the graduation mark.

2.3. HPLC preparations and analyses

Quantitative and qualitative analyses of *Allium* species' flowers were performed using HPLC method developed by Kusterer and Keusgen [80]. Volatile sulfur compounds in *Allium* bulb extracts were analyzed by means of HPLC by newly developed methods. Same HPLC methods were used to fractionate *Allium* extracts for bioactivity assays and later to isolate volatile sulfur compounds found in bioactive fractions.

2.3.1. Quantitative and qualitative analyses of Allium flower extracts derivatized with OPA

In order to derivatise *Allium* flower extracts, 4.93 ml of OPA reagent and 50 mL of 2-methyl-2-propanethiol were added into flasks, containing *Allium* flower extracts. Flasks were kept in the dark for 30 minutes. Subsequently, 20 µl of 1M iodoacetamide solution were added to the flasks. The flaks were incubated for 2 more minutes. Thereafter, the extracts were filtered using syringe with attached syringe filters. First, extract filtration was performed through 0.45 µm pore size filter following filtration with 0.2 µm pore size filter. Derivatized extracts were diluted with distilled water in 1:1 ratio before quantitative HPLC analysis. Separation was performed using Merck 7000 HPLC system and Nucleodur 100-5 C18ec column (250 x 4 mm, 5 microns). The injection volume was 20 µl. An HPLC gradient used to analyze samples displayed in Table 2.4. Same gradient

program was used for qualitative HPLC/MS analysis. A column with smaller diameter (2 mm) was used. For quantitative analysis eluents were acetonitrile HPLC grade (Eluent A) and 50 mM phosphate buffer pH 6.5 (Eluent B). For qualitative HPLC/MS analysis eluents were acetonitrile HPLC grade (Eluent A) and 50 mM ammonium acetate buffer pH 6.5 (Eluent B). Peaks were identified using a diagram proposed by Kusterer (Figure 2.1). The diagram shows retention times of amino acids, CSOs and their γ-glutamyl derivatives based on the gradient analysis program. The amounts of cysteine sulfoxides and amino acids were calculated by using external standard method. For this, known concentrations of OPA-derivatized alliin were measured using quantitative HPLC method. Isocratic HPLC method shown in Table 2.5 was used for the HPLC measurement of alliin standard for calibration curve. Calibration graph was drawn using alliin chromatogram peak area and concentration data. The UV/VIS detector was set to 334 nm for all measurements of OPA derivatized *Allium* extracts.

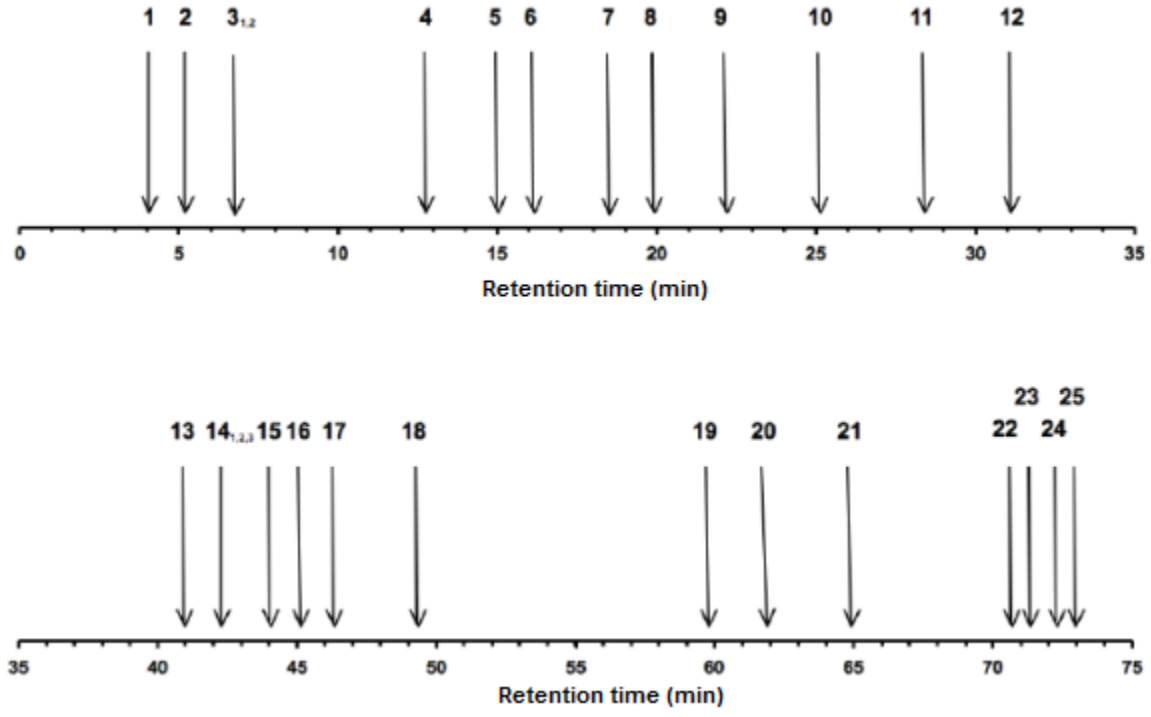

Figure 2.2. Retention times of amino acids and CSO identified previously by Kusterer [81]. 1. Aspartic acid, 2. Glutamic acid, 3.1 γ-Glutamyl-Isoalliin, 3.2 γ-Glutamyl-Homoisoalliin, 4. Asparagine, 5. Histidine, 6. Glutamine, 7. (+)-Methiin, 8.

Serine, 9. S-(2-Pyridyl)-Cysteinsulfoxid, 10. Arginine, 11. Threonine, 12. Glycine, 13. (+)-Alliin, 14.1 (+) - Isoalliin, 14.2 (+)-Marasmin, 14.3 γ-Glutamyl-S-(1-Butenyl)-Cystein, 15. Tyrosine, 16. Alanine, 17. Propiin, 18. S-(3-Pyrrolyl)-Cysteinsulfoxid, 19. (+)-Homoisoalliin, 20. (+)-Butiin, 21. Valine, 22. Tryptophane, 23. Phenylalanine, 24. Isoleucine, 25. Leucine

Table 2.4. The gradient program used for cysteine sulfoxide and amino acid analysis [80].

Time (min)	Eluent A (%)	Eluent B (%)
0	22	78
20	22	78
49	25	75
50	25	75
54	29	71
55	29	71
63	32	68
65	37	63
75	37	63
75,1	22	78
85	22	78

Table 2.5. The isocratic program used for calibration curve.

Time (min)	Eluent A (%)	Eluent B (%)
0	30	70
20	30	70

2.3.2. Bioactivity guided fractionation of A. aflatunense, A. stipitatum, A. rosenorum and A. pallens

30 grams of bulbs of *A. aflatunense*, *A. stipitatum*, *A. pallens* and *A. rosenorum* were extracted with ethyl acetate (1000 mL) using procedure described above. Prior to injection, the extracts were dissolved in 40% or 45% methanol solution to obtain final concentration of 90 mg/ml. The resulting solution was filtered through syringe filters (0.45 µm and 0.2 µm). Injection volume was 1 ml. Waters HPLC system (600 E System controller and a Waters 991 PDA) was used for separation. A VP 250/16 Nucleodur 100-5 C18 EC column (250mm x 16mm) was used. UV detection was performed at 254 nm. Gradient HPLC programs used for analysis of the samples displayed in Table 2.6 for *A. aflatunense,* Table 2.7 for *A. stipitatum* and Table 2.8 for *A. pallens* and *A. rosenorum* extracts.

Number of collected fractions for *A. aflatunense* was 7, for *A. stipitatum* was 5, for *A. pallens* was 5 and for *A. rosenorum* was 5. Collected fractions were subjected to liquid-liquid extraction with ethyl acetate aliquots 10 min each with application of salting out method. For this, saturated sodium chloride solution (NaCl solution) was prepared. For each 50 ml of a collected fraction 150 ml of the NaCl solution was added. The final mixture was subjected to liquid-liquid extraction with ethyl acetate three times. Each time 100 ml of ethyl acetate was used for 10 minutes. Combined ethyl acetate layers were evaporated under reduced pressure until near dryness with further evaporation using nitrogen gas. The residues were stored at -20 ºC until further processing. IC_{50} values of fractions were determined using XTT cell proliferation assay.

Table 2.6. The gradient preparative HPLC program used to isolate fractions of *A. aflatunense*

Time (min)	Flow rate (mL/min)	MeOH %	H_2O %
0	8.0	40	60
2.00	8.0	40	60
40.00	8.0	95	5

Table 2.7. The gradient preparative HPLC program used to isolate fractions of *A. stipitatum*

Time (min)	Flow rate (mL/min)	MeOH %	H2O %
0	7.0	45	55
2.00	7.0	45	55
30.00	7.0	95	5

Table 2.8. The gradient preparative HPLC program used to isolate fractions of *A. pallens* and *A. rosenorum*

Time (min)	Flow rate (mL/min)	MeOH %	H_2O %
0	7.0	40	60
2.00	7.0	40	60
30.00	7.0	95	5

2.4. Bioactivity tests

To assess anticancer activity of *Allium* extracts, a standard pre-clinical “drug discovery” process was applied. To describe briefly, during first step, several *Allium* EtOAc extracts were screened using XTT cell proliferation assay in order to determine the most cytotoxic species. Human bladder cancer cell lines T24 and UMUC3 and human fibroplast healthy cells HFF were obtained from Department of Urology and Child Urology at University Hospital of Giessen and Marburg and used in the assay. As a result of the extract screening, four most cytotoxic species were determined and further analyzed using propidium iodide (PI) flow cytometry cell death assay, real-time polymerase chain reaction (real-time PCR) and Western blot technique using T24 and HFF cells. Next, fractions were isolated from the four extracts and screened for their antiproliferative activity against T24 cells. Active fractions were evaluated using XTT antiproliferative assay, real-time PCR and Western Blot analyses. Structures of compounds found in active fractions were elucidated using high resolution mass spectroscopy (HR-MS) and proton nuclear magnetic resonance (^{1}H-NMR) techniques. A diagram representing the flow of the whole process is depicted in Figure 2.2.

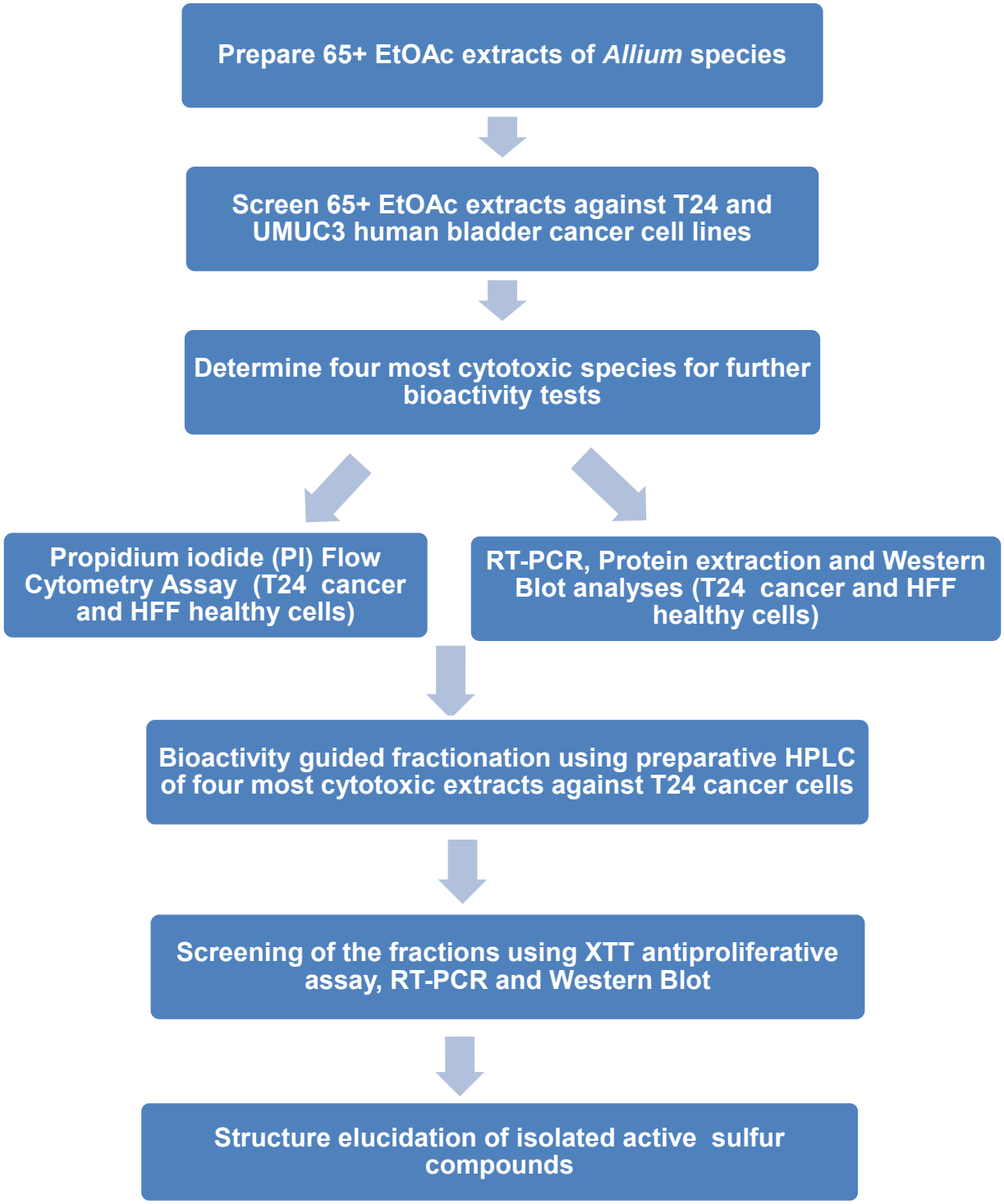

Figure 2.3. A diagram representing whole bioactivity test process applied in this research

2.4.1. Human bladder cancer cell lines (T24 and UMUC3) and human fibroblast cell line (HFF-1)

Human bladder cancer cell lines T24 and UMUC3 and human foreskin fibroblast HFF-1 were kept under standard conditions. The cells were cultured in 75 cm^2 tissue culture flasks and grown at 37°C under a humidified 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2% penicillin-streptomycin (10,1000 U/ml penicillin and 10 mg/ml streptomycin).

2.4.2. XTT cell proliferation assay

To determine extracts with the highest cytotoxic activity, firstly, T24 and UMUC3 cells were seeded in triplicates in 96-well plates with 2500 cells/well for T24 and 6000 cells/well for UMUC3 and incubated for 24 h before treatment. Extracts and compounds were added in wells dose dependently (1.04-33.33 µg/ml). Doxorubicin was used as a positive control. After 48 hours of treatment, cell supernatant was removed and XTT reagent was added. Plates were incubated for 4 hours with XTT reagent. Absorbance was measured at 405 nm with 650 nm as a reference, using microplate reader. IC_{50} values were calculated using Microsoft Excel. The four most cytotoxic *Allium* extracts were tested against HFF-1 cells.

2.4.3. Flow cytometry analysis of cell death in T24 cells treated with EtOAc extracts of A. aflatunense, A. stipitatum, A. rosenorum and A. pallens

T24 cells were seeded in 6-well plates at $15x10^4$cells/well in triplicates and incubated for 24 hours before treatment. Following treatment with EtoAc extracts of A*. aflatunense* (8 µg/ml), *A. stipitatum* (37.38 µg/ml), *A. rosenorum* (28.36 µg/ml), *A. pallens* (6.68 µg/ml) for 48 h, cells were trypsinized and counted to confirm cell count. Afterwards, cells were centrifuged at 1000 rpm for 10 mins, and washed twice with PBS. Cells were suspended in propidium iodide, transferred into Eppendorf tubes and stored on ice in

the dark for 30 min. Flow cytometric analysis was performed using Attune Acoustic Focusing Cytometer. Results were analyzed using Attune Cytometric Software v. 1.2.5.

2.4.4. RNA and protein analytics

Total RNA and proteins of cells were isolated with TriFast reagent according to the manufacturers' protocol. For quantitative real-time PCR, 1 µg RNA was treated by DNAse I and cDNA synthesis was performed with random hexamer primers and M-MLV reverse transcriptase in 20 µl volume. 1 µl cDNA was submitted to SYBR green based real-time PCR. Cycling conditions were: 95°C, 15m, followed by 50 cycles 95°C, 15s, 58°C, 30s, 72°C, 30s. Target mRNA levels are displayed as -ΔCt value relative to TATA-Binding Protein (TBP) reference mRNA. The following primer sets (Table 2.9, Biomers, Germany) were employed (forward sequence, +; reverse sequence, -).

Table 2.9. Primer sets employed in real-time PCR analysis of mRNA extracted from cancer cells treated with *Allium* extracts.

Primer Name	**Forward Sequence, +**	**Reverse Sequence, -**
HO-1	CCT ACA CAC CAG CCA TGC AGC	GTG GCA CTG GCA ATG TTG GG
Nrf2	TGC TTT CAT AGC TGA GCC CAG TAT C	TCC ATA GCT GGA AGA TTC CAC TGA G
CDKN1a	AAG ACC ATG TGG ACC TGT CAC TGT C	GGA TTA GGG CTT CCT CTT GGA GAA G
ß-actin	TAT CCA GGC TGT GCT ATC CCT GTA C	TTC ATG AGG TAG TCA GTC AGG TCC C

For Western-blot analysis, protein concentration was determined (Pierce BCA Protein Assay, Thermo Scientific, Rockford, USA), and 40 µg of each sample was subjected to SDS-PAGE and transferred to polyvinylidene fluoride membrane by electroblot. The membranes were blocked at room temperature for 1.5 h. Primary antibodies for hemeoxygenase-1 (HO-1) (dilution 1:1000) and β-actin (dilution 1:5000) were added and incubated overnight at 4°C in tris-buffered saline with 0,1% Tween containing 5% dry milk. Then, secondary horseradish peroxidase coupled anti-rabbit immunoglobulin (dilution 1:2000) was added for band detection with enhanced chemiluminescent luciferase kit by an Fluorchem IS-8900 imager system.

3. RESULTS

3.1. Quantitative and qualitative HPLC analysis of CSOs and amino acids in flowers of Allium spp.

In this investigation, flowers of *Allium* species were extracted using methanol and water as was described in Section 2.1.2. As most of the amino acids have poor UV absorption, pre-column derivatization with OPA improves their sensitivity and separation in HPLC column. After derivatization with OPA, CSOs and amino acids can be detected at 334 nm wavelength. OPA derivatized *Allium* flower extracts were screened for all previously reported cysteine sulfoxides and amino acids by means of HPLC and HPLC/MS. Retention times of peaks of amino acids and CSOs, obtained from measurements were compared to the retention times of peaks identified and reported earlier by Kusterer [81]. Identity of peaks was confirmed by means of mass spectroscopy. The best separation of the peaks was observed in quantitative analysis using phosphate buffer as one of the eluents. Ammonium acetate buffer was used in qualitative HPLC/MS measurements. This resulted in slight shifts of retention times of tryptophane, phenylalanine, leucine and isoleucine peaks. Therefore, it was not possible to surely assign peaks for those amino acids and they were checked only qualitatively for the presence of their *m/z* values. Retention times of tyrosine and alanine are very close to each other and their peaks at different times did not separate clearly or only one of the amino acids was present, thus reported amount is either a total amount of both or of the present one. Standard deviations were calculated based on average data collected from three HPLC measurements (n=3).

3.1.1. Total CSO content in samples

All quantitative data regarding amounts of CSOs found in the flowers of *Allium* species is presented in Table 3.1. The highest CSO content was found to be 7.05% in *A. rosenorum* (4293). The only CSO found in this sample was methiin, being responsible for the whole CSO content. Another species of *A. rosenorum* (1886) had total CSO

amount of 0.12%. Two CSOs were found in the latter sample, which were methiin-0.11% and S-(2-pyrollyl)-L-CSO-0.01%. Extract of *A. trautvetterianum* (6275) flower, collected from Tajikistan in 2015, had the lowest amount of methiin being equal to 0.04%. The total CSO content of *A. trautvetterianum* (6275) was equal to 0.12%.

A. trautvetterianum (6275) was also the only sample with butiin amount within calculation range, which was equal to 0.08%. Amount of butiin in *A. suworowii* (4287) was out of calculation range. In no other samples butiin was detected.

(+)-Marasmine was present in *A. jesdianum* (1082)-0.03%, *A. jesdianum* (1222)-0.12%, *A. protensum* (4282)-0.15%*, A. protensum* (4291)-0.18%*, A. nevskianum* (5451)-0.07%, and *A. gypsaceum* (4280)-1.46%, which was the highest among all.

(-)-Marasmine was present in *A. gypsaceum* (4280) in amount of 0.14%, making it a sample to contain both isomers of marasmine. Extract of *A. nevskianum* (5451) flower also had (-)-marasmine, but the amount was out of calculation range.

Extracts of *A. stipitatum* (1177) flower was the only sample to bear 2-pyridyl *N*-oxide-L-cysteine sulfoxide with amount of 0.09%.

A red pigment precursor, *S*-(2-pyrollyl)-L-cysteine sulfoxide was most abundant in flower of *A. hollandicum* (1653) with amount of 0.66%. Other species to have the red pigment precursor were *A. jesdianum* (1222)-0.55%, *A. jesdianum* (3951)-0.01%, *A. jesdianum* (1083) -0.07%, *A. jesdianum* (3953)-0.07%, *A. rosenorum* (2530)-0.16%, *A. rosenorum* (1886)-0.01%, *A. hollandicum* (2802) -0.07%, *A. hollandicum* (2800) -0.01%, *A. suworowii* (4287)-0.28%, *A. macleanii* (2415)-0.10%.

3.1.2. Total amino acid content in samples

All quantitative data regarding amounts of CSOs found in the flowers of *Allium* species is presented in Table 3.2. All samples had minimum one of tryptophane, phenylalanine, leucine or isoleucine amino acids.

The highest amino acid content was found to be 29.93% in *A. rosenorum* (4293). The amounts of other amino acids are as follows: aspartic acid -1.02%, glutamic acid-1.56%, asparagine-5.22%, histidine-1.25%, glutamine-10.68%, serine-2.89%, arginine-3.31%, threonine-0.97%, no glycine, tyrosine/ alanine-1.54, valine-1.39%.

Arginine amount was highest in *A. rosenorum* (4293) with 3.3%. In *A. rosenorum* (1886) the amount of arginine was only 0.03%.

Extracts of *A. trautvetterianum* (6275) and *A. jesdianum* (3951) flowers had lowest amounts of amino acids with 0.55% and 0.52% respectively.

Extracts of *A. suworowii* (4287) and *A. keusgenii* (1198) flowers had highest amounts of aspartic acid with 2.60% and 2.48% respectively.

The amount of valine was highest in *A. derderianum* (1207)-3.09%, which also had the highest tyrosine/alanine amount of 3.51%.

Table 3.1. Distribution of CSOs in several *Allium* species from subgenus *Melanocrommyum*

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TAX NR. + PLANT NAME	MATERIAL WEIGHT (mg)	(-)-METHIINE %	STD(%)	(+)-METHIINE %	STD(%)	2-PYRIDYL N-OXIDE CSO %	STD(%)	(-) MARASMINE %	STD(%)	(+)-MARASMINE %	STD(%)	S-(2-PYROLLYL)-L-CSO %	STD(%)	BUTIIN %	STD(%)	TOTAL CSO
4293 *A. rosenorum*	**310**	-	-	7.046	7.11	-	-	-	-	-	-	-	-	-	-	7.046
1653 *A. hollandicum*	**787.3**	TR	-	3.398	0.20	-	-	-	-	-	-	0.655	0.36	-	-	4.053
1222 *A. jesdianum*	**724.3**	TR	-	2.819	1.85	-	-	-	-	0.124	2.63	0.547	1.77	-	-	3.490
4283 *A. giganteum*	**600**	-	-	2.914	1.37	-	-	-	-	-	-	-	-	-	-	2.914
4296 *A. suworowii*	**610**	-	-	1.720	3.86	-	-	-	-	-	-	0.454	5.56	-	-	2.175
4280 *A. gypsaceum*	**510**	-	-	0.545	3.11	-	-	0.141	0.14	1.463	0.38	-	-	-	-	2.150
4287 *A. suworowii*	**490**	TR	-	1.897	2.63	-	-	-	-	-	-	-	-	TR	-	1.897
1198 *A. keusgenii*	**467.7**	-	-	1.882	3.72	-	-	-	-	-	-	-	-	-	-	1.882
4282 *A. protensum*	**340**	TR	-	1.131	5.61	-	-	-	-	0.154	1.55	0.284	3.22	-	-	1.569
4291 *A. protensum*	**320**	TR	-	0.464	2.97	-	-	-	-	0.183	0.16	-	-	-	-	0.648
4285 *A. caspium*	**610**	TR	-	0.597	1.87	-	-	-	-	-	-	-	-	-	-	0.597
5451 *A. nevskianum*	**616**	-	-	0.477	2.20	-	-	TR	-	0.075	0.01	-	-	-	-	0.552
1148 *A. moderense*	**858**	-	-	0.536	7.27	-	-	-	-	-	-	-	-	-	-	0.536
1207 *A. derderianum*	**754.3**	-	-	0.488	2.99	-	-	-	-	-	-	-	-	-	-	0.488
2530 *A. rosenorum*	**1067**	-	-	0.322	0.42	-	-	-	-	-	-	0.157	1.64	-	-	0.479
1082 *A. jesdianum*	**555**	-	-	0.438	0.96	-	-	-	-	0.025	15.49	-	-	-	-	0.463
1177 *A. stipitatum*	**1883.5**	TR	-	0.277	0.80	0.088	4.01	-	-	-	-	-	-	-	-	0.365
3953 *A. jesdianum*	**911**	-	-	0.232	0.32	-	-	-	-	-	-	0.071	0.19	-	-	0.303
2802 *A. hollandicum*	**921**	-	-	0.211	1.28	-	-	-	-	-	-	0.071	4.96	-	-	0.283
2415 *A. macleanii*	**973**	-	-	0.155	10.14	-	-	-	-	-	-	0.098	10.23	-	-	0.253
1083 *A. jesdianum*	**1072**	-	-	0.181	0.11	-	-	-	-	-	-	0.072	0.23	-	-	0.253
2800 *A. hollandicum*	**719**	-	-	0.147	1.78	-	-	-	-	-	-	0.015	0.81	-	-	0.162
3951 *A. jesdianum*	**1494**	-	-	0.145	0.33	-	-	-	-	-	-	0.009	10.18	-	-	0.154
1886 *A. rosenorum*	**892**	-	-	0.108	0.81	-	-	-	-	-	-	0.012	7.48	-	-	0.120
6275 *A. trautvetterianum*	**452**	-	-	0.040	2.63	-	-	-	-	-	-	-	-	0.080	4.28	0.116

Table 3.2. Distribution of amino acids in several *Allium* species from subgenus *Melanocrommyum.*

TAX NR. + PLANT NAME	MATERIAL WEIGHT (mg)	ASPARTIC ACID %	STD(%)	GLUTAMIC ACID %	STD (%)	ASPARAGINE %	STD(%)	HISTIDINE %	STD(%)	GLUTAMINE %	STD(%)	SERINE %	STD(%)	ARGININE %	STD(%)	THREONINE %	STD(%)	GLYCIN %	STD(%)	TYROSINE/ ALANINE %	STD(%)	VALINE %	STD(%)	TOTAL A.A.
4293 *A.rosenorum*	310	1.020	2.62	1.557	2.39	5.219	4.97	1.352	3.86	10.678	2.37	2.892	3.85	3.307	4.50	0.974	3.55	0.000	0.00	1.538	2.93	1.392	2.77	29.930
4287 *A. suworowii*	490	2.600	0.58	1.414	0.77	3.514	0.26	0.461	4.25	2.377	4.68	1.015	1.18	0.863	1.71	0.488	0.14	0.222	1.26	1.386	1.09	0.820	2.73	15.161
1198 *A. keusgenii*	467.7	2.479	4.25	1.270	1.90	0.777	1.54	0.000	0.00	1.578	4.88	1.882	3.72	0.523	3.14	0.534	1.73	0.000	0.00	1.729	1.61	1.263	0.65	12.063
1207 *A. derderianum*	754.3	0.570	0.91	1.820	2.95	0.397	1.02	0.132	1.50	0.491	5.42	0.260	0.29	0.220	4.17	0.667	2.94	0.301	2.95	3.513	1.20	3.086	1.39	11.456
1148 *A. moderense*	858	1.689	1.60	0.755	2.73	2.411	2.51	0.468	4.62	1.620	1.48	0.822	3.17	0.467	3.80	0.423	1.60	0.147	1.92	0.576	5.54	0.778	2.84	10.155
4282 *A. protensum*	340	0.410	3.29	0.706	1.46	1.997	4.53	0.399	4.88	1.980	2.52	1.155	5.10	1.818	1.26	0.327	0.94	0.000	0.00	0.740	0.00	0.384	5.64	9.916
4285 *A. caspium*	610	0.312	0.84	0.838	6.99	0.516	1.79	TR	0.00	4.281	3.27	0.823	9.35	0.243	8.71	0.274	10.61	0.000	4.72	1.270	7.61	0.700	4.65	9.257
4291 *A. protensum*	320	0.407	0.69	0.730	0.81	1.925	1.93	0.384	0.78	2.753	1.15	0.352	2.92	0.503	0.27	0.288	2.02	0.199	1.03	0.451	0.15	0.434	0.10	8.427
4296 *A. suworowii*	610	0.804	5.71	0.762	6.42	0.652	8.97	0.430	6.97	1.507	3.94	0.789	2.10	0.487	0.96	0.360	2.55	0.303	1.31	1.261	0.27	1.035	1.18	8.390
1222 *A. jesdianum*	724.3	0.600	1.22	0.603	2.10	0.687	0.63	0.127	5.98	1.658	2.72	1.221	1.83	0.786	0.95	0.393	0.50	0.162	0.42	0.637	1.73	0.632	0.72	7.507
4283 *A. giganteum*	600	0.345	2.11	0.573	3.55	0.727	0.78	0.000	0.00	1.029	0.62	0.999	3.18	0.763	1.58	0.261	3.67	0.000	0.00	1.026	2.57	0.842	3.11	6.587
4280 *A. gypsaceum*	510	0.000	0.00	0.400	1.41	0.561	2.76	0.145	11.24	2.619	1.14	0.777	0.35	0.579	1.86	0.398	1.00	0.136	0.79	0.257	1.66	0.240	2.79	6.136
5451 *A. nevskianum*	616	0.219	6.25	0.450	1.61	0.610	1.65	0.237	5.96	1.357	1.38	0.332	0.44	0.150	0.63	0.103	5.49	0.068	0.31	0.203	0.14	0.195	2.31	3.925
1653 *A. hollandicum*	787.3	0.162	4.78	0.531	1.92	0.273	0.73	0.135	7.29	1.037	0.75	0.202	2.34	0.117	0.80	0.164	3.94	0.062	1.32	0.426	3.11	0.217	1.51	3.326
1177 *A. stipitatum*	1883.5	0.356	4.63	0.687	0.83	0.149	1.31	0.000	0.00	0.193	4.08	0.278	1.91	0.049	0.97	0.069	0.34	0.000	0.00	0.182	1.68	0.106	0.58	2.076
1082 *A. jesdianum*	555	0.086	1.77	0.144	1.29	0.026	0.59	TR	0.00	0.688	0.48	0.000	0.00	0.302	1.31	0.073	0.73	0.048	0.33	0.204	7.93	0.166	3.27	1.736
2802 *A. hollandicum*	921	0.027	2.12	0.108	0.16	0.052	1.52	TR	0.00	0.344	0.22	0.269	1.10	0.044	0.52	0.026	1.26	0.013	0.77	0.102	2.95	0.103	1.23	1.088
2530 *A. rosenorum*	1067	0.141	0.72	0.032	0.83	0.053	1.53	TR	0.00	0.138	0.56	0.485	0.40	0.026	1.54	0.025	0.89	0.023	2.13	0.097	0.86	0.060	0.43	1.081
3953 *A. jesdianum*	911	0.035	1.73	0.070	0.81	0.086	2.00	TR	0.00	0.337	0.17	0.190	0.30	0.069	0.67	0.040	0.13	0.018	0.07	0.045	0.16	0.112	1.12	1.001
2415 *A. macleanii*	973	0.057	12.18	0.089	1.47	0.117	10.11	TR	0.00	0.350	10.05	0.104	10.14	0.049	3.93	0.021	15.68	0.000	0.00	0.067	10.70	0.054	12.47	0.916
1886 *A. rosenorum*	892	0.034	1.00	0.052	1.32	0.038	0.01	TR	0.00	0.250	0.73	0.270	0.80	0.029	16.82	0.022	11.83	0.015	12.06	0.108	0.92	0.080	0.27	0.896
1083 *A. jesdianum*	1072	0.024	0.07	0.074	0.08	0.062	0.03	TR	0.00	0.253	0.01	0.145	0.10	0.034	0.30	0.035	0.82	0.016	0.65	0.078	0.16	0.102	0.76	0.822
2800 *A. hollandicum*	719	0.017	7.02	0.100	3.17	0.034	0.59	TR	0.00	0.201	0.04	0.221	1.70	0.022	0.59	0.014	2.68	0.011	8.39	0.063	1.10	0.059	0.76	0.742
6275 *A. trautvetterianum*	452	0.037	1.17	0.10	1.89	0.09	1.07	TR	0.00	0.12	1.03	0.04	2.36	0.03	4.23	0.01	3.20	0.000	0.00	0.07	0.08	0.09	6.20	0.545
3951 *A. jesdianum*	1494	0.027	0.53	0.065	0.70	0.051	0.05	TR	0.00	0.216	1.05	0.000	0.00	0.027	0.33	0.028	3.49	0.020	0.42	0.045	2.48	0.042	0.02	0.521

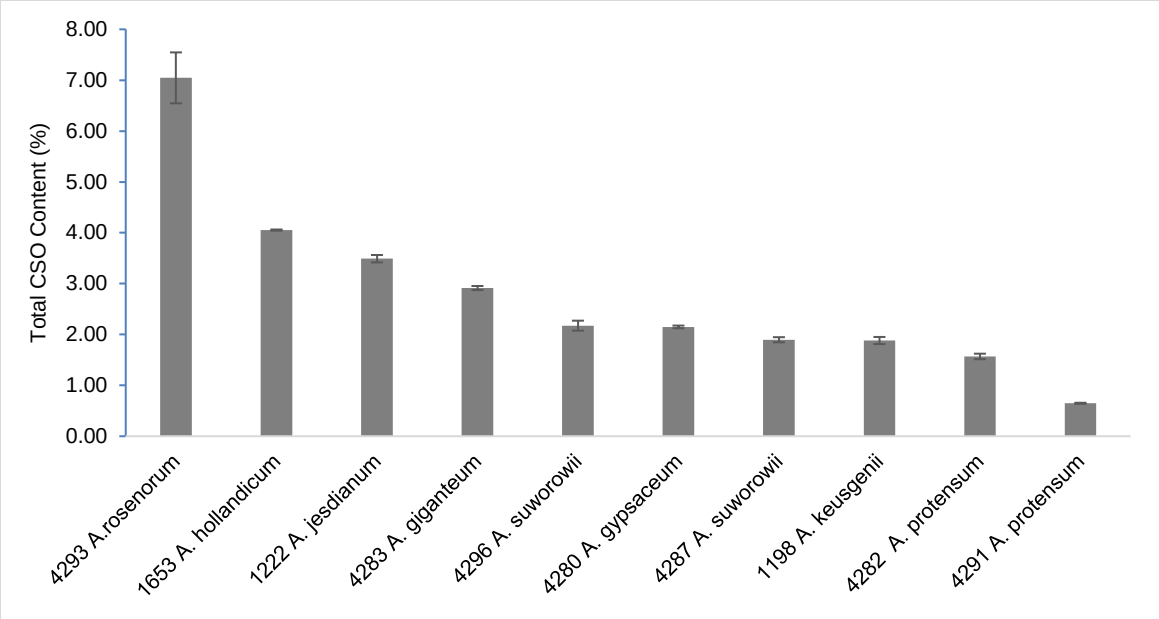


Figure 3.1. Ten samples with the highest total CSO content (%)

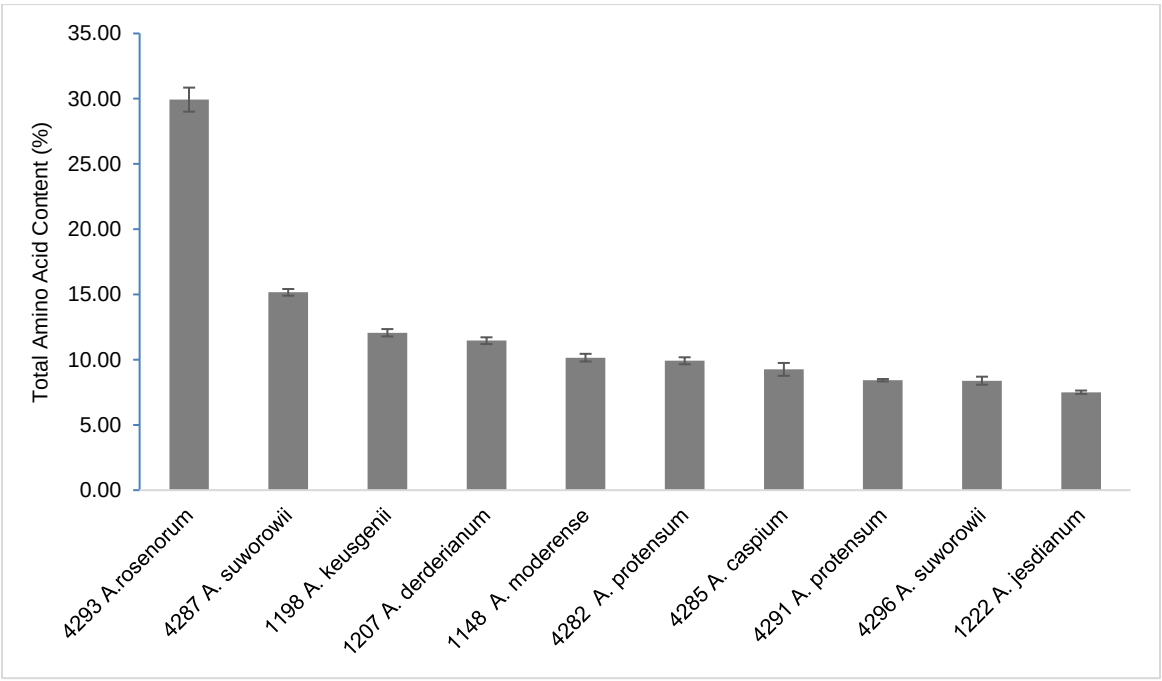


Figure 3.2. Ten samples with the highest total amino acid content (%)

3.2. Bioactivity against cancer cells

3.2.1. Inhibition of cell proliferation by Allium EtOAc extracts in T24 and UMUC3 cells

To characterize the effects of *Allium* extracts, T24 and UMUC3 cells were treated dose dependently for 48 hours. After 48 hours, the cytotoxicity was assessed using XTT assay and expressed as IC_{50}. Results are presented in three groups. Tested subjects with IC_{50}<=1.0 µg/ml against minimum 1 cell line labeled as “very active”, tested subjects with 1.0 µg/ml<IC_{50}<16.67 µg/ml against minimum one cell line labeled as “moderately active” and tested subjects with IC_{50} ≥16.67 µg/ml labeled as “inactive” within tested concentration range.

Table 3.3. IC50 (µg/ml) values of “very active” tested subjects against T24 and UMUC3 cancer cells.

		T24		UMUC3	
TAX/ACC No.	**MATERIAL**	**mean**	**SD**	**mean**	**SD**
	Doxorubicin	0.19	0.03	0.23	0.02
	Dipyrithione	0.41	0.11	0.62	0.05
1178	*A. aflatunense*	0.18	0.00	0.70	0.02
7002	*A. stipitatum*	0.38	0.02	0.65	0.08
IRAN-Commer.	*A. stipitatum*	0.92	0.13	2.46	0.04

Table 3.4. IC50 (µg/ml) values of “moderately active” tested subjects against T24 and UMUC3 cancer cells.

		T24		UMUC3	
TAX/ACC	**MATERIAL**	**mean**	**SD**	**mean**	**SD**
2390	*A. pallens*	1.67	0.06	2.38	1.12
	Aldrithiol-2	3.73	0.21	10.57	0.30
2256	*A. rosenorum*	7.09	0.24	3.38	0.15
Commercial	*A. sativum*	8.79	0.44	12.42	0.69
3208	*A. nutans*	10.82	0.54	6.32	0.10
2996	*A. platyspathum*	11.01	1.30	6.01	0.15
2800	*A. hollandicum*	13.73	0.59	10.16	0.07
0779	*A. karataviense*	14.10	0.55	7.22	0.60
5015	*A. oreoprasum*	14.35	0.42	14.42	0.61
3548	*A. senescens*	14.39	0.68	12.47	1.42

Table 3.5. IC50 (μg/ml) values of “inactive” tested subjects against T24 and UMUC3 cancer cells.

		T24		UMUC3	
TAX/ACC No.	**ALLIUM**	**mean**	**SD**	**mean**	**SD**
0985	*A. paniculatum*	23.75	0.3	>33.3	
3183	*A. strictum*	31.49	1.72	>33.3	
1482	*A. tuberosum*	>=33.3		>33.3	
5410	*A. ramosum*	16.31	1.06	>=33.3	
1017	*A. atropurpureum*	>33.3		28.71	2.77
5011	*A. fedschenkoanum*	24.47	0.8	>33.3	
7051	*A. oreoprasum*	>33.3		>33.3	
7052	*A. oreoprasum*	>33.3		>33.3	
7053	*A. talassicum*	>33.3		>33.3	
6178	*A. pskemense*	>33.3		>33.3	
3513	*A. angulosum*	>33.3		>33.3	
3558	*A. victorialis*	>33.3		>33.3	
5738	*A. stellerianum*	>33.3		19.14	0.32
1642	*A. spirale*	>33.3		32.87	0.51
3740	*A. denudatum*	16.65-33.3		15.98	0.56
0192	*A. schoenoprasum*	31.57	1.32	>33.3	
10001	*A. schoenoprasum*	>33.3		>33.3	
1732	*A. rupestre*	>33.3		>33.3	
3193	*A. rubens*	>33.3		>33.3	
5309	*A. rotundum*	>33.3		>33.3	
5316	*A. pictistamineum*	>33.3		>33.3	
0068	*A. cernuum*	>33.3		>33.3	
5193	*A. cornutum*	>33.3		>33.3	
3187	*A. obliquum*	>33.3		>33.3	
2672	*A. maximowiczii*	>33.3		>33.3	
1513	*A. lusitanicum*	>33.3		>33.3	
3200	*A. altaicum*	>33.3		>33.3	
0126	*A. rotundum*	23.16	4.56	30.36	1.88

		T24		UMUC3	
TAX/ACC No.	**ALLIUM**	**mean**	**SD**	**mean**	**SD**
5301	*A. rupestre Steven*	15.35	2.37	25.09	2.43
2584	*A. douglasii*	25.02	3.23	28.39	1.29
0469	*A. flavum*	>33.3		>33.3	
2023	*A. canadense*	>33.3		>33.3	
2218	*A. macleanii* (flower)	30.89	1.89	16.14	0.2
5451	*A. nevskianum* (flower)	>33.3		>33.3	
0882	*A. oreophilum*	>33.3		>33.3	
5372	*A. sphaerocephalon*	>33.3		>33.3	
	Marasmius alliaceus	>33.3		>33.3	
	Tulbaghia violacea (Stalk)	>33.3		>33.3	
	Tulbaghia violacea (Bulb)	>33.3		>33.3	
7017	*A.mirrum*	>33.3		>33.3	
6275	*A. trautvetterianum*	>33.3		>33.3	
6268	*A. carolinianum*	>33.3		>33.3	
	Black paste-like extract from *Allium carolianum*	>33.3		>33.3	

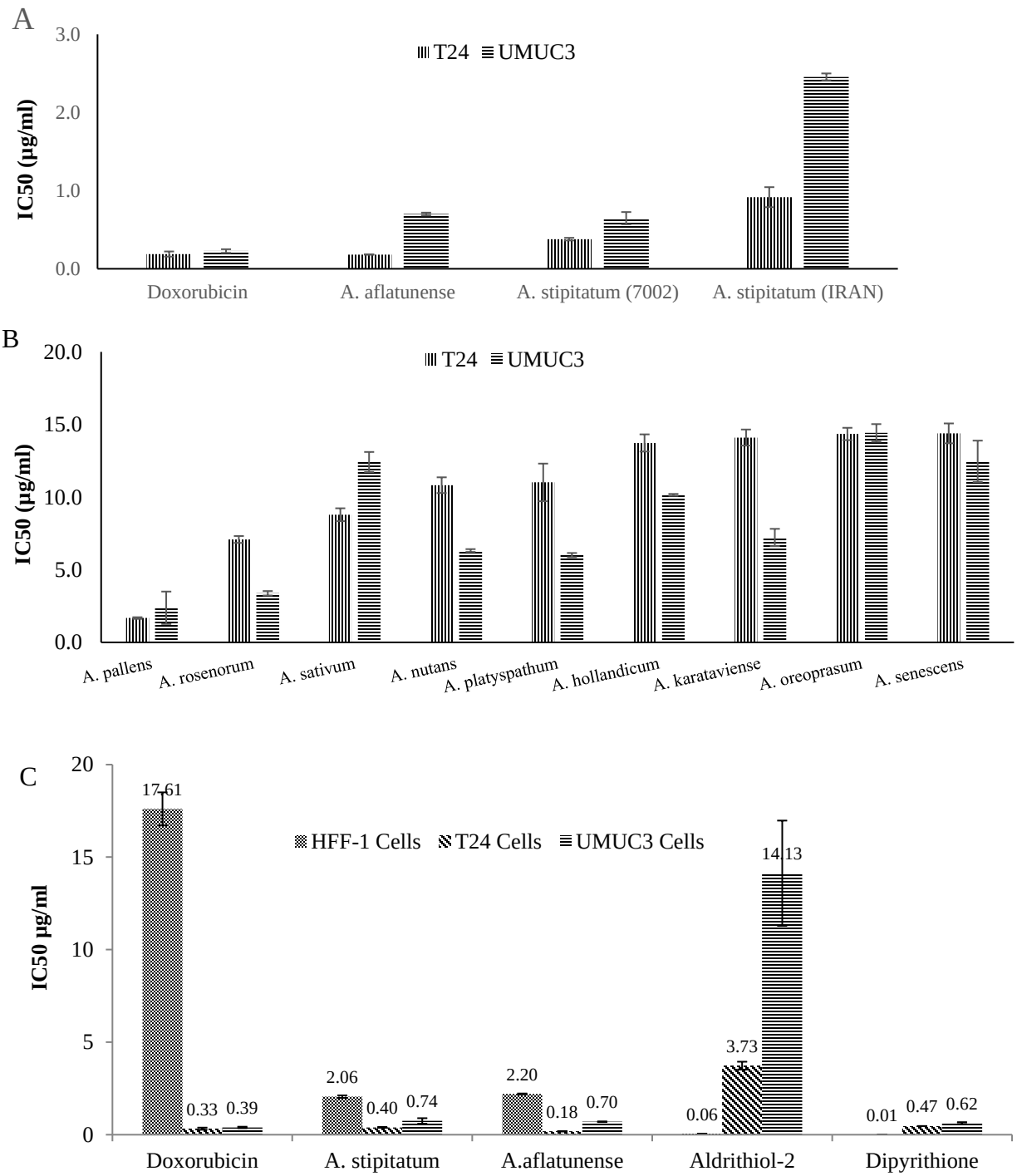


Figure 3.3. Cytotoxicity of *Allium* extracts, Aldrithiol-2 and dipyrithione against T24 and UMUC3 cells. Doxorubicin was used as positive control. (A) Tested subjects with IC_{50}<=1.0 µg/ml against minimum one cell line. (B) Tested subjects with 1.0 µg/ml<IC_{50}<16.67 µg/ml against minimum one cell line. (C) IC_{50} (µg/ml) values of *A. stipitatum*, *A. aflatunense*, doxorubicin, 2,2′-dipyridyl disulfide (Aldrithiol-2) and dipyrithione against T24, UMUC3 cancer and healthy HFF-1 cells

The group of very active test subjects included extracts of *A. aflatunense* (IPK Gatersleben) with IC_{50} values of 0.18 µg/mL for T24 and 0.70 µg/mL for UMUC3 cell lines, *A. stipitatum* (Afghanistan) with IC_{50} values of 0.40 µg/mL for T24 and 0.74 µg/mL for UMUC3 cell lines and *A. stipitatum* (Iran) with IC_{50} values of 0.92 µg/mL for T24 and 2.46 µg/mL for UMUC3 cell lines respectively. Compound dipyrithione had IC_{50} values of 0.41 µg/mL for T24 and 0.62 µg/mL for UMUC3 cell lines. The IC_{50} values of doxorubicin were 0.19 µg/mL for T24 and 0.23 µg/mL for UMUC3 cell lines (Figure 3.3, Table 3.3).

The group of moderately active test subjects included *A. pallens* (IC_{50} of 1.67 µg/mL for T24 and 2.38 µg/mL for UMUC3), Aldrithiol-2® (IC_{50} of 3.73 µg/mL for T24 and 10.57 µg/mL for UMUC3), *A. rosenorum* (IC_{50} of 7.09 µg/mL for T24 and 3.38 µg/mL for UMUC3), *A. sativum* (IC_{50} of 8.79 µg/mL for T24 and 12.42 µg/mL for UMUC3), *A. nutans* (IC_{50} of 10.82 µg/mL for T24 and 6.32 µg/mL for UMUC3), *A. platyspathum* (IC_{50} of 11.01 µg/mL for T24 and 6.01 µg/mL for UMUC3), *A. hollandicum* (IC_{50} of 13.73 µg/mL for T24 and 10.16 µg/mL for UMUC3), *A. karataviense* (IC_{50} for 14.10 µg/mL for T24 and 7.22 µg/mL for UMUC3), *A. oreoprasum* (5015, IC_{50} of 14.35 µg/mL for T24 and 14.42 µg/mL for UMUC3), *A. senescens* (IC_{50} of 14.39 µg/mL for T24 and 12.47 µg/mL for UMUC3) (Table 3.4).

Inactive test subjects, which were virtually non-toxic against both cell lines, as they had cell viability of 90% or more even at the highest concentration, are as follows: EtOAc bulb extracts of *A. paniculatum*, *A. strictum*, *A. tuberosum*, *A. ramosum*, *A. atropurpureum*, *A. fedschenkoanum*, 7051 *A. oreoprasum*, 7022 *A. oreoprasum*, *A. talassicum*, *A. pskemense*, *A. angulosum*, *A. victorialis*, *A. stellerianum*, *A. spirale*, *A. denudatum*, 0192 *A. schoenoprasum*, 10001 *A. schoenoprasum*, 1732 *A. rupestre*, *A. rubens*, *A. rotundum, A. pictistamineum*, *A. cernuum, A. cornutum*, *A. obliquum*, *A. maximowiczii*, *A. lusitanicum*, *A. altaicum*, *A. rotundum*, 5301 *A. rupestre*, *A. douglasii*, *A. flavum*, *A. canadense*, *A. macleanii* (flower), *A. nevskianum* (flower), *A. oreophilum*, *A. sphaerocephalon*, *Marasmius alliaceus*, *Tulbaghia violacea* (Stalks), *Tulbaghia*

violacea (Bulbs), *A. mirum*, *A. trautvetterianum*, *A. carolinianum*, Black paste-like extract from *A. carolianum* (Table 3.5).

3.2.2. Flow cytometry assay results of EtOAc extracts of Allium spp.

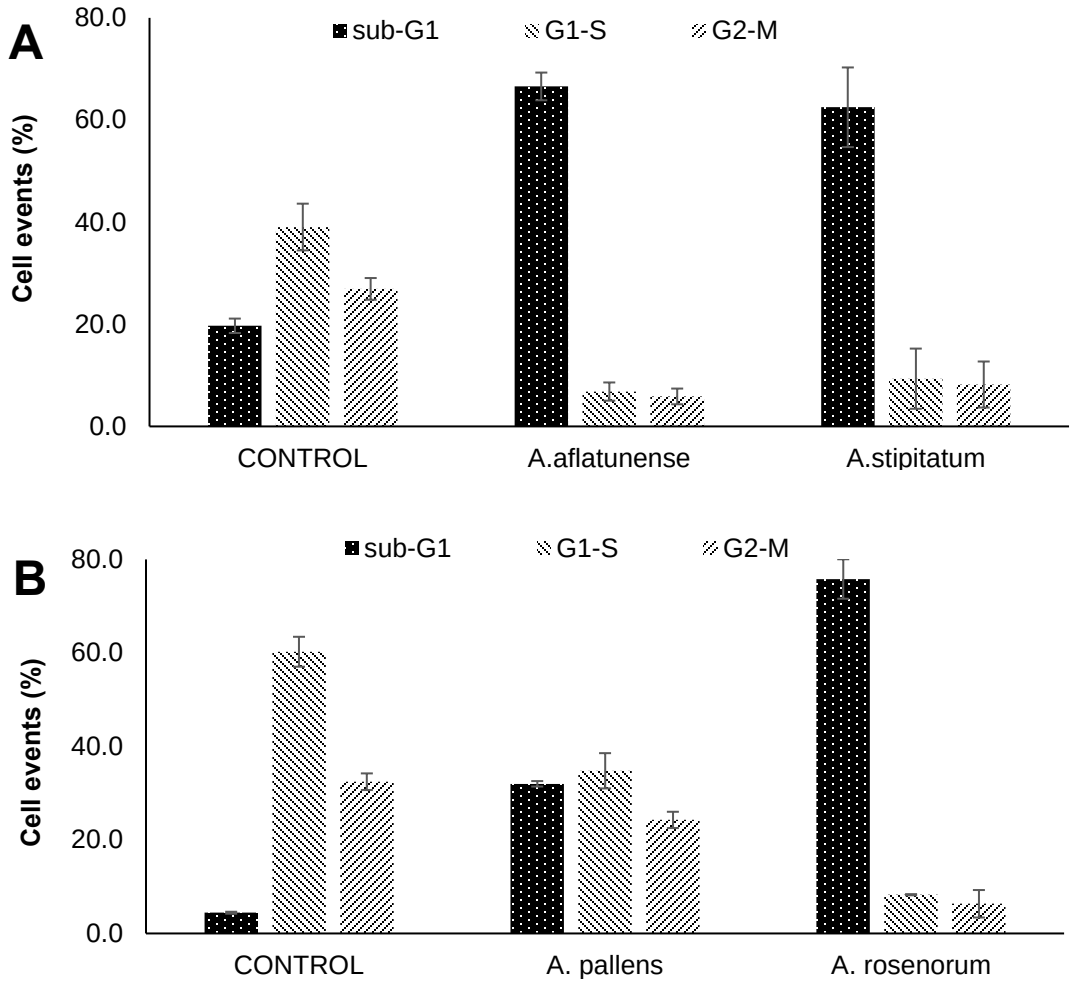


Figure 3.4. The effect of EtOAc extracts of *A. aflatunense* and *A. stipitatum* (Graph A) and *A. rosenorum* and *A. pallens* (Graph B) on sub-G1 group in T24 cells. The T24 cells were incubated with the extracts for 48 h, following with harvest and analysis for cell death by flow cytometry as described in Materials and Methods. Data represents mean ± SD of three experiments.

To determine mechanism of action of four most cytotoxic *Allium* extracts, T24 cells were treated for 48 h with the extracts and processed for flow cytometry. Administration of EtOAc extracts of *A. aflatunense*, *A. stipitatum, A. rosenorum* and *A. pallens* lead to a significant accumulation of T24 cells in a sub-G1 population characterized by having DNA content less than cells confined in G1 phase of the cell cycle. Ongoing death processes cause the reduction of DNA content. The percentage of sub-G1 events in cells treated with *A. pallens* and *A. rosenorum* were 32.00% and 75.81% respectively, compared to 4.43% in untreated T24 control cells. The percentage of sub-G1 events in cells treated with *A. aflatunense* and *A. stipitatum* extracts were 66.57% and 62.49% respectively, compared to 19.70% in untreated T24 control cells (Figure 3.4).

3.2.3. Allium extracts upregulates Nrf2, HO-1, and CDKN1A mRNA

The EtOAc extracts of *A. aflatunense*, *A. stipitatum*, *A. rosenorum* and *A. pallens* were tested for their mechanisms of action for Nrf2 and its target gene, HO-1, and CDKN1a in T24 cells.

Nrf2 mRNA was upregulated only by *A. stipitatum* and *A. pallens*; HO-1 mRNA, by *A. stipitatum*. *A. rosenorum*, *A. pallens* and *A. aflatunense*; Cdkn1a mRNA, by *A. stipitatum*, *A. pallens*, *A. rosenorum* and *A. aflatunense*. (Figure 3.5).

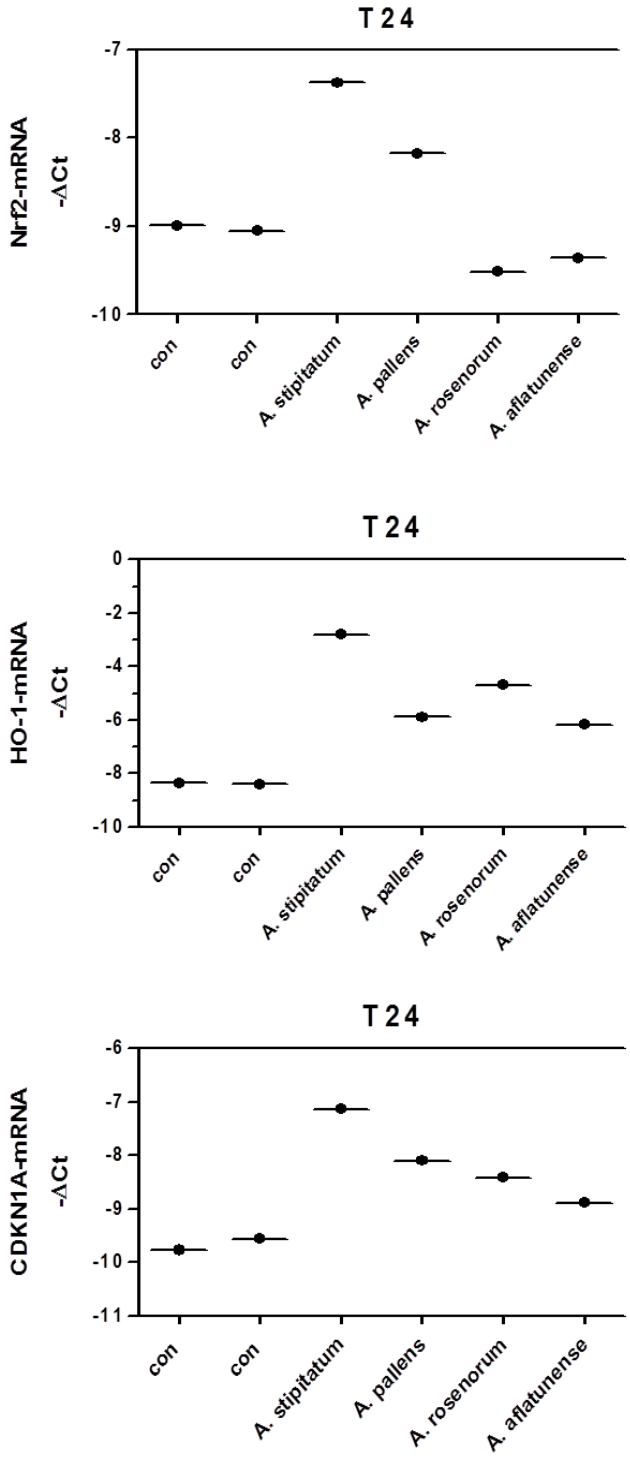


Figure 3.5. The effect of *A. aflatunense*, *A. stipitatum*, *A. rosenorum*, *A. pallens* EtOAc extracts on upregulation of Nrf2, HO-1, CDKN1a mRNA in T24 cells after 48 hours of treatment.

3.3. Bioactivity guided fractionation of four most cytotoxic Allium spp EtOAc extracts

Four most cytotoxic extracts were fractionated using preparative HPLC method. As a result, seven fractions were collected from *A. aflatunense*, four fractions from *A. stipitatum*, five fractions from *A. rosenorum* (Figure 3.9) and five fractions from *A. pallens* (Figure 3.8). All fractions were tested for their bioactivity against T24, UMUC3 and HFF-1 cells using XTT cell proliferation assay. The value of IC_{50} all fractions from *A. pallens* and *A. rosenorum* against all three cell lines was >33.33µg/ml. Thus, they were not further investigated.

Fractions collected from *A. aflatunense* were numbered from 1 through 7 (Figure 3.6). Fractions 2 and 3 from *A. aflatunense* were combined before the cell proliferation assay as compound with same *m/z* was detected in both fractions in mass spectrometer analysis. The resulting fraction was marked as Fraction 23. Fractions 23, Fraction 4 and Fraction 5 exhibited significant toxic effect against T24 and UMUC3 cancer cells. Fraction 1 may be considered inactive as IC50 values against T24 and UMUC3 cells are very close to the maximum applied concentration. Fraction 7 may be classified as “moderately active” with IC50 values of 13.37 µg/ml and 13.83 µg/ml against T24 and UMUC3 cell lines respectively. Fractions 23, 4, 5 and 6 exhibited strong toxic activity against T24 cells with IC50 values less than 1.0406 µg/ml. Both fractions 23 and 4 had IC50 values less than 1.0406 µg/ml against UMUC3 cells. Fractions 5 and 6 had values of 1.19 µg/ml and 1.37 µg/ml against UMUC3 cells respectively.

Fractions collected from *A. stipitatum* were numbered from 1 through 4 (Figure 3.7). Fractions 1 and 2 were inactive against all cell lines with IC50 values greater than 33.33 µg/ml. Fractions 3 and 4 were toxic against T24 cells and had IC50 values of 2.08 µg/ml and 3.09 µg/ml respectively. Same fractions were relatively less active against UMUC3 cells and had IC50 values of 8.20 µg/ml for fraction 3 and 12.27 µg/ml for fraction 4. When tested against healthy HFF-1 cells, fractions 3 and 4 had high IC50 values of 16.35 µg/ml and 24.79 µg/ml respectively.

Table 3.6. IC50 (µg/ml) values of fractions collected from *A. aflatunense* using preparative HPLC. Values represent mean ± SD (n=3).

Fr/ Cell line	Fr 1	Fr 23	Fr4	Fr 5	Fr 6	Fr 7
HFF-1	>33.33	3.20 (±0.45)	2.24 (±0.77)	12.18 (±3.65)	10.83 (±2.33)	>33.33
T24	28.70 (±6.55)	<1.0406	<1.0406	<1.0406	<1.0406	13.37 (±1.06)
UMUC3	30.25 (±4.32)	<1.0406	<1.0406	1.19 (±0.06)	1.37 (±0.24)	13.83 (1.62)

Table 3.7. IC50 (µg/ml) values of fractions collected from *A. stipitatum* using preparative HPLC. Values represent mean ± SD (n=3).

Fr/ Cell Line	Fr 1	Fr 2	Fr 3	Fr 4
HFF-1	>33.33	>33.33	16.35 (±3.25)	24.79 (±1.41)
T24	>33.33	>33.33	2.08 (±0.28)	3.09 (±0.28)
UMUC3	>33.33	>33.33	8.20 (±2.49)	12.27 (±1.13)

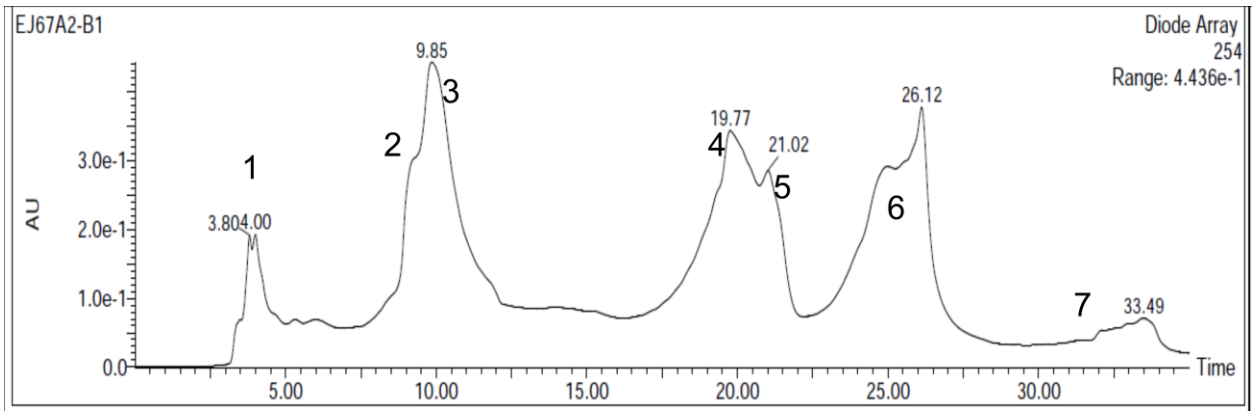


Figure3.6. Fractions collected from EtOAc extract of *A. aflatunense* by means of preparative HPLC. Total 7 fractions were collected and used in XTT cell proliferation assay.

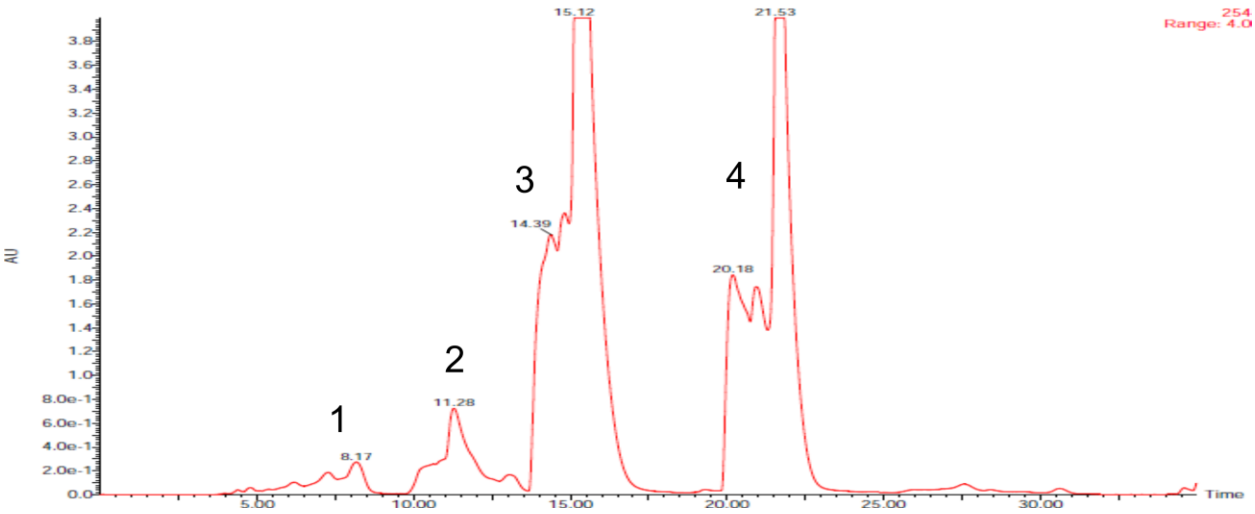


Figure 3.7. Fractions collected from EtOAc extract of *A. stipitatum* by means of preparative HPLC. Total 4 fractions were collected and used in XTT cell proliferation assay.

Fractions collected from *A. rosenorum* and *A. pallens* exhibited almost no activity even at their highest tested concentrations. It was also not possible to obtain clear mass spectrums of the fractions for compound prediction.

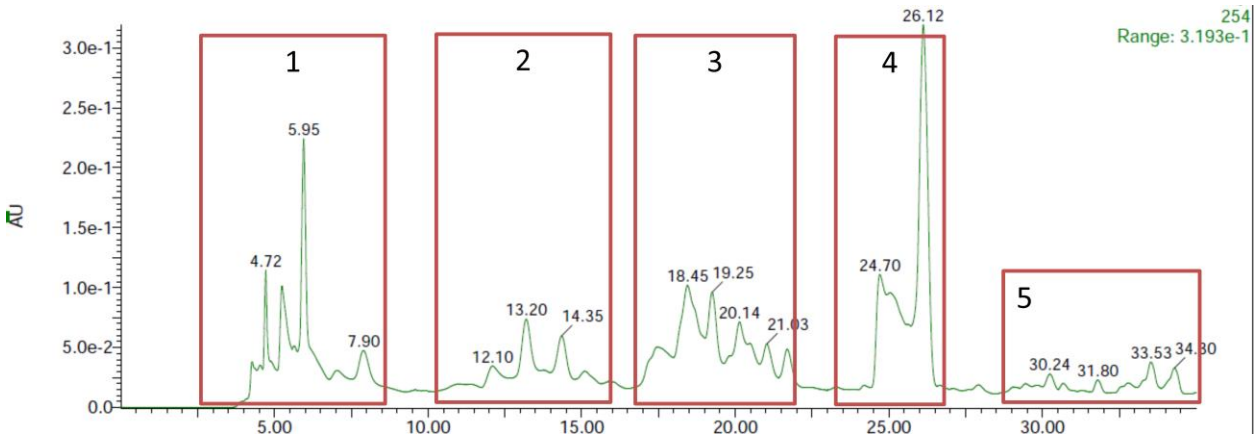


Figure 3.8. Fractions collected from EtOAc extract of *A. pallens* by means of preparative HPLC. Total 5 fractions were collected and used in XTT cell proliferation assay.

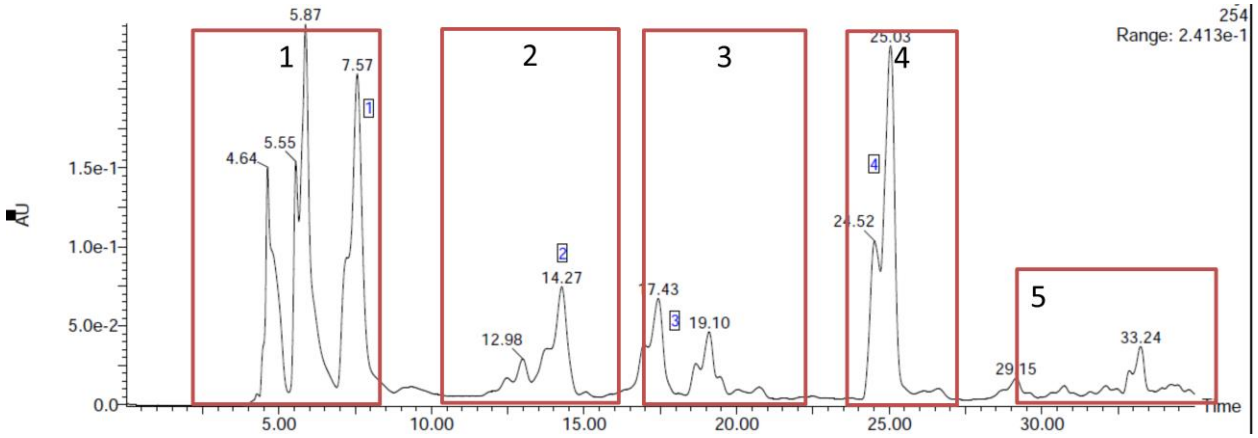


Figure 3.9. Fractions collected from EtOAc extract of *A. rosenorum* by means of preparative HPLC. Total 5 fractions were collected and used in XTT cell proliferation assay.

3.4. Fractions from A. stipitatum induce expression of hemeoxygenase-1 (HO-1) in T24 cells

The effect of fractions F3 and F4 from *A. stipitatum* on upregulation of HO-1 mRNA and expression of HO-1 proteins in T24 cells is shown in Figure 3.10. Both fractions were

tested at two different concentrations of 0.25 µg/ml and 0.5 µg/ml. Only fraction F3 at concentration of 0.5 µg/ml was very active and significantly upregulated HO-1 mRNA. Fraction F4 had very low activity at both concentrations. Western Blot analysis showed that both fractions F3 and F4, at concentration of 0.5 µg/ml, induced expression of HO-1 protein in T24 cells (Figure 3.11).

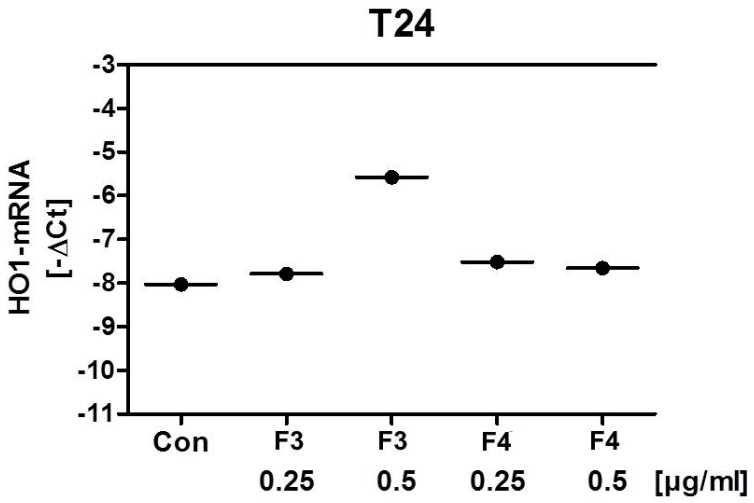


Figure 3.10. Real-time PCR results of upregulation of HO-1 mRNA in T24 cells by *A. stipitatum* fractions F3 and F4. (Con is negative untreated control).

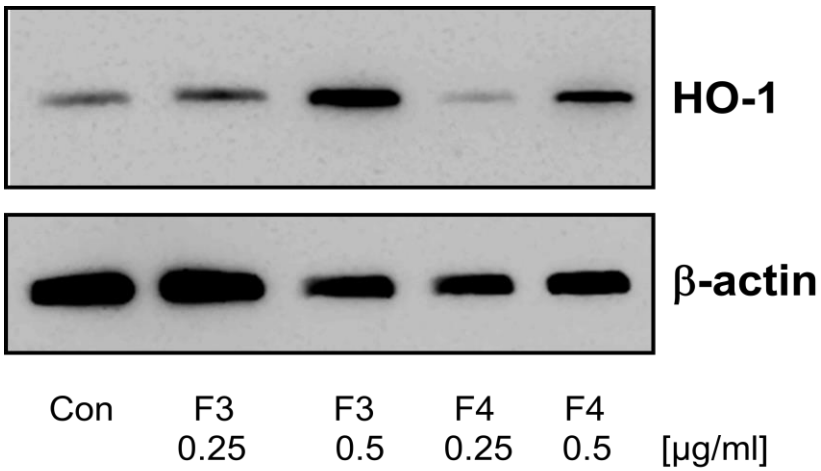


Figure 3.11. Western Blot results of HO-1 protein expression in T24 cells by *A. stipitatum* fractions F3 and F4. (Con is negative untreated control).

Fraction F3 has strongly and fraction F4 slightly induced expression of HO-1 proteins in Western blot analysis. Both fractions F3 and F4 from *A. stipitatum* induced upregulation of HO-1 and Cdkn1a mRNA in T24 cells (data for Cdkn1a not shown).

3.5. Amino acid and CSO content of 7002 A. stipitatum and 1178 A. aflatunense bulbs

Bulbs of 7002 *A. stipitatum* and 1178 *A. aflatunense* were analyzed for their amino acid and CSO content. Bulb weights were 533.1 mg for *A. stipitatum* and 242.4 mg for *A. aflatunense*. Results are presented in Table 3.8 as percentage of compounds related to the fresh weight of the bulbs. Structures of CSOs are given in Figure 1. It can be observed from the results that both *A. stipitatum* and *A. aflatunense* have common cysteine derivatives which are methiin, S-(2-pyridyl) cysteine N-oxide and marasmin. The total amount of these three cysteine derivates is higher in *A. aflatunense* (1.220%) than in *A. stipitatum* (0.489%). Bulbs of *A. stipitatum* were found to have two more cysteine sulfoxides, alliin and isoalliin, with total amount of 0.327%, which were not present in *A. aflatunense* bulbs. Due to unclear separation of methiin and serine peaks in a chromatogram, a sum value of both compounds is given.

	Allium stipitatum		*Allium aflatunense*	
Amino acid/CSO	**Weight (%)**	**RSD (%)**	**Weight (%)**	**RSD (%)**
Aspartic acid	0.050	1.48	0.122	4.60
Glutamic acid	0.089	0.37	0.237	2.86
Asparagine	0.030	0.88	2.639	2.17
Glutamine	0.044	0.31	0.256	4.58
Methiin+Serine	**0.349**	**0.91**	**0.567**	**3.15**
S-(2-pyridyl)cysteine N-oxide	**0.074**	**1.87**	**0.541**	**2.42**
Threonine	0.030	1.88	0.262	4.68
Alliin	**0.127**	**5.71**	**-**	**-**
Isoalliin	**0.200**	**1.28**	**-**	**-**
Marasmin	**0.066**	**2.97**	**0.111**	**3.84**
Alanine/Tyrosine	0.047	9.33	0.161	1.64
Valine	present		present	
Tryptophane	present		present	
Phenylalanine	present		present	
Leucine/Isoleucine	present		present	

Table 3.8. CSOs and amino acids found in 7002 *A. stipitatum* and 1178 *A. aflatunense* by means of HPLC and HPLC/MS. Amounts of the compounds presented as weight percentage (Weight (%)) of compounds related to the fresh weight of the bulbs with their relative standard deviation (RSD (%)) (n=3).

3.6. Structure elucidation of some fractions from A. stipitatum and A. aflatunense

Fractions F3 and F4, isolated from *A. stipitatum*, were identified using HPLC-MS, HR-MS and H^1-NMR spectroscopic data. Spectrograms presented in the Appendix sections.

Fraction F3, from *A. stipitatum*, when subjected to HPLC-MS analysis, revealed a [M+H^+] ion at *m/z* 174.3, a [M+CH_3OH+H^+] ion at *m/z* 206.2 and a [2M+H^+] ion at *m/z* 347.4.

A high-resolution MS analysis indicated a [M+H^+] ion at *m/z* 174.0046 and a [M+Na^+] ion at *m/z* 195.9863. Molecular formula was suggested to be $C_6H_8NOS_2$.

In the H^1-NMR spectra, a singlet at δ 2.47(s, 3H), indicated a methyl group (3H), suggesting it was isolated from the aromatic nucleus. Four hydrogens were present at δ 8.34 (dq, J = 6.4, 0.6 Hz, 1H), δ 8.05 (dd, J = 8.4, 1.3 Hz, 1H), δ 7.65 (m, 1H) and δ 7.36 (m, 1H).

Fraction F4, from *A. stipitatum*, when subjected to HPLC-MS analysis, revealed a [M+H^+] ion at *m/z* [M+H^+]=220.1, a [M+CH_3OH+H^+] ion at *m/z* 252.4 and a [2M+H^+] ion at *m/z* 439.0.

A high-resolution MS analysis indicated a [M+H^+] ion at *m/z* 219.9947 and a [M+Na^+] ion at *m/z* 241.9769. Molecular formula was suggested to be $C_7H_{10}NOS_3$.

In the H^1-NMR spectra, again a singlet at δ 2.27 indicated a methyl group, suggesting it was isolated from the aromatic nucleus. Another singlet at δ 3.99 (2H) representing methylene attached to sulfurs was present. Four hydrogens were present at δ 8.33 (dd, J = 6.4, 0.7 Hz, 1H), δ 8.06 (1H), δ 7.64 (m, 1H) and δ 7.35 (m, 1H).

When compared to data reported by O'Donnel [31], fraction F3 from *A. stipitatum* may be identified as 2-(methyldithio)pyridine-N-oxide (PNO) and F4 as 2-[(methylthiomethyl)dithio]pyridine-N-oxide (MTPNO) (Figure 1.4).

Two fractions from *A. aflatunense* could be only tentatively identified due to insufficient amount of biological material. Identification was based only on HPLC-MS spectrogram and comparing elution times of a standard compounds and the isolated fraction. One fraction was tentatively identified as 2,2′-dithio-bis-pyridine-N-oxide (dipyrithione, DPT) and another one as 2-[(methylthiomethyl)dithio]pyridine-N-oxide (Figure 1.4).

4. DISCUSSION

4.1. Essential amino acid composition of flowers from subgenus Melanocrommyum

Flowers of species from genus *Allium* subgenus *Melanocrommyum* were analyzed by means of HPLC method for their essential amino acid content. Glutamate is an anion of glutamic acid and therefore glutamic acid in *Allium* species may be considered as a precursor in arginine biosynthesis, as presented in the Section 1.3. Based on study by Schuphan and Schwerdtfeger [37], arginine amount will increase as size of *A. cepa* bulb increases from June through August, getting ready for the storage period. The amount of arginine in bulbs will decrease as bulbs start to sprout. It is possible to assume that as plant enters flowering stage, essential amino acids, serving as plant's nitrogen storage will mobilize to the flower, therefore leaving the bulb with minimum amount of amino acids. Due to small number of samples within each section, it is not possible to provide statistically significant results for scientific discussion and conclusion. Nevertheless, some data and discussion are provided when available.

4.1.1. Allium rosenorum

Flower extract of *A. rosenorum* (4293) had the highest total amount of amino acids of 29.93%. Almost one third of the amino acid content was provided by glutamine with 10.68%. Asparagine and arginine were second and third most abundant amino acids with amounts of 5.22% and 3.31%, respectively. Bulb samples of *A. rosenorum* (4293), labeled (a) and (b), were investigated by Kusterer [12] and reported total amino acid content found to be 0.537%. The interesting point is that amount of glutamine (0.2027%) in the bulb samples was also the highest among other amino acids. Amount of arginine in the bulbs was 0.0079%, asparagine-0.0943% and aspartic acid-0.0846%. In the flowers, amount of asparagine was little less than half of glutamine amount, a relation which can be observed in Kusterer's bulb data as well, where amount of asparagine is also little less than half of glutamine amount. In the bulb extract, amount

of arginine wasn't third highest in contrary to the data from flower extract. This can be explained with the bulb collection time, which is usually *Allium*'s flowering and not a storage period. Also, reporting almost 30% of total amount of amino acids in the flower can be arguable, since the number is too high, and no similar results were obtained in any study performed in several years in Prof. Dr. Keusgen's lab. In general, the amino acid composition of flowers can be totally different from bulbs, because the bulb is a storage organ and the flower is a reproduction organ. Water content is a serious problem at the time of extraction. Therefore, while the absolute amount of CSOs and amino acids is questionable, the pattern of present CSOs and amino acids and their amounts relative to their total amounts are reliable distinguishing criteria and should be used in this case.

In other two samples of *A. rosenorum* the total amount of amino acids was much lower with 1.08% (Acc. Nr. 2530) and 0.90% (Acc. Nr. 1886) (Figure 4.1). The most abundant amino acid was serine with amounts of 0.48% (Acc Nr. 2530) and 0.27% (Acc. Nr. 1886). Glutamine was also of lower content with amounts 0.14% (Tax Nr. 2530) and 0.25% (Tax Nr. 1886) (Figure 4.1.).

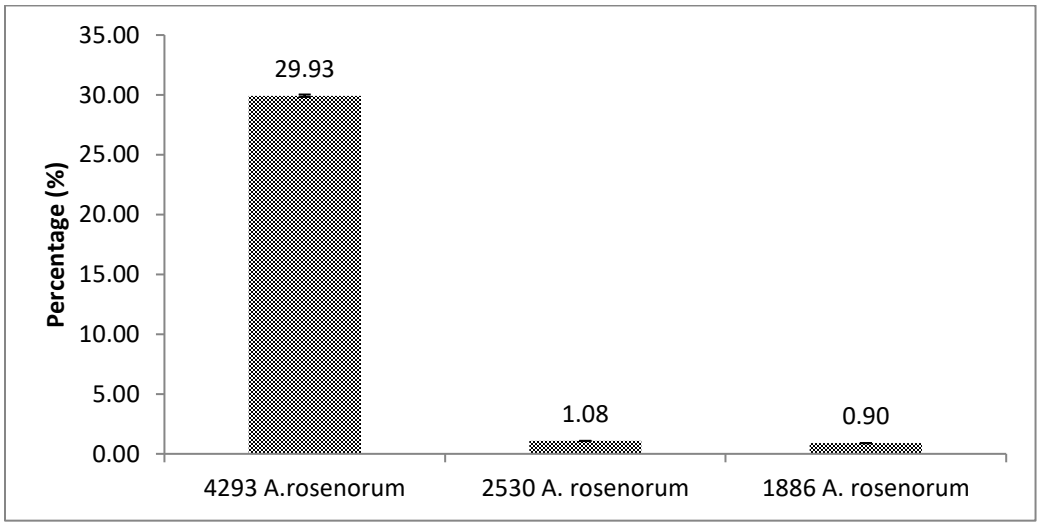


Figure 4.1. Total amino acid amounts in flower extracts of three species *A. rosenorum* with Tax Nr. 4293, 2530 and 1886.

4.1.2. Section Compactoprason

The amount of total amino acid percentage among species of section *Compactoprason* was in *A. giganteum* (4283). The amount was 6.57%, which is drastically higher than in two other species, *A. macleanii* (2415)-0.92% and *A. trautvetterianum* (6275)-0.57% (Figure 4.2). The last two species were also among other six species with the lowest total amino acid amount and had the amount less than 1%.

Amino acids with highest amounts in *A. giganteum* flower extract were glutamine and serine with 1.03% and 1.00%, respectively. Arginine was fifth most abundant amino acid with 0.763%, coming after tyrosine/alanine and valine. Assuming that arginine is among major nitrogen storage amino acids, three reasons may be proposed for low arginine amount. First reason, is that the flower was in its early stage and therefore biosynthesis is still ongoing and will increase eventually. Second reason is that flowering was close to seed producing stage and arginine was mobilized to forming seeds. Third reason is that glutamine is preferred to arginine as the primary source of nitrogen in the flower. In flower extracts of *A. macleanii and A. trautvetterianum* glutamine was also major amino acid, while arginine was placed at lower orders of abundance list. It may be speculated that glutamine may serve as a major nitrogen storage in flowers of section *Compactoprason*.

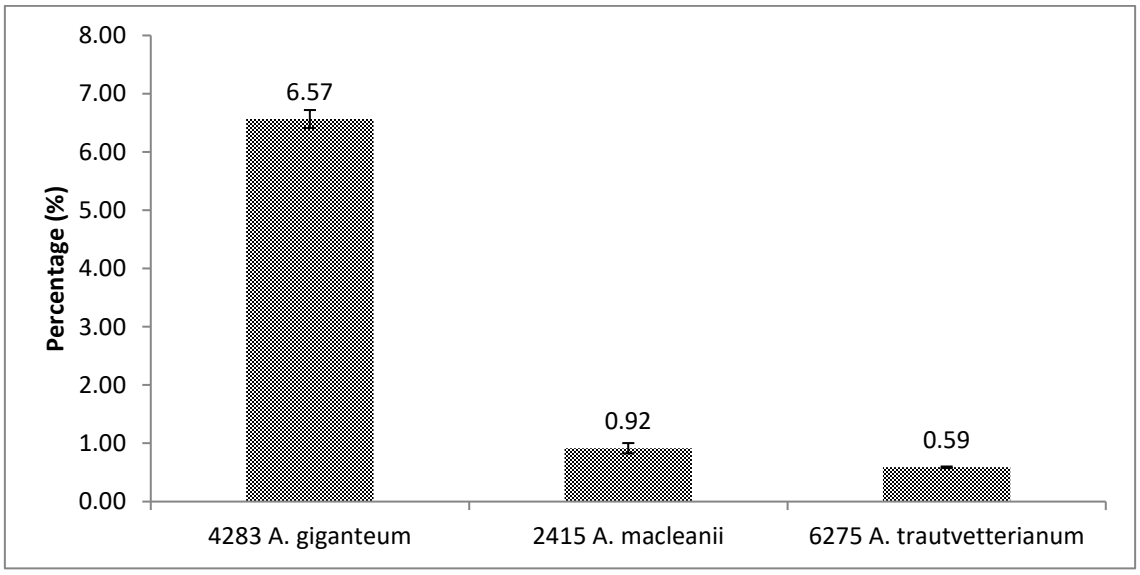


Figure 4.2. Total amino acid amounts in flower extracts of species *A. giganteum*, *A. macleanii* and *A. trautvetterianum*

4.1.3. Section Acanthoprason

Only one representative of section *Acanthoprason* was present. Total amino acid content of *A. derderianum* (1207) was 11.46%, making it fourth highest on the list. Valine had the highest amount among present amino acids in the extract and the amount was 3.09%. Mixture of tyrosine and alanine was also very high with the amount of 3.51%. Due to incomplete separation of the peaks it was difficult to calculate the two amino acids separately. Second most abundant essential amino acid was glutamic acid with 1.820%. Arginine had the lowest amount of 0.22%. As glutamic acid is the main precursor in arginine biosynthesis, it may be assumed that the flowers were collected at early stages of the biosynthesis and not enough arginine was produced. The plant was collected in Iran (2007) [82].

4.1.4. Section Kaloprason

The difference between the species of section *Kaloprason* was not as drastic as in previous ones. Flower extracts of *A. protensum* species with taxon identifiers 4282 and 4291 had total amino acid amount of 9.92% and 8.43%, respectively, and *A. protensum* (4282) being the extract with highest total amino acid amount. Flower extract of *A. caspium* (4285) was second highest with 9.26% in the section, and *A. nevskianum* (5451) had the lowest amount of 3.92% (Figure 4.3).

Glutamine was major essential amino acid in all species with *A. caspium* having highest amount of 4.28%. Arginine (1.818%), asparagine (1.997%) and glutamine (1.980%) amounts were close in 4282 *A. protensum*. Amount of glutamic acid (0.706%) was less than half of arginine's amount in species 4282, which may suggest a shift in arginine biosynthesis reaction towards product formation. In all other species amounts of

glutamic acid were higher than amounts of arginine. Species of section *Kaloprason* may also prefer glutamine as a primary form of nitrogen storage.

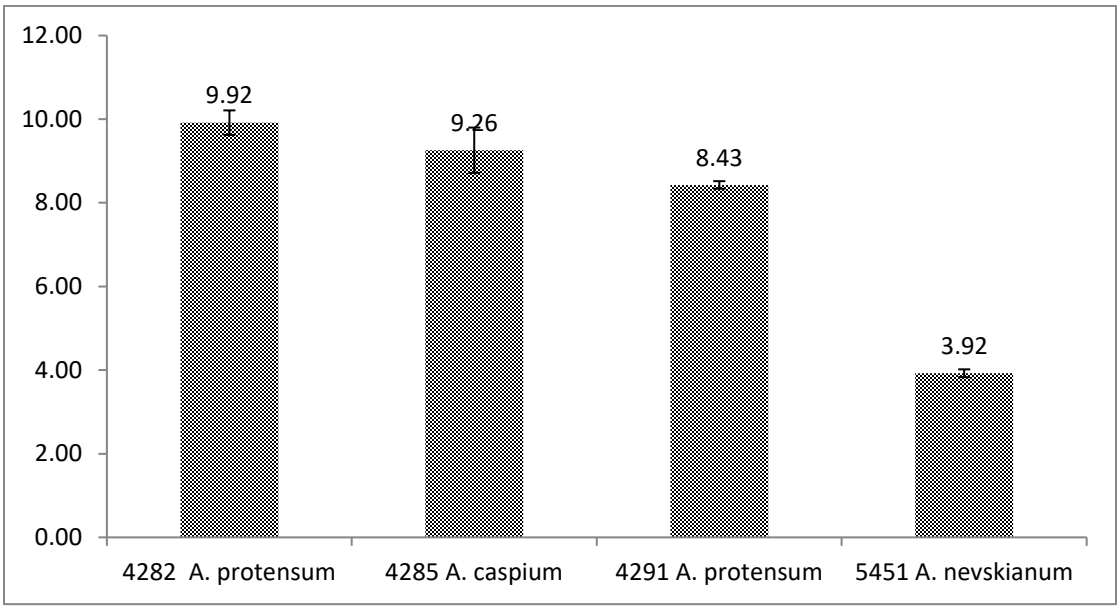


Figure 4.3. Total amino acid amounts in flower extracts of species *A. protensum* (4282 and 4292), *A. caspium* and *A. nevskianum*.

4.1.5. Section Megaloprason

Two representatives of section *Megaloprason* were same species with different taxon identifiers. Total amino acid amount in 4287 *A. suworowii* (15.16%) was almost twice as high as in the 4296 *A. suworowii* (8.39%), and was second highest amount among all other analyzed species (Figure 4.4). Asparagine (3.514%), aspartic acid (2.600%) and glutamine (2.377%) amounts were highest in species 4287, while in species 4296 glutamine (1.507%) was highest followed by tyrosine/alanine (1.261%) and valine (1.035%). In both species amount of glutamic acid was almost twice as higher than amount of arginine, suggesting the idea that *A. suworowii* species may prefer asparagine and glutamine rather than arginine as nitrogen storage compounds.

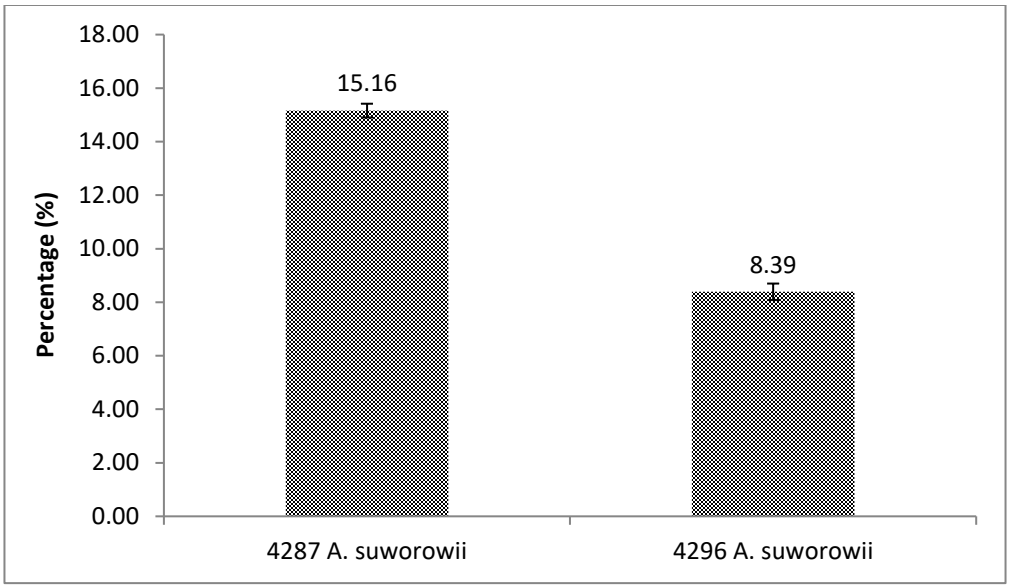


Figure 4.4. Total amino acid amounts in flower extracts of species 4287 *A. suworowii* and 4296 *A. suworowii*.

4.1.6. Section Melanocrommyum

Total amino acid amount in two species in section *Melanocrommyum* content was among highest overall. Extract of *A. keusgenii* had 12.06% and *A. moderense* had 10.16% total amino acid amount (Figure 4.5). While in *A. moderense*, asparagine was the major amino acid with amount of 2.41%, in *A. keusgenii* aspartic acid dominated the list with 2.48%. Glutamine amounts in both species were close with *A. moderense* having 1.620% and *A. keusgenii* having 1.578%. Glutamic acid and arginine amounts were also close with amounts of 0.755% and 0.467%, respectively, for *A. moderense* and 1.270% and 0.523%, respectively, for *A. keusgenii.*

In results reported by Kusterer [81], the flower extract of *A. keusgen*ii had asparagine with highest amount and glutamine shared second place with glutamic acid as both had second highest amount; in *A. moderense* flower extract asparagine was major and glutamine had second highest amount. Same report contains data for bulbs and flowers of 1198 *A. keusgenii* and 1148 *A. moderense*, where amounts of almost all essential

amino acids in flower extract are higher than in bulb extract. For example, in 1198 *A. keusgenii*, amount of aspartic acid is almost ten (10) times higher, glutamic acid is minimum 4 (four) times higher, asparagine is minimum 11 (eleven) times higher, glutamine is minimum 5 (five) times higher and arginine is minimum 9 (nine) times higher in the flower extract than in the bulb extract. In 1148 *A. moderense*, amount of aspartic acid is 1.5 times higher, asparagine is almost 1.9 time higher, glutamine is 1.9 times higher, arginine is minimum 12 times higher in the flower extract than in the bulb extract. Amount of glutamic acid is almost equal in the flower and the bulb extract. Obviously, ratio of flower to bulb amino acids is much greater in *A. keusgenii* than in *A. moderense.* For this reason, it is worth noting the water amount of plant material. The 1198 *A. keusgenii* flower extract Kusterer labeled as “T”, which means “trocken” in German and “dried” in English. To prepare extracts of the species 1198, Kusterer used 0.72 g of the dried flower and 0.3 g of the bulb. To prepare extracts of the 1148 *A. moderense*¸ Kusterer used 1.30 g of the fresh flower and 0.54 g of the bulb. Difference in water contents of the plant parts in both species may be a reason of greater difference in amino acid amounts between *A. keusgenii* parts than between *A. moderense* parts. Collection times of the samples is another factor affecting amounts of amino acids. In addition, it may be just a specificity related to these samples only, thus more data from controlled experiments is required for a general statement. It may be worth considering Kusterer’s findings as an additional supporting evidence to support idea of mobilization of essential amino acids from bulbs to flowers.

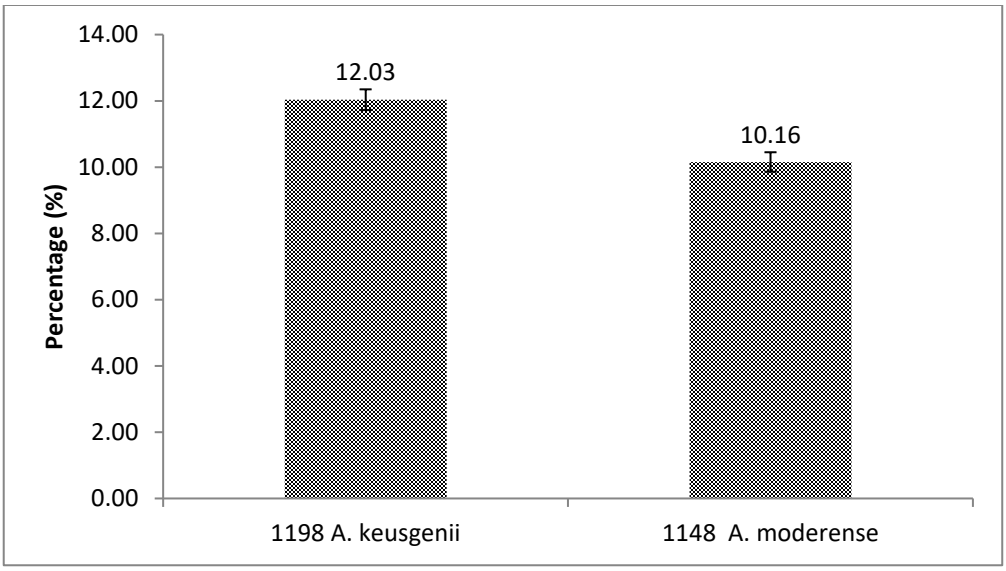


Figure 4.5. Total amino acid amounts in flower extracts of species *A. keusgenii* and *A. moderense.*

4.1.7. Section Popovia

Extract of flower of *A. gypsaceum* (4280) was the only species in section *Popovia*. The total amount of the extract was 6.14%, which put the species in the middle of the list among other extracts. The major amino acid in the extract was glutamine with amount of 2.62%. Probably, *A. gypsaceum* prefers glutamine as the nitrogen storage compound. Amount of glutamic acid (0.40%) was less than amount of arginine (0.579%) which may be an indicator of initiation of arginine biosynthesis period of arginine. Amount of asparagine (0.561%) was close to the amount of arginine.

4.1.8. Section Procerallium

Section *Procerallium* was the section with highest number of tested species. The extract of *A. rosenorum* (4293) had highest amount of total amino acids, 29.93%, within the section and among all tested species. The extracts of *A. rosenorum* (1886), *A. jesdianum* (1083 and 3951) and *A. hollandicum* (2800) had total amino acid amounts

less than 1% and were among six extracts with the lowest total amino acid values overall (Figure 4.6). For the discussion below 4293 *A. rosenorum* data is excluded as erroneous. While all samples had glutamine as major or second major amino acid, 1177 *A. stipitatum* had glutamic acid (0.69%) and aspartic acid (0.36%) as major amino acids. Amount of arginine in species 1177 was very low (0.05%), so possibly its production did not start yet.

In flowers of 2530 *A. rosenorum*, 1886 *A. rosenorum* and 2800 *A. hollandicum* serine was major amino acid followed by glutamine. In 2530 *A. rosenorum* amount of glutamine was equal to the amount of aspartic acid.

In 1222 *A. jesdianum* and 1082 *A. jesdianum* amounts of glutamic acid were less than arginine's; and in 2530 *A. rosenorum* and 3953 *A. jesdianum* amounts of glutamic acid and arginine were equal. These findings may be indicating initiation of arginine's biosynthesis in the flowers of species.

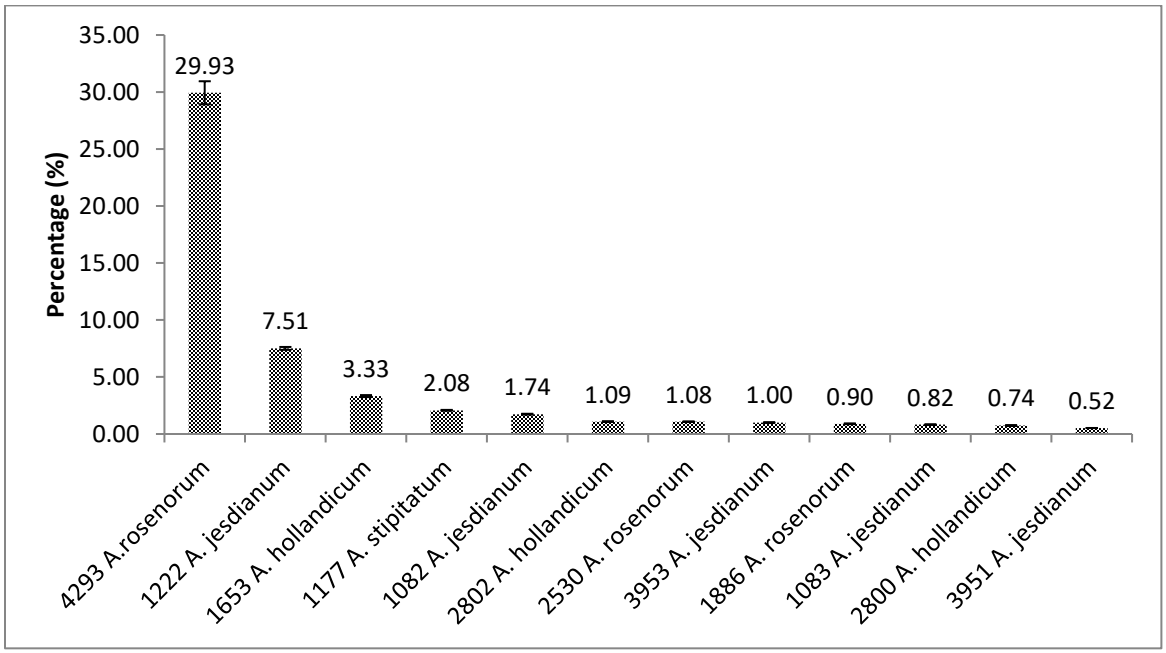


Figure 4.6. Total amino acid amounts in flower extracts of species of section *Procerallium*.

4.2. Cysteine sulfoxide composition of flowers from subgenus Melanocrommyum

Flowers of species from genus *Allium* subgenus *Melanocrommyum* were analyzed by means of HPLC method for their cysteine sulfoxide. Methiin was present in all analyzed flower extracts. In most cases methiin was major CSO found, while in cases like in flower of *A. trautvetterianum*, amount of butiin was twice as higher than amount of methiin. Samples were discussed within sections. Data from corresponding bulbs earlier analyzed by Dr. Kusterer in AK Keusgen, also discussed when available.

4.2.1. Methiin

Methiin, is the only CSO which was found in all tested flower extracts. Moreover, methiin was present in virtually all bulb samples previously analyzed in a working group of Prof. Dr. M. Keusgen (data not shown).

Cleavage of methiin by alliinase enzyme yields several products which include dimethyl disulfide sulfoxide (MMTSI), dimethyl disulfide sulfone (MMTSO), dimethyl disulfide and methanethiol (Figure 4.7). While dimethyl disulfide found in the air above fields of *Brassicas* during growing and flowering seasons, MMTSI and MMTSO are considered to be phytoalexins, which act as antibacterial or antiyeast upon tissue wounding [83].

Methiin is the simplest CSO and yields some volatile sulfur compounds, when cleaved by alliinase enzyme. Volatile organic compounds synthesized by plants can have various functions such as attracting pollinators, communication among plants alarming of a predator, herbivore deterrent and etc [84–87]. As methiin present in most *Allium*s, and some Brassicaceae, abundance of methiin in flowers may be due to a need for constant production of volatile sulfur compounds for certain purposes, which also have characteristic ‘cabbagy’ or ‘fresh onion’ odor [88].

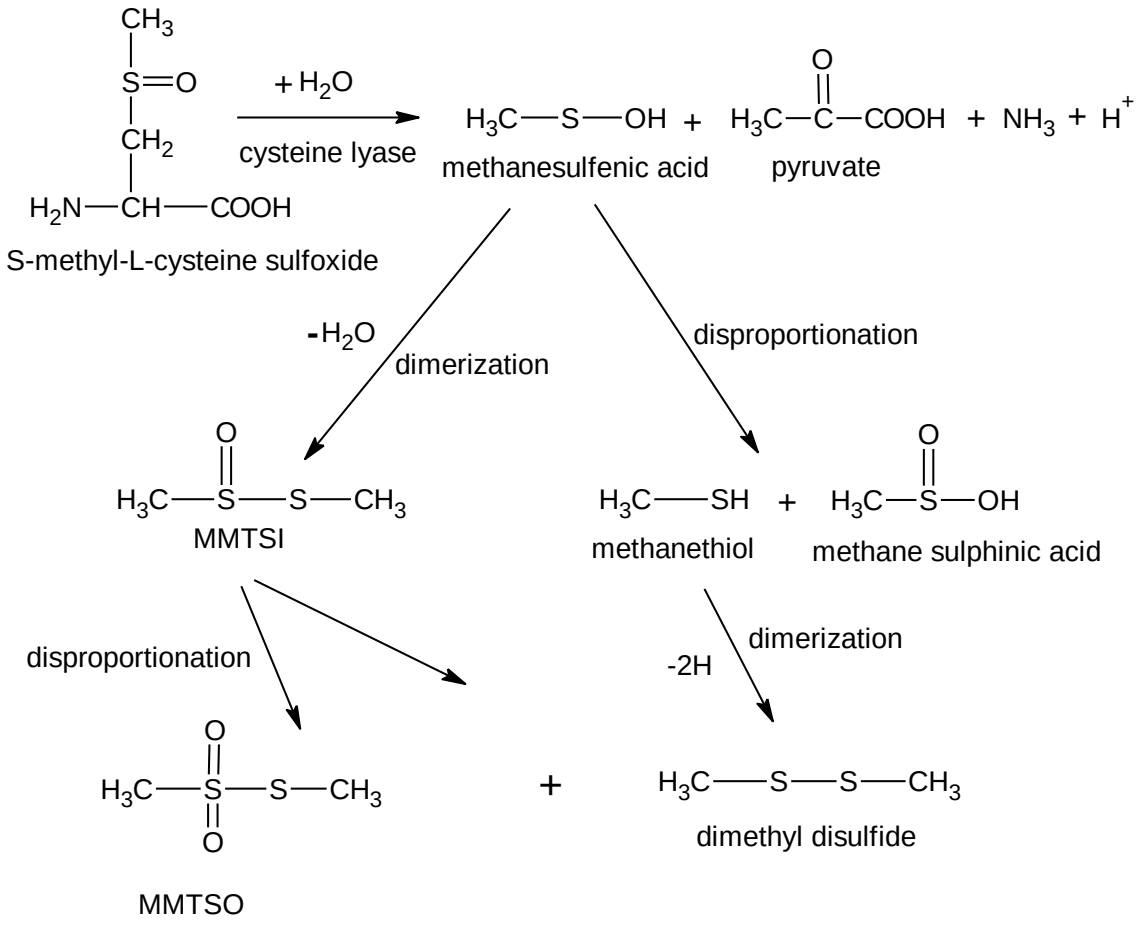


Figure 4.7. Breakdown products of SMCSO. Metabolic scheme outlining the action of cysteine lyase on SMCSO leading to the formation of several secondary bioactive products via the release of the highly reactive intermediate, methane sulphenic acid. MMTSI: S-methyl methanethiosulphinate (dimethyl disulphide sulphoxide), MMTSO: S-methyl methanethiolsulphonate (dimethyl disulphide sulphone) [83].

4.2.2. Allium rosenorum

The highest amount of cysteine sulfoxides (CSOs) and amino acids was present in *A. rosenorum* (4293). The total CSO content was 7.05% and total amino acid content was 29.93% of the fresh material weight. The only CSO found in the extract was methiin, with the amount of 7.05%, which is also the total CSO amount found. Previously, bulbs of *A. rosenorum* (4293) were investigated by Kusterer [12]. Two bulb samples, labeled

(a) and (b), were analyzed and total CSO content was found to be 0.089% and 0.112% respectively. Only two CSOs reported to be present in the bulb, methiin and *S*-(2-pyrrolyl)-L-cysteine sulfoxide. In sample a, methiin and *S*-(2-pyrrolyl)-L-cysteine sulfoxide were found 0.020% and 0.069% respectively, in sample b, the amounts were 0.021% and 0.101% respectively. In the chromatogram of 4293 flower extract (Figure 4.8), methiin and serine did not elute separately, so manual separation using best effort was done as shown in the Figure 4.8.

In *A. rosenorum* (1886), the total CSO amount was only 0.12%. Methiin and *S*-(2-pyrrolyl)-L-cysteine sulfoxide were the only CSOs present in the extract with 0.11% and 0.01%, respectively. Same CSOs were present in bulbs, whereas amounts of the CSOs were related inversely, with methiin being in higher amount.

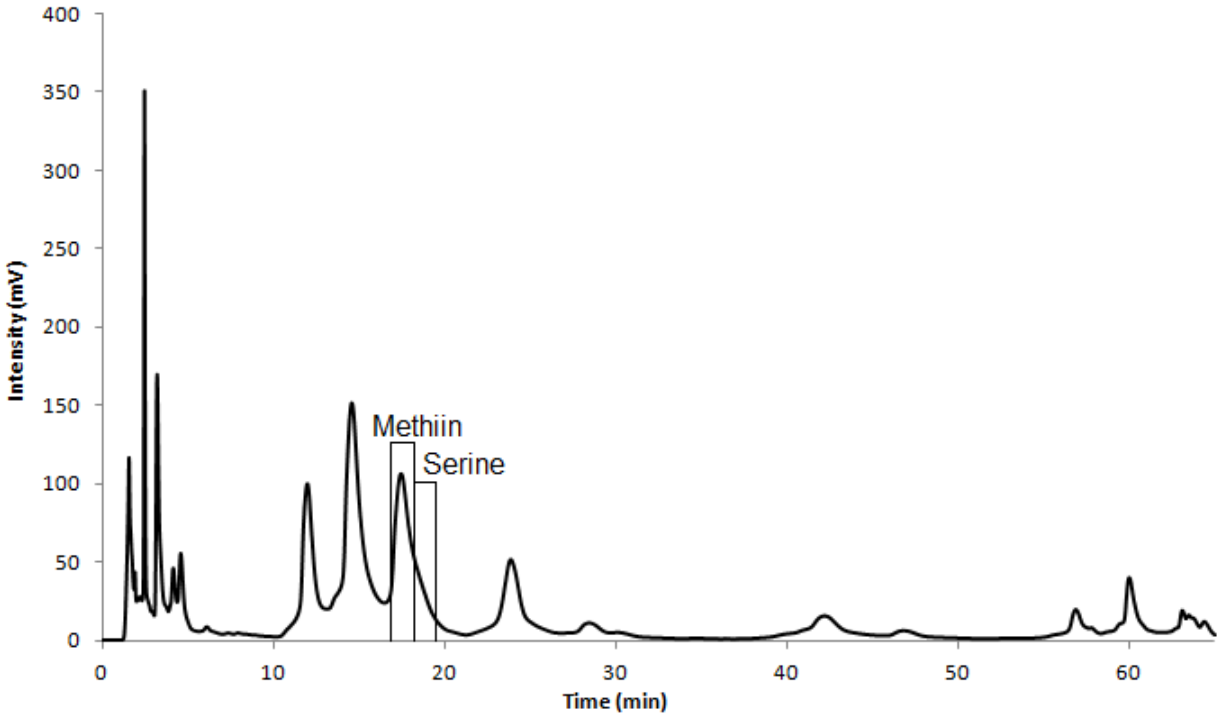


Figure 4.8. Chromatogram of OPA derivatized 4293 *A. rosenorum* flower extract.

4.2.3. Section Compactoprason

Out of three species in section *Compactoprason*, *A. giganteum* had highest amount of CSO with 2.91% (Figure 4.9), which was also fourth highest amount overall. Other two species, *A. macleanii* (2415)-0.25% and *A. trautvetterianum* (6275)-0.12%, were among species with lowest amounts of CSO.

Methiin (2.91%) was the only CSO found in *A. giganteum*, and *A. trautvetterianum* was the only extract to have butiin (0.08%) within calculation range. In *A.macleanii*, methiin was major CSO with 0.16%, whereas in *A. trautvetterianum*, amount of butiin (0.08%) was twice as high of the methiin amount of 0.04%. One of the reasons of low amount of CSOs in *A. trautvetterianum* may be due to relatively small sampling amount (453 mg) compared to *A. macleanii* (973 mg) and *A. giganteum* (600 mg), while recommended amount of source material for flower is 1000 mg. Water content of *A. giganteum* may also be a reason of high CSO percentage, as this flower extract was prepared and frozen several years ago by Dr. Jan Kusterer (AK Keusgen). It is possible that flowers of *A. giganteum* had relatively lower amounts of water at the time of extraction. Kusterer had previously reported amounts of CSOs in bulbs of 2 accessions of *A. macleanii* (6240 and 6256) [81]. Origin of all 3 *A. macleanii* species (2415, 6240 and 6256) happen to be collected in Tajikistan, while flowers of *A. macleanii* (2415) were collected from culture collection at IPK Gatersleben. When compared to CSO data of *A. macleanii* Acc. No. 6240 and 6245 bulbs, CSO percentage of *A. macleanii* (2415) flowers is lower. Amounts of total CSOs in bulbs were 0.3594% and 0.7881% in accessions 6240 and 6256, respectively, compared to 0.25% in *A. macleanii* (2415). Although weights of analyzed bulbs were not significantly different, the difference between total CSO percentages were proposed to be due to high water content of pollinated bulbs of accession 6240, while bulbs of accession 6256 were still in the middle of their flowering stage. Marasmin was not found in flowers of *A. macleanii* (2415), which is in correlation with bulbs (Acc. No. 6240 and 6256) data reported by Kusterer. Neither was it detected in flowers of *A. giganteum* and *A. trautvetterianum*. S-(2-pyrollyl)-L-CSO was found in flowers of *A. macleanii* (2415) accounting for 40% of total CSOs. This finding is in

correlation with Kusterer's data, although the amount of same CSO in bulbs of accessions 6240 and 6256 was accounting for 84.23% and 82.44% of the total CSO content. Kusterer reports that only methiin and *S*-(2-pyrollyl)-L-CSO were found in bulbs of accessions 6240 and 6256, which is in correlation with findings for flowers of *A. macleanii* (2415).

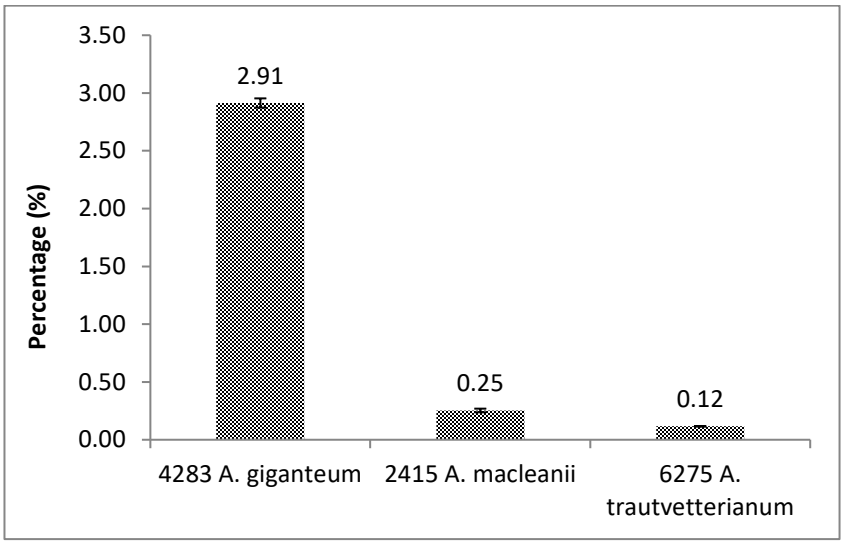


Figure 4.9. Total CSO amounts in flower extracts of *A. giganteum* (4283), *A. macleanii* (2415) and *A. trautvetterianum* (6275).

4.2.4. Section Acanthoprason

There was only one species in the section *Acanthoprason*, *A. derderianum* (1207). The species 1207 was collected in Iran at height of over 2750 m, along with other species in this section at various heights (1850 m - 2750 m) [81]. The total CSO amount in the flower extract was 0.49%, and it was the amount of methiin, which was the only CSO detected in the extract. When compared to previously analyzed bulb sample by Kusterer et al (2011) [12], it can be seen that total amount of CSOs in flowers, almost ten times more than in the bulb (0.059%), but flowers lacked marasmin and S-(2-pyrrolyl)- L-cysteine sulfoxide, which were present in bulbs in the amounts of 0.045% and 0.06%

respectively. Methiin amount in the bulb was almost ten times less than in the flowers and amount was 0.04%. In previous

The bulb of 1207 was collected during flowering season, and although for a solid statement more experiments and data are required, it is possible to speculate that methiin biosynthesis was mainly taking place in the flower. Methiin has been suspected to serve as a phytoalexin at key growth stages [83], therefore higher amount of methiin in the flower and being the only CSO in the flower, may be due to the same reason.

4.2.5. Section Kaloprason

Total amount of CSOs in *A. protensum* (4282) was the highest in the section species and more than twice as high as the amount in *A. protensum* (4291), *A. caspium*(4285) and *A. nevskianum* (5451) (Figure 4.10).

Only *A. nevskianum* lacked (-)-methiin, while it was present in other three samples, but in amounts out of calculation range. Both isomers of marasmin were present in *A. nevskianum*, and amount of (-)-marasmin was out of calculation range. Kusterer has reported CSO content of bulbs of two other species of *A. nevskianum* (4286 and 4288) [81]. Total CSO content was very low (0,045%). Methiin was major component in both accessions. Marasmin and S-(2-pyrrolyl) - L-cysteine sulfoxide were present only species 4286. Bulb of *A. alexianum* (4242) was found to have methiin, marasmin and S-(2-pyrrolyl) - L-cysteine sulfoxide, with methiin and S-(2-pyrrolyl) - L-cysteine sulfoxide being two major components.

Both species of *A. protensum* had (+)-marasmin in amounts, more than twice as high as the amount in *A. nevskianum*. Flower of *A. protensum* (4282) was the only one to contain S-(2-pyrrolyl) - L-cysteine sulfoxide, second major CSO after methiin. It may be suggested that in bulbs and flowers of species of *Kaloprason* section marasmin may be second, after methiin, most common CSO, with (-)-marasmin isomer found in flowers.

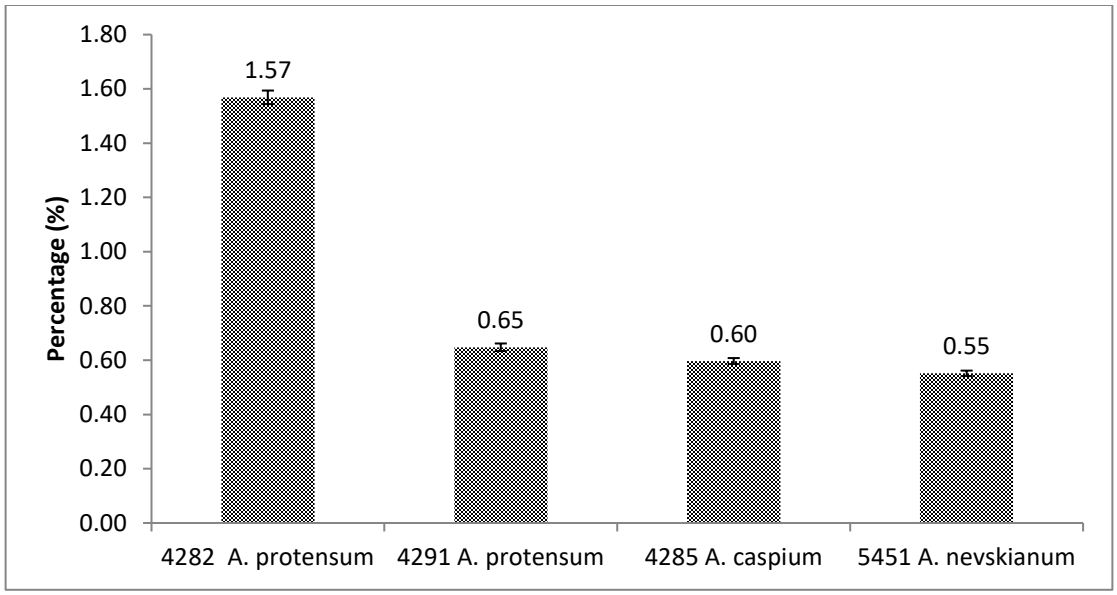


Figure 4.10. Total CSO amounts in flower extracts of *A. protensum* (4282 and 4291), *A. caspium* (4285) and *A. nevskianum* (5451).

4.2.6. Section Megaloprason

Two same species with different accession numbers were present in this section. Extracts of *A. suworowii* 4296 and 4287 had total CSO amounts of 2.17% and 1.90%, respectively (Figure 4.11). While in each of the species (+)-methiin was present and was major CSO, (-)-methiin was present only in species with 4287 accession number and in amount out of calculation range. In *A. suworowii* 4296, additionally only S-(2-pyrollyl)-*L*-CSO (0.45%) was present, whereas in the 4287 butiin was third and final CSO, which was also out of calculation range.

In previously analyzed bulbs of *A. suworowii* with different accession numbers (4247 and 4276-supposedly *A. suworowii*) [12], both samples contained marasmin, being a major CSO in the extract. Marasmin was absent in the flowers in current investigation. All bulb samples contained methiin, and S-(2-pyrrolyl)- L-cysteine sulfoxide was found only in bulbs of the sample 4276, in the amount of 0.110%, which was significantly higher than the amount detected in the flowers. Total CSO content of bulbs was either

slightly higher (4247-2.251%) or less (4276-0.572%) than the amount in the flowers. Apparently, in flower extracts no marasmin was detected, while in bulbs marasmin was major compound. Butiin was only found in one flower extract and not in bulbs.

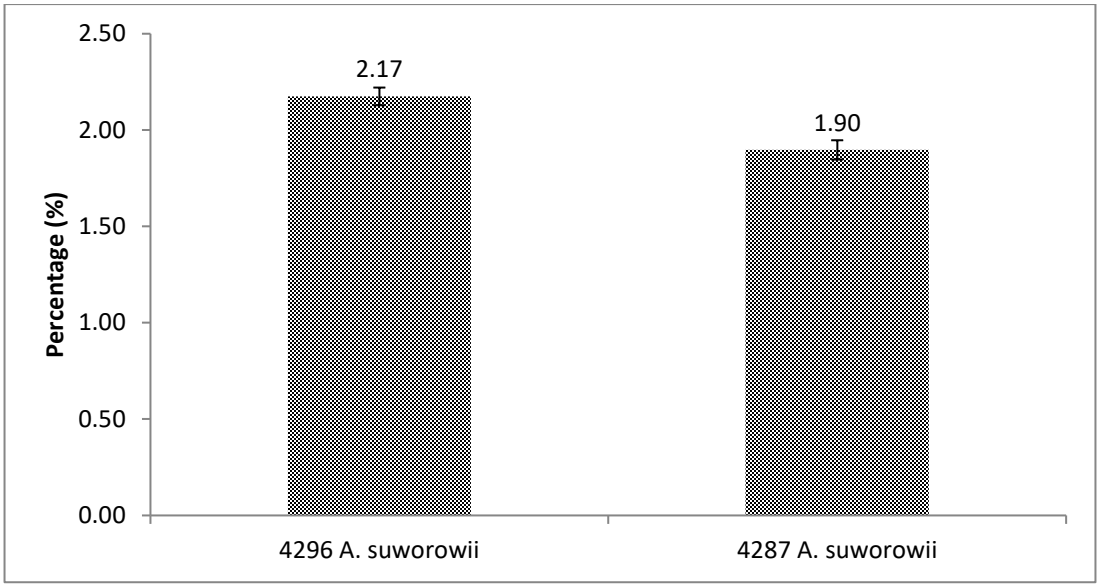


Figure 4.11. Total CSO amounts in flower extracts of *A. suworowii* 4296 and 4287.

4.2.7. Section Melanocrommyum

The difference in total CSO amounts between two representatives of the section was about three times higher in *A. keusgenii* (1198) than in *A. moderense* (1148) (Figure 4.12). In both species methiin was the only CSO present.

When compared to previous results of bulb analysis by Kusterer et al [12], total CSO amount in the flowers of *A. keusgenii* is more than ten times higher than in the bulb, although in the bulb besides methiin, marasmin and S-(2-pyrrolyl)- L-cysteine sulfoxide are present. In the bulb of *A. keusgenii* methiin was still a CSO with highest amount.

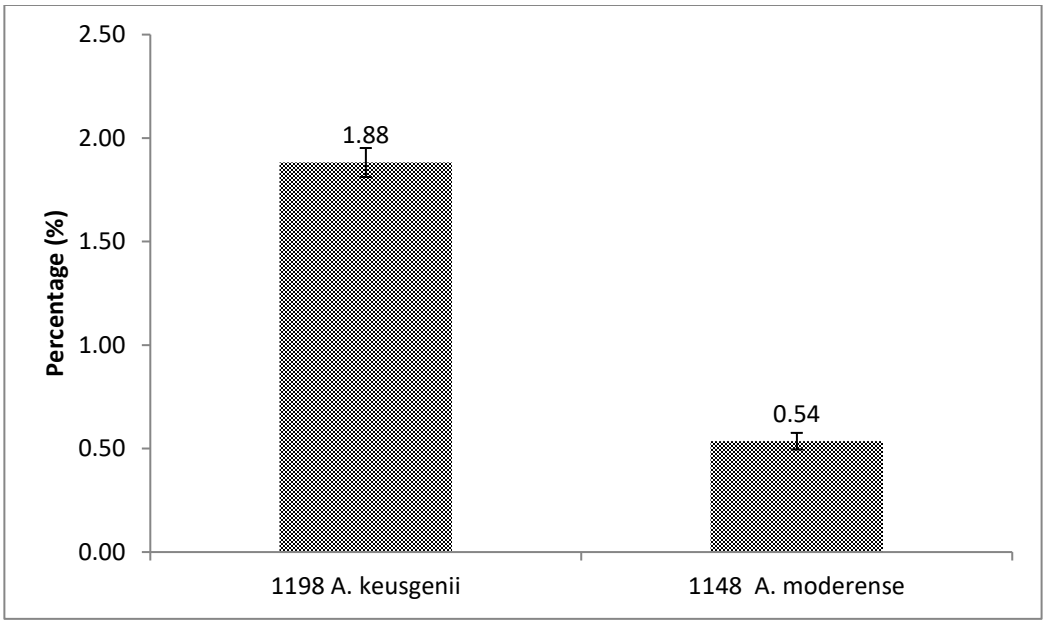


Figure 4.12. Total CSO amounts in flower extracts of *A. keusgenii* (1198) and *A. moderense* (1148).

4.2.8. Section Popovia

The only representative of the section, *A. gypsaceum*, had the highest amount of (+)-marasmin (1.46%) of almost 68% of the total amount of CSOs. The extract also contained high amounts of (-)-marasmine with the amount of 0.14%. (+)-Methiin amount was also very high (0.55%).

4.2.9. Section Procerallium

As it was already mentioned earlier, *A. rosenorum* (4293) had the highest total amount of CSOs, although methiin was the only CSO present, accounting for the total amount. The flower extracts of *A. rosenorum* (4293), *A. hollandicum* (1653) and *A. jesdianum* (1222) were the top three extracts with highest total CSO amount of 7.05%, 4.05% and 3.49% (Figure 4.13).

In the extract of *A. hollandicum* (1653), S-(2-pyrrolyl)-L-cysteine sulfoxide was second highest CSO accounting for 16.29% of the extract's total CSO amount. The absolute amount of S-(2-pyrrolyl) - L-cysteine sulfoxide in the extract was also the highest among all tested extracts. Methiin was accounting for the rest of total CSO amount of the extract. The extract also contained (-)-methiin with amount out of calculation range.

In the extract of *A. jesdianum* (1222), total amount of CSOs was distributed among (+)-methiin, (+)-marasmin and S-(2-pyrrolyl)-L-cysteine sulfoxide. (-)-Methiin was also present in amount out of calculation range. (+)-Methiin was major CSO of the extract with amount of 80.80% of the total CSO amount of the extract. S-(2-pyrrolyl)-L-cysteine sulfoxide was second major CSO with of 15.75% of the total CSO amount. The amount of (+)-marasmin was 3.44% of the total CSO amount. Species with taxon identifiers 1222, 1082 and 1083 belong to *A. jesdianum* ssp. *angustitepalum*; 3951 and 3953 belong to *A. jesdianum* ssp. *jesdianum.* Subspecies 1222 had highest amount of all CSOs, and 1082 lacked S-(2-pyrrolyl)-L-cysteine sulfoxide and 1083 lacked marasmin. While all tested *A. jesdianum* species were cultivated at and collected from IPK Gatersleben, the origin of 1222 is Tajikistan and origin of species 1082 and 1083 is Afghanistan. Species 3951 and 3951 are both from Iran and they only had methiin and S-(2-pyrrolyl)-L-cysteine sulfoxide. It seems not possible to compare to all *A. jesdianum* bulbs analyzed by Kusterer [81], as some of the analyzed species seem to undergo taxonomical changes. Species 1172, 1178, and 1180 have been renamed to *Allium remediorum* (R.M. Fritsch) R.M. Fritsch with taxon identifiers 6635, 6637 and 6638, respectively [82]. Origin of all three species is Iran. Origin of 4241 (TAX 6527) *Allium jesdianum* subsp. *angustitepalum* bulb is Uzbekistan and its extract had S-(2-pyrrolyl)-L-cysteine sulfoxide as a major CSO and methiin second most abundant. No other CSOs were reported for this species. Flower extract of 1083 (subsp. *angustitepalum*) also had only methiin and S-(2-pyrrolyl)-L-cysteine sulfoxide, but with methiin being major CSO. Among all analyzed flowers of *Procerallium* species, only flower of 1222 *Allium jesdianum* Boiss. & Buhse subsp. *angustitepalum* was found to carry marasmin and S-(2-pyrrolyl)-L-cysteine sulfoxide together in addition to methiin. Bulb of same species reported by Kusterer [81] to carry only S-(2-pyrrolyl)-L-cysteine sulfoxide in addition to

methiin. The amount of S-(2-pyrrolyl)-L-cysteine sulfoxide in flower was 100-fold of the one in the bulb.

The extracts of *A. rosenorum* (2530), *A. jesdianum* (1082), *A. stipiatum* (1177), *A. jesdianum* (3953), *A. hollandicum* (2802), *A. jesdianum* (1083), *A. hollandicum* (2800), *A. jesdianum* (3951), *A. rosenorum* (1886) had total amount of CSOs below 1% and ranging from 0.48% (*A. rosenorum*, 2530)-0.12% (*A. rosenorum*, 1886).

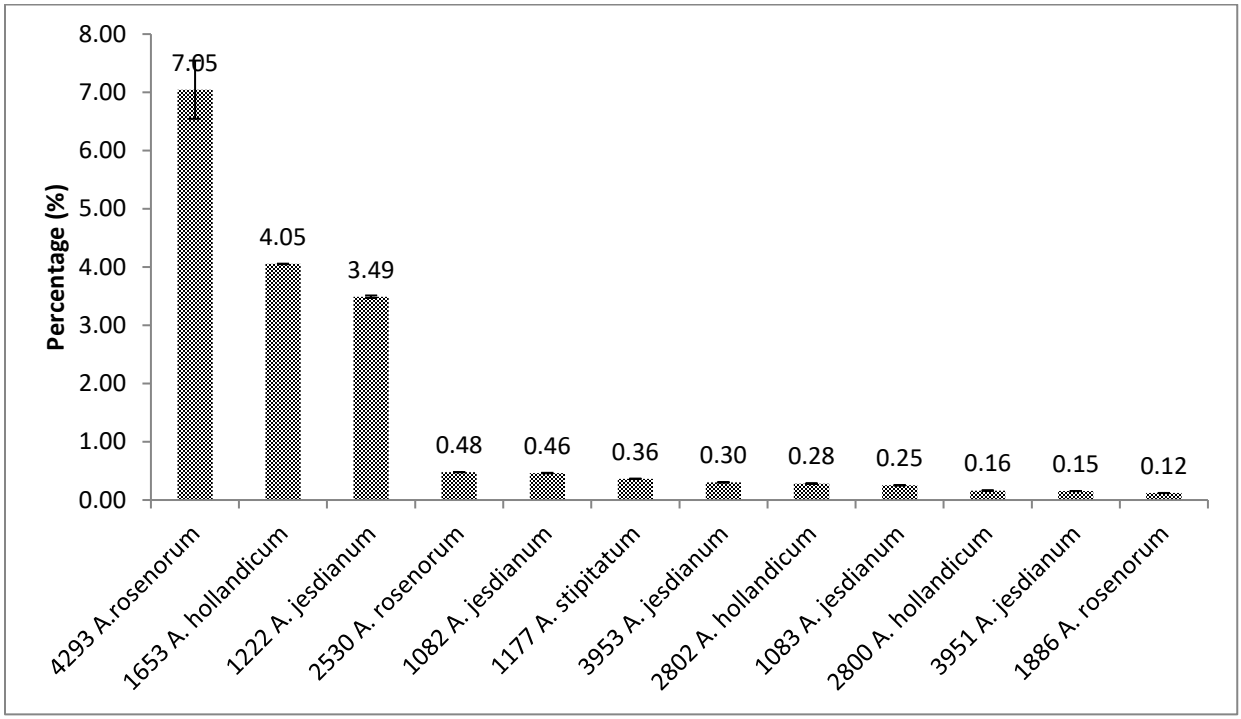


Figure 4.13. Total CSO amounts in flower extracts of species of section *Procerallium*

When compared to CSO amounts in related bulb extracts (Kusterer [81]), while total CSO content among bulbs of various accessions of *A. stipitatum* (1086, 1090, 1240 and 4238) was between 0,2144%-0,4235%, the amount in flower of 1177 was 0.36%, which is comparable to the average amount of CSOs in the bulbs. Contrary to variety of CSOs in bulbs, in the flower only methiin and S-(2-pyridyl)-cysteine N-oxide were found, while in the bulbs marasmin was present in addition to the mentioned ones. In the flower extract trace amounts of (-)-methiin were detected, which was not reported to be

present in the bulbs. The distribution percentage of CSOs was also different between flower and bulbs. In bulbs, S-(2-pyridyl)-cysteine N-oxide was main CSO with amounts between 45.85%-74.43% and the methiin's amount being the second highest, but in the flower of 1177, the amount of S-(2-pyridyl)-cysteine N-oxide was only 24.32% and methiin being the most abundant with 75.68%.

4.3. Anticancer activity of Allium species

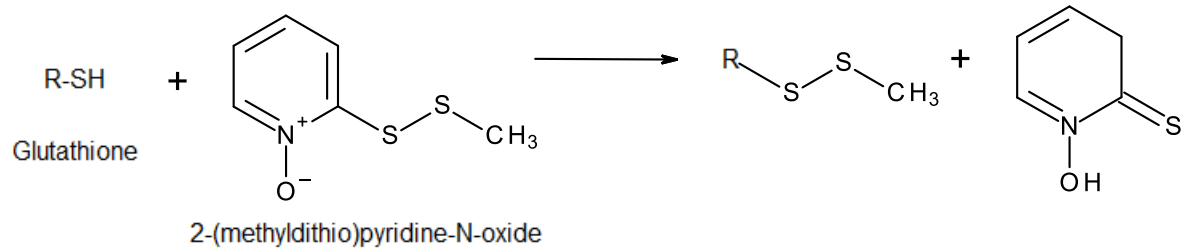
In this study, we screened several EtOAc extracts obtained from bulbs or flowers of *Allium* species and two commercially available compounds, previously reported to be present in *A. stipitatum*, for cytotoxicity effect against T24 and UMUC3 human bladder cancer cells lines. Extracts of *A. aflatunense* and *A. stipitatum* were found to be the most cytotoxic, and comparable to the effect of doxorubicin, a standard chemotherapy drug. Four most cytotoxic extracts (*A. stipitatum*, *A. aflatunense, A. pallens and A. rosenorum*) were subjected to flow cytometry analysis. Significant reduction of DNA content was recorded, which shows death processes taking place during treatment of T24 cells with the extracts. In a quest to find cytotoxic compounds, responsible for the bioactivity, and then to understand their mechanism of action, same four extracts were evaluated using bioactivity guided fractionation. While *A. stipitatum* and *A. aflatunense* each had 2 and 3 cytotoxic fractions respectively, fractions of *A. pallens* and *A. rosenorum* had no effect within tested dose range. The reason of inactivity of fractions from last two plants may be due to high instability of compounds or absence of active compounds in isolated fractions, since only fractions detectable at wavelength of 254 nm were isolated. Thus, further investigation of compounds from *A. pallens* and *A. rosenorum* is advised. After extract of *A. stipitatum* strongly upregulated Nrf2, HO-1, and CDKN1a mRNAs, we decided to determine the extract's bioactive fractions responsible for the mechanisms of action. F1 fraction, identified as PNO, was more selective to cancer cells than healthy cells, while fraction F2, identified as MTPNO, was more toxic to healthy rather than cancer cells. Both compounds showed to act as oxidants and cell cycle blockers by upregulating heme oxygenase (HO-1) and CDKN1a

mRNA in T24 cells. Although, compounds PNO and MTPNO have previously demonstrated significant antiproliferative activity against MCF7 breast carcinoma, A49 non-small-cell lung carcinoma cancer cells and HT29 human colon adenocarcinoma cells[31], no information about mechanism of action was provided up to date. Considering our findings and previous studies [73,89–92], we suggest that both compounds act by depleting glutathione (GSH) in cancer cells.

The role of glutathione (GSH) in cancer cells is complex and an elevated level of GSH was observed in various cancer types, suggesting a function of also an antioxidant. GSH shown to protect cancer cells by interfering with the cytotoxic action of numerous anticancer drugs, including cisplatin, adriamycin, melphalan and taxol [93]. Ovarian cancer cells exposed to cisplastin have been reported increasing degrees of drug resistance via increasing synthesis of glutathione [94]. Therefore, it is possible to assume that blocking glutathione synthesis or depleting glutathione in cancer cells will remove one of the guard shields protecting them. As reported before, depletion of GSH will result in increased intracellular concentration of reactive oxygen species (ROS) which may lead to apoptosis [95,96]. Also, depletion of GSH in rat brains results in induction of HO-1 mRNA and proteins [97]. Diallyl disulfide, one of garlic's main components, induces apoptosis through the induction of reactive oxygen species [98]. Previous studies have also reported diallyl disulfide and diallyl trisulfide to induce accumulation of Nrf2 proteins [74].

Both fractions F1 and F2 from *A. stipitatum*, with compounds PNO and MTPNO respectively, induced accumulation of HO-1 proteins in T24 cells at a concentration of 0.5 µg/ml, with relatively greater accumulation at PNO band compared to the MTPNO (Figure 3.11). Fraction F1, with compound PNO, significantly induced upregulation of HO-1 mRNA in treated T24 cells, while fraction F2, with compound MTPNO, didn't show significant effect when compared to control (Figure 4).

Reaction 1



Reaction 2

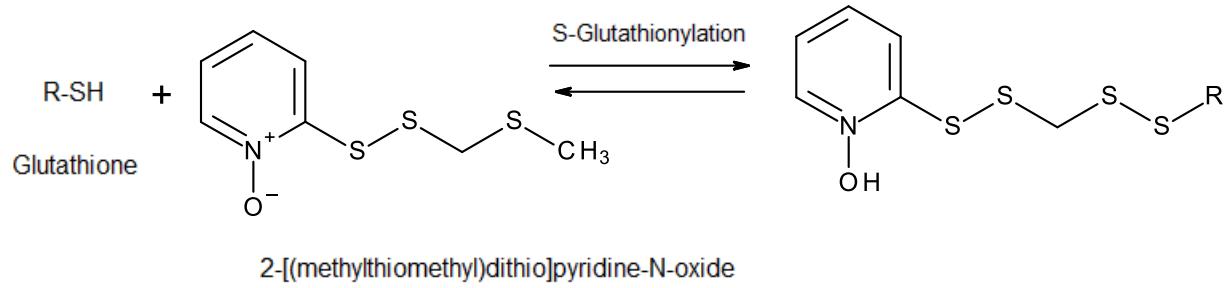


Figure 4.14. Reactions 1 and 2 show mechanisms through which compounds PNO and MTPNO may result in glutathione depletion in T24 cancer cells. While Reaction 1 goes to completion, Reaction 2 may be reversible due to S-glutathionylation process.

Reactions 1 and 2 (Figure 4.14) propose interaction between glutathione and PNO and MTPNO respectively. It is proposed that Reaction 1 is completed in one direction and probably results in serious depletion of glutathione. The ratio of GSH (reduced) and its disulfide, GSSG (oxidized), contributes to the redox potential of the cell and thereby contributes to redox homeostasis. Induced oxidative stress, which results in depletion of GSH, leads to decreased GSH (reduced)/GSSG (oxidized) ratio (Figure 4.15) [99]. Therefore, as the depletion of GSH increases, S-glutathionylation process (Figure 4.14) will kick in, trying to prevent serious depletion of the GSH.

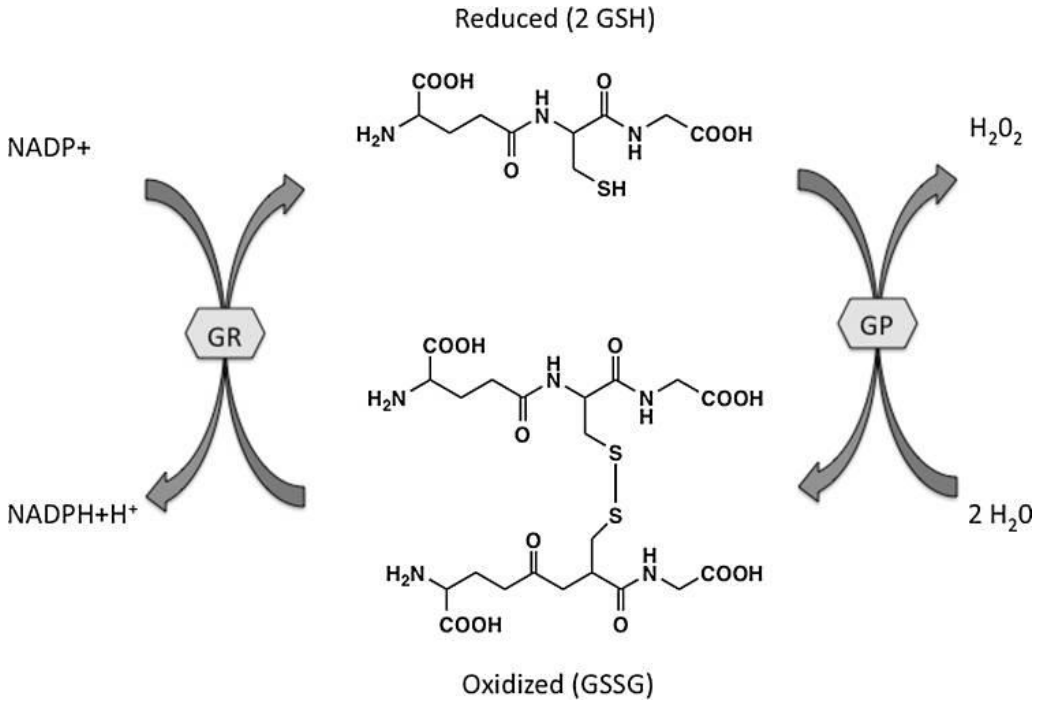


Figure 4.15. Glutathione as a biological redox buffer. The ratio of GSH/GSSG reflects the redox capacity of the cell. The ratio is kept in balance through oxidation/reduction reactions involving GSH peroxidase and GSH reductase. Reactive oxygen species-/reactive nitrogen species induced changes that decrease GSH lead to cell death via apoptosis or necrosis. GP, GSH peroxidase; GR, GSH reductase; GSH, reduced glutathione; GSSG, oxidized glutathione [99].

It is possible that Reaction 2 (Figure 4.14) may be reversible via S-Glutathionylation [100,101], which may explain relatively lower signal of HO-1 band in Western Blot analysis produced by MTPNO as well as not being able to induce higher levels of upregulation of HO-1 mRNA when compared to PNO. A negative feedback mechanism may be proposed, which also reverses upregulation of HO-1 mRNA.

The high IC_{50} value of the fractions may be due to the possibility that compounds of the *A. stipitatum* extract exhibit synergistic effect when acting altogether, which results in lower IC_{50} values than the ones of individual components.

Extract of *A. aflatunense* found to upregulate HO-1 and Cdkn1a mRNA, although yielding much lower signals than the ones of *A. stipitatum* extract. It was found also that *A. aflatunense* extract possesses compounds DPT and MTPNO. These findings, combined with the findings from *A. stipitatum* extract and compounds, lead to the opinion that PNO has higher oxidative stress potential than MTPNO, and PNO and MTPNO together may exhibit stronger effect. In addition, it may be suggested, that PNO and MTPNO acting together will result in upregulation of Nrf2 mRNA, the effect, which was observed in the trials with whole extract and not with compounds separately.

In summary, it was demonstrated that EtOAc extracts of *A. aflatunense* and *A. stipitatum* exhibit strongest toxic activity against T24 and UMUC3 cells, among many other *Allium* species, even prevailing activity of very well studied garlic. Extract of *A. stipitatum* found to upregulate Nrf-2 and HO-1 mRNA. Main bioactive compounds of *A. stipitatum* extract found to be PNO and MTPNO, which act as strong oxidants and eventually trigger cancer cells' defense mechanism, since upregulation of HO-1 mRNA and expression of same proteins was observed when the cells were treated with the compounds. This upregulation is believed to be a result of GSH depletion in cancer cells.

5. CONCLUSION

Species of genus *Allium* have been used since ancient times as food and folk medicine. In this study flowers of genus *Allium* subgenus *Melanocrommyum* were investigated. While significant taxonomic discovery was not observed, several species had higher CSO and essential amino acid content in flowers than previously was reported in bulbs. Results differed even among same species collected from different sites. Flower extract of *Allium rosenorum* (4293) while had highest total amount of CSOs with 7.05%, the only CSO which was present was methiin. The amino acid content was also highest in the same sample with almost 30% of fresh material weight. Due to unusually high amounts of CSO and amino acids, the results for species 4293 may be considered erroneous, thus additional trial is suggested. When compared to the results of bulb analysis by Kusterer, the total CSO amount was around 0.1% and had two reported CSOs, methiin and *S*-(2-pyrrolyl)-L-cysteine sulfoxide with around 1:4 ratio. When same *A. rosenorum* species compared it is possible to see huge difference in CSO and essential amino acid amounts among them. Flowers of *A. rosenorum* (2530) had total CSO amount of 0.48% and flowers of *A. rosenorum* (1886) had 0.12%, which was the lowest amount among all tested species. Meanwhile, both species had *S*-(2-pyrrolyl)-L-cysteine present. Similar findings confirm the fact that the chemical content of the plants will be different based on collection sites, thus it may be not possible to use it for taxonomic purposes. Fact of presence of methiin in all flower samples lending support to the idea that methiin may be the key CSO which plays role during flowering period, and volatile sulfur compounds formed as products of methiin cleavage may have significant contribution with various functions.

Allium flower extracts were found to have aspartic acid, asparagine, arginine, glutamine and glutamic acid as major essential amino acids. It is not possible to generalize specific preference for any of those as nitrogen storage compound per section due to small number of species. Additional controlled trials with higher number of samples is required for this purpose.

Analyzed *Allium* bulbs were collected during their flowering period for proper identification of the species, thus their CSO and amino acid content is usually less than the bulbs' which enter storage period. In early spring, nearly the entire bulb is used to produce green plant parts and the flower. If the leaves are fully developed, new storage started inside the bulb. Therefore, during that period flowers may carry higher amount of the mentioned compounds when compared to their bulbs.

Several epidemiological studies have been performed along with numerous *in vitro* experiments which investigated effect of garlic and onions for their canceroprotective and antitumor effects. In fact, only a few garlic and onion species were investigated. Rich genus *Allium* may have high potential in this area, considering that its species, especially subgenus *Melanocrommyum* have been extensively used in Central Asia since ancient times. As a result of this investigation, several *Allium* species extracts were screened for their antiproliferative activity against T24 and UMUC3 human bladder cancer cells. Main species of focus were found to be *A. stipitatum* (7002) and *A. aflatunense* (1178). They found to be active by killing cancer cells by inducing apoptosis and cell cycle arrest. When evaluated for their molecular targets, it was found that *A. stipitatum* and *A. aflatunense* both upregulate HO-1 mRNA, thus acting as reactive oxygen species, since HO-1 mRNA may be triggered as a cell protection mechanism against oxidative stress. Compounds extracted from active fractions from *A. stipitatum,* 2-(methyldithio)pyridine-N-oxide (compound 1) and 2-[(methylthiomethyl)dithio]pyridine-N-oxide (compound 2) were found to be responsible for upregulation of HO-1 mRNA, with compound 1 being more active than compound 2. Previous investigations provide enough evidence that glutathione found in cells reacts with sulfur compounds to restore redox balance of a cell. Therefore, for this investigation it is assumed that both compounds result in cells death via glutathione depletion. HO-1 proteins are synthesized to prevent oxidative stress caused by the compounds. Because of glutathione depletion, Cdkn1a mRNA may also be upregulated causing cell cycle arrest which leads to apoptosis. Apoptosis in the cells is also confirmed by PI flow cytometry assay in which significant reduction of DNA content was recorded, which shows death processes taking place during treatment of T24 cells with the extracts.

Based on findings of this research 1178 *A. aflatunense* extract may be considered as the most toxic to T24 and UMUC3 cancer cells. Extracts of 7002 *A. stipitatum* and *A. stipitatum* (Iran) are second and third toxic extracts, respectively, against T24 and UMUC3 cancer cells. All three *Allium* extracts showed almost no selectivity against HFF-1 healthy cells.

The list of remaining most toxic *Allium* extracts can be presented as follows in the order of decreasing toxicity against T24 and UMUC3 cancer cells: *A. pallens*, *A. rosenorum*, *A. sativum*, *A. nutans*, *A. platyspathum*, *A. hollandicum*, *A. karataviense, A. oreoprasum*, *A. senescens*. More details already presented in Table 3.4.

There is plenty of data regarding anticancer effect of sulfur compounds found in *Allium* species. This study is a first one to provide information on mechanism of action of compounds in *A. stipitatum.* In addition, it is proposed that *A. aflatunense* also has two same compounds which are present in *A. stipitatum.* Different ratios of the compounds result in differences in mechanisms of actions and cytotoxicity level overall. Since cancer is a complex disease that involves multiple mechanisms, handling some of the extracts as a whole may be more effective than individual compounds. With 750 species present in genus *Allium* there is a lot of room for future research.

6. REFERENCES

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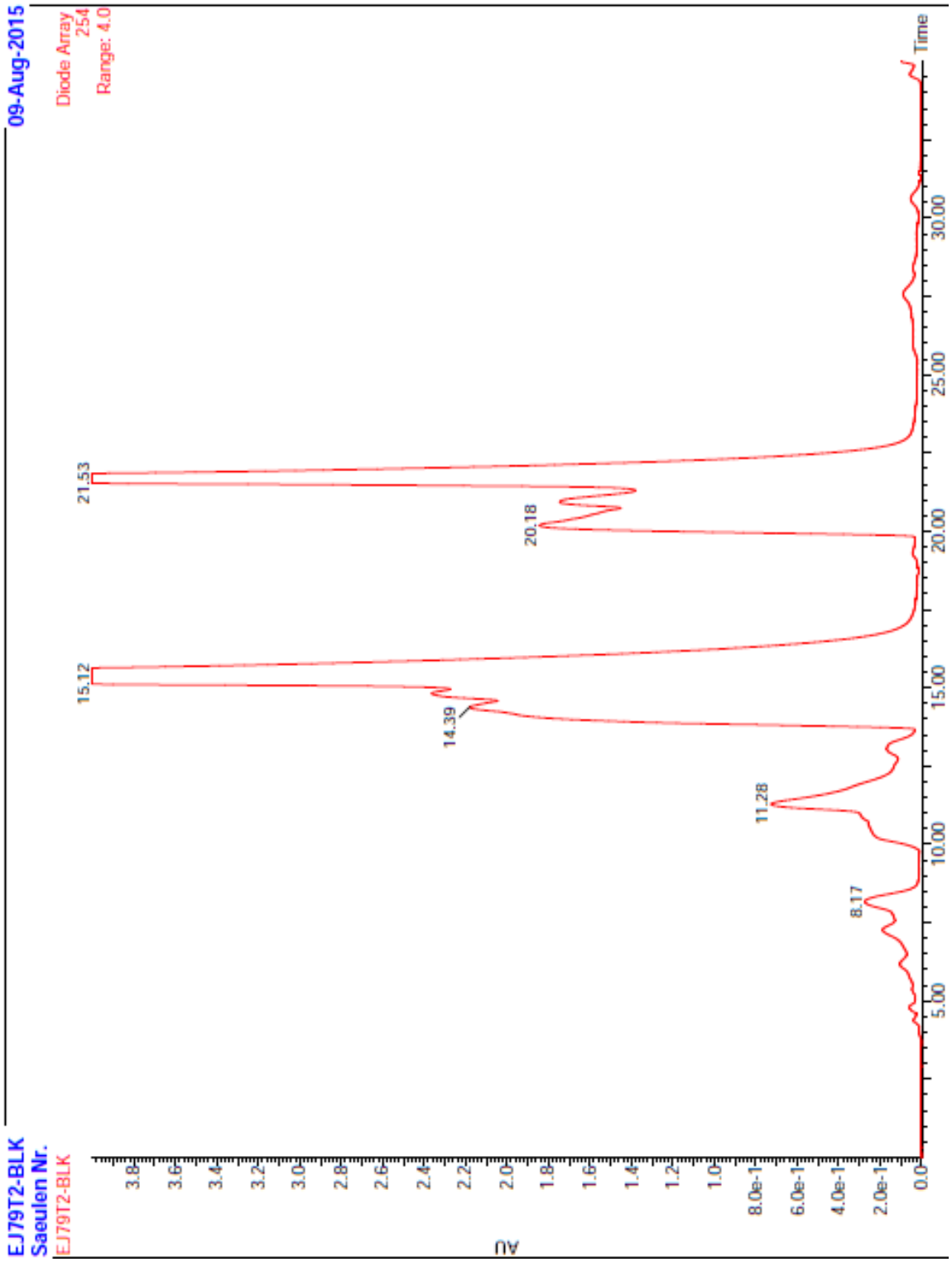
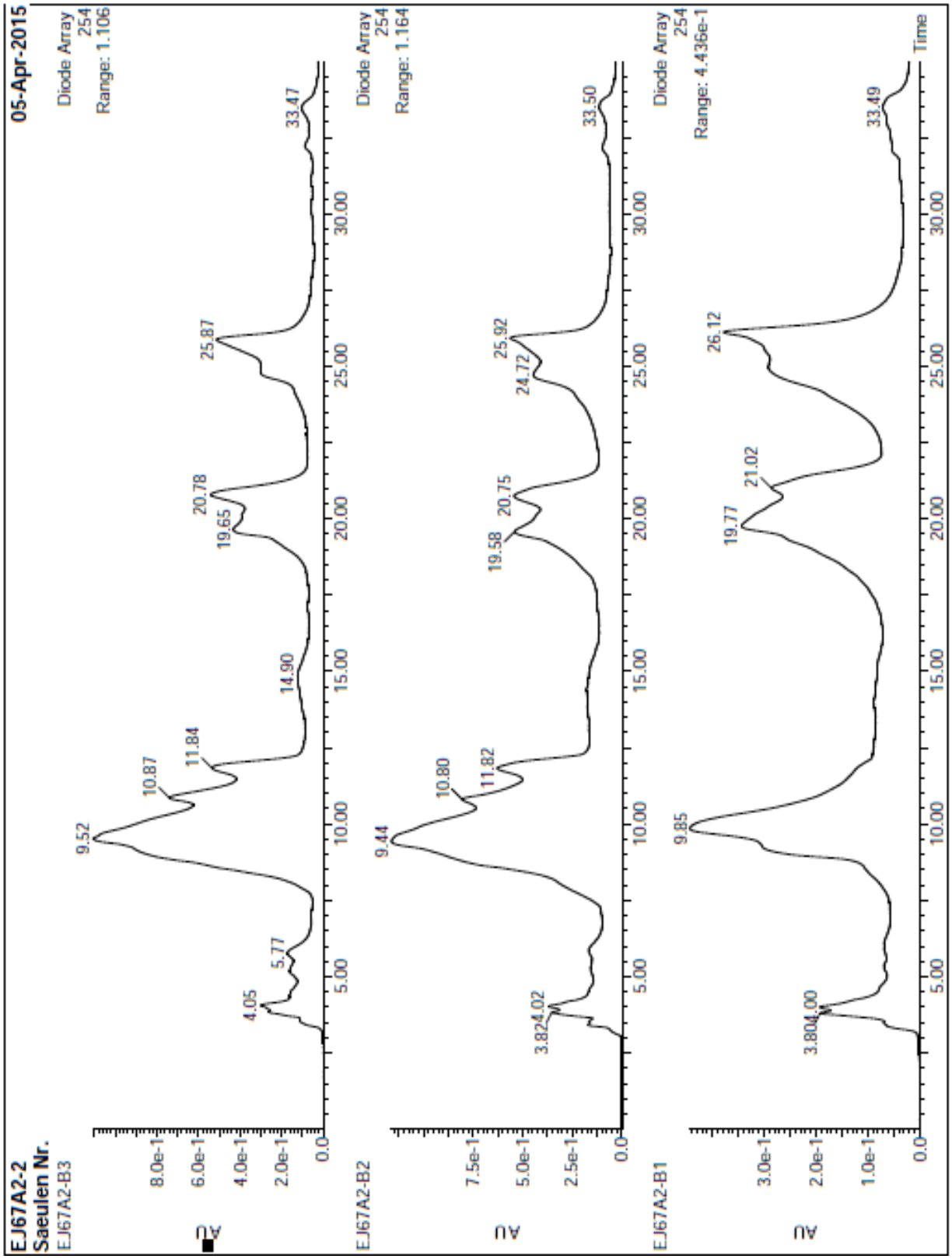
7. APPENDIX

7.1. Spectroscopic and chromatographic data

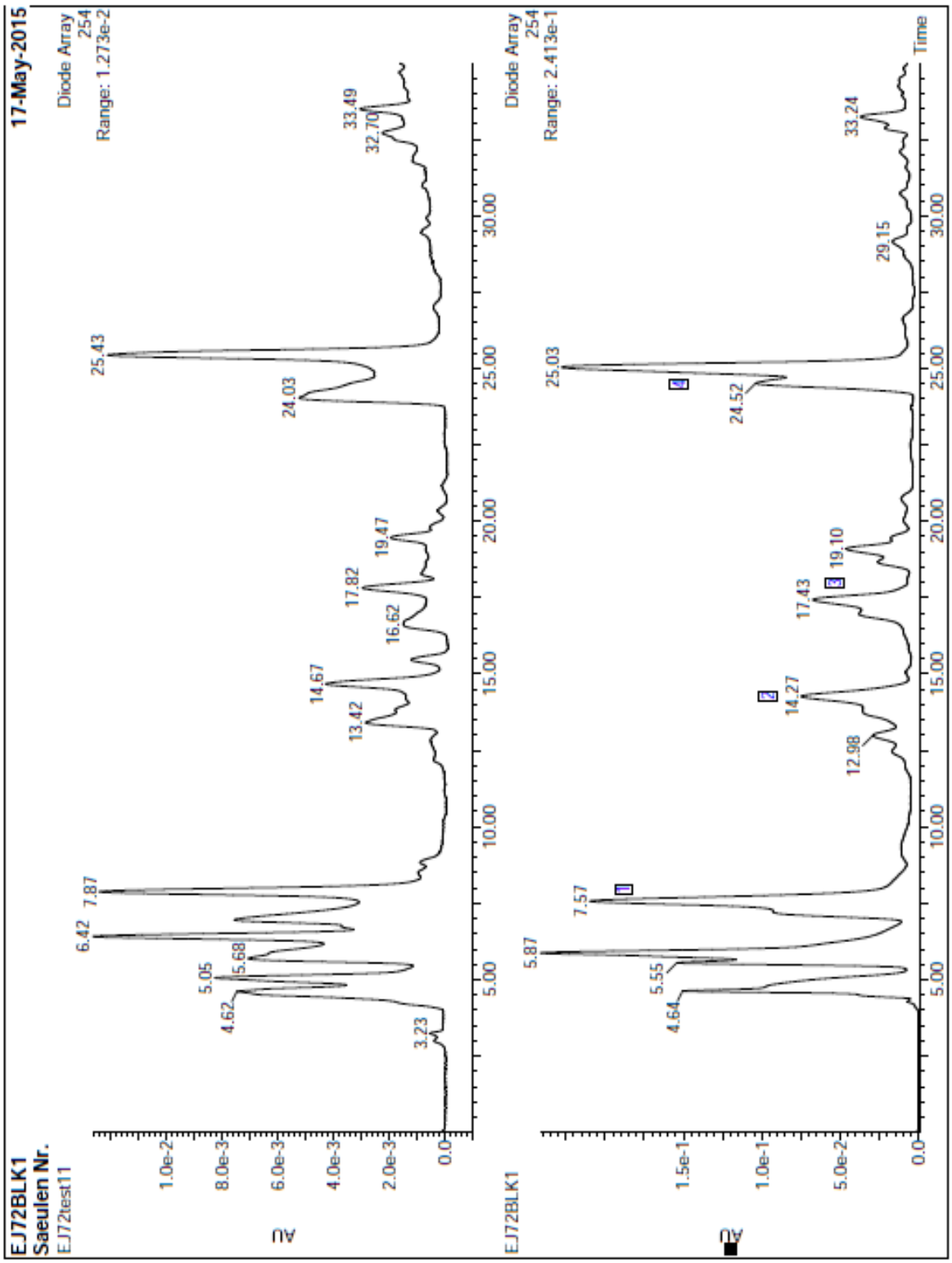
Preparative HPLC Chromatogram of 7002 *A. stipitatum* EtOAc bulb extract



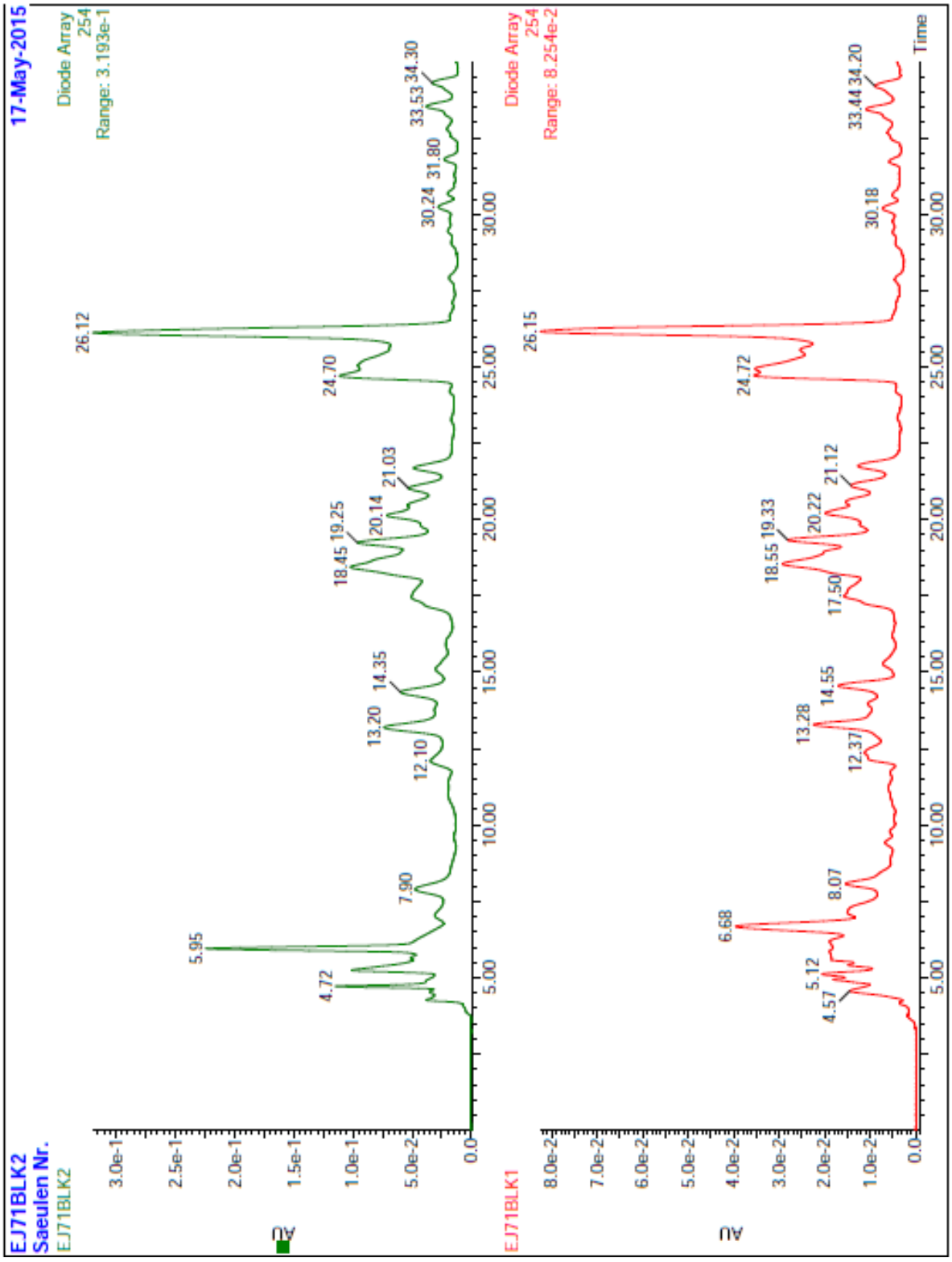
Preparative HPLC Chromatogram of 1178 *A. aflatunense* EtOAc bulb extract



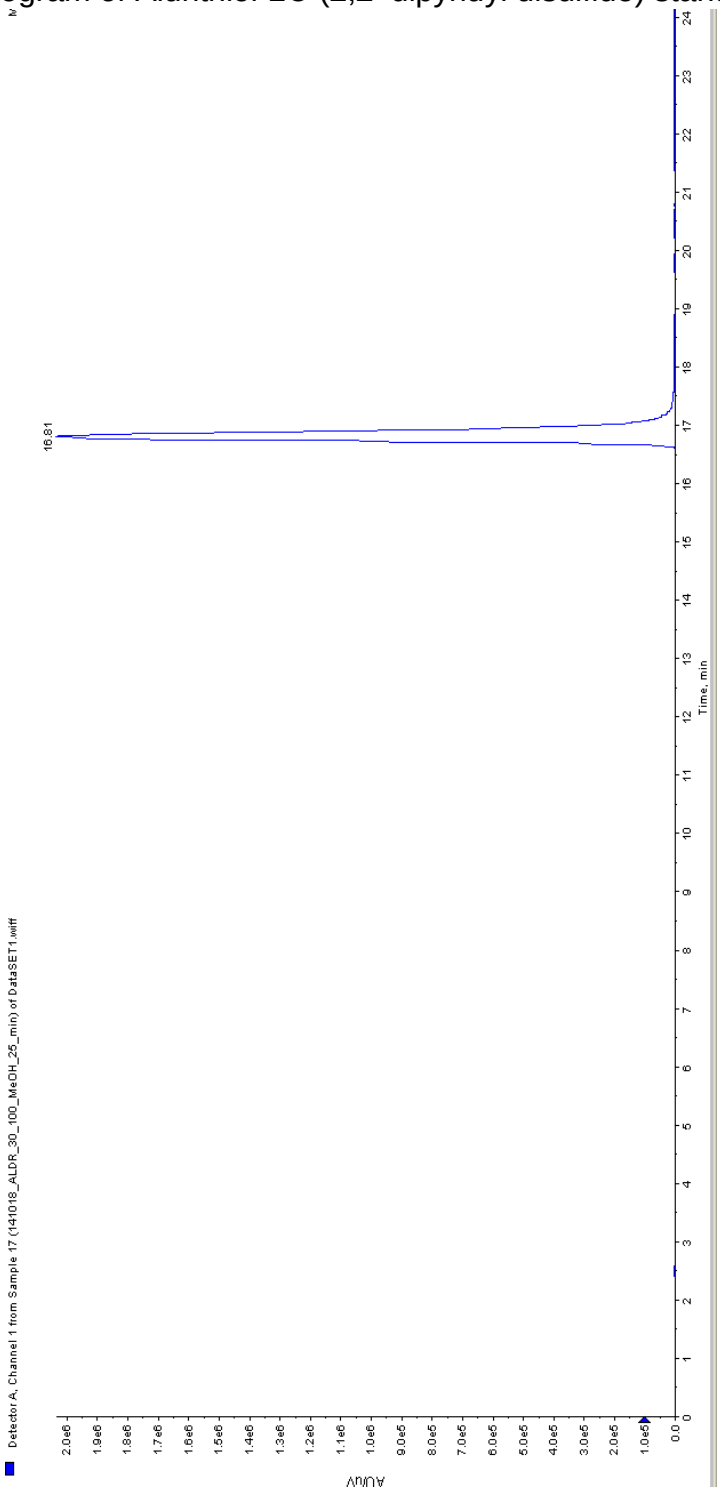
Preparative HPLC Chromatogram of 2256 *A. rosenorum* EtOAc bulb extract



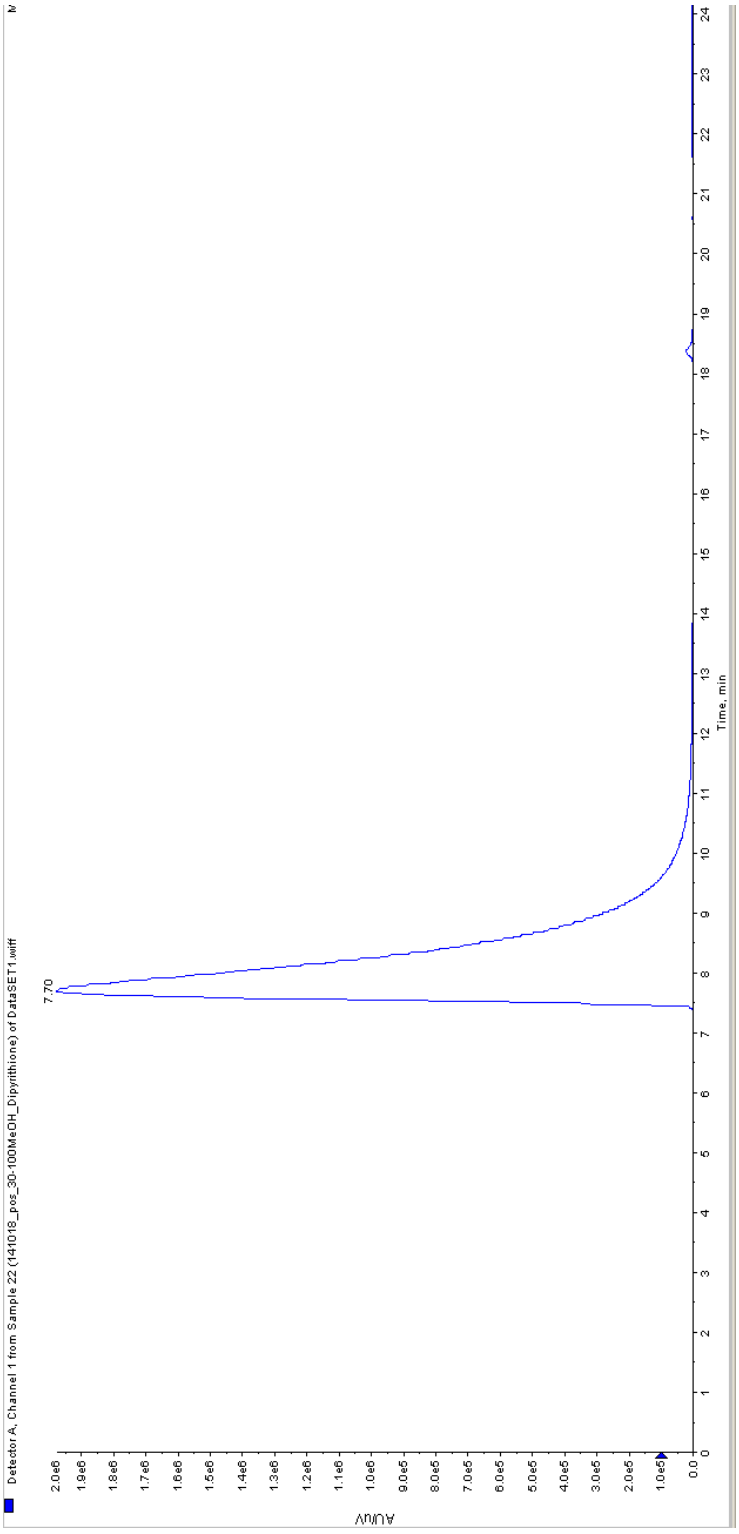
Preparative HPLC Chromatogram of 2390 *A. pallens* EtOAc bulb extract



HPLC Chromatogram of Aldrithiol-2® (2,2′-dipyridyl disulfide) standard

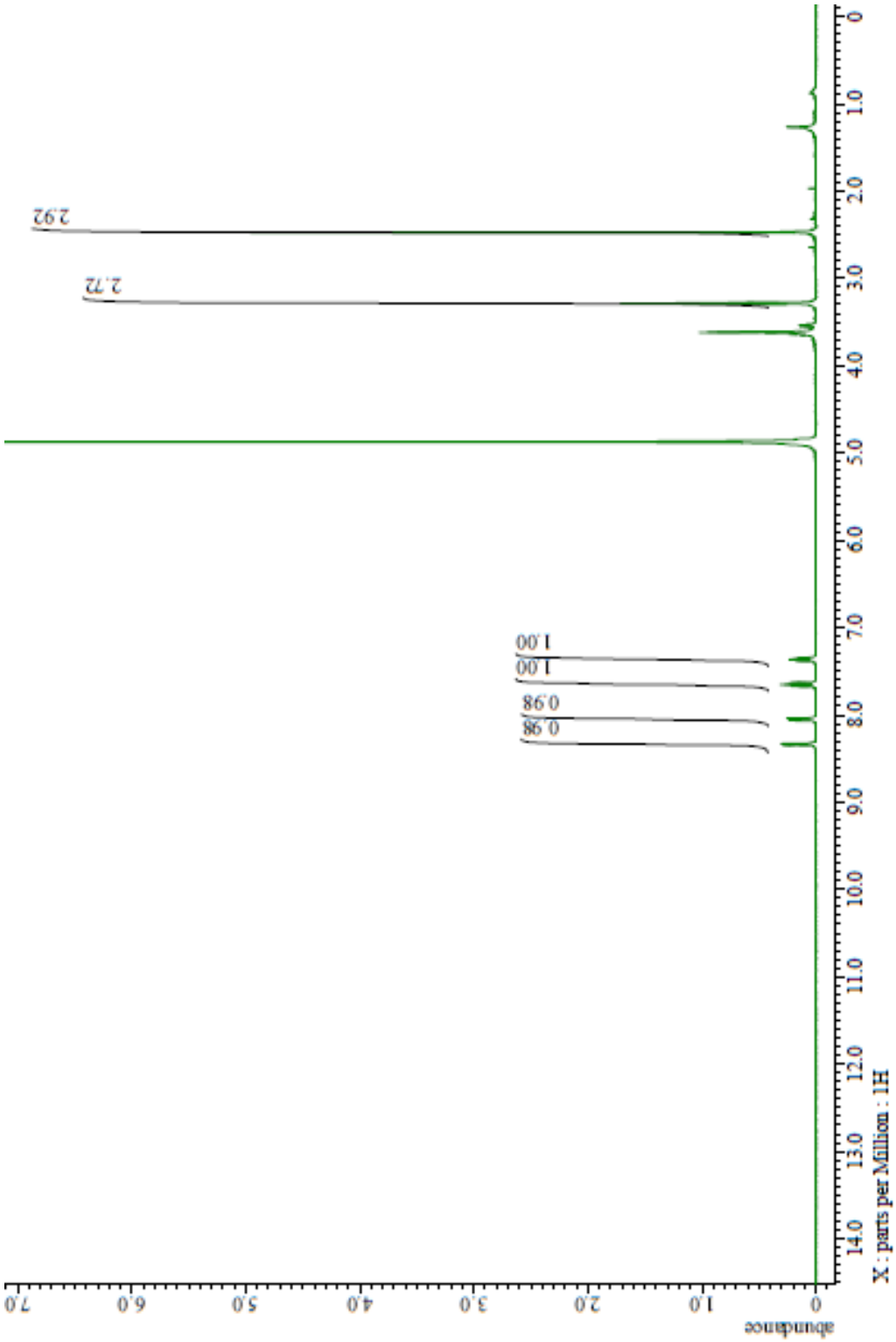


HPLC Chromatogram of dipyrithione (2,2'-dithiobis(pyridine-N-oxide)) standard



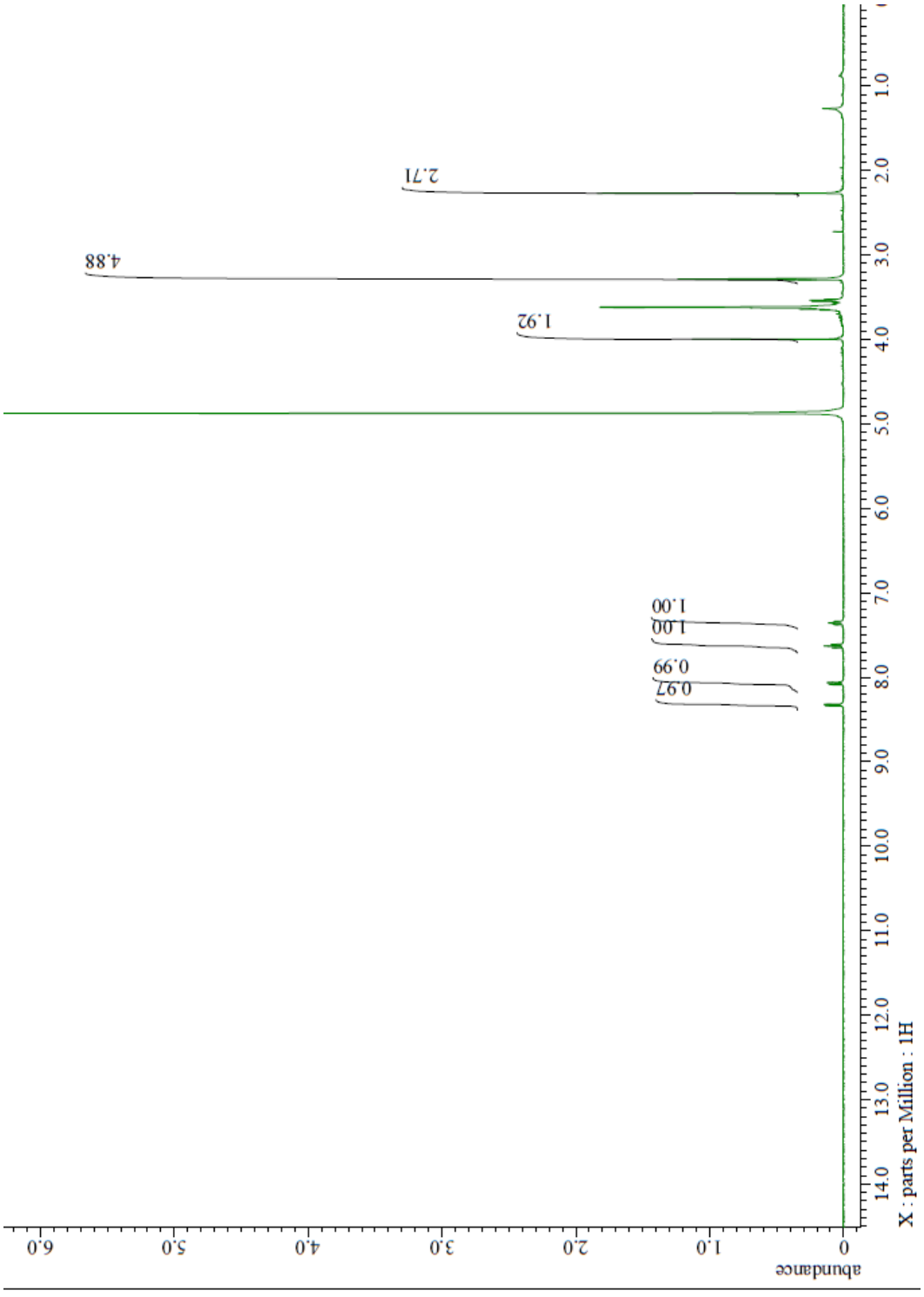
NMR Chromatogram of fraction 3 from 7002 *A. stipitatum* EtOAc bulb extract

(2-(methyldithio)pyridine N-oxide)

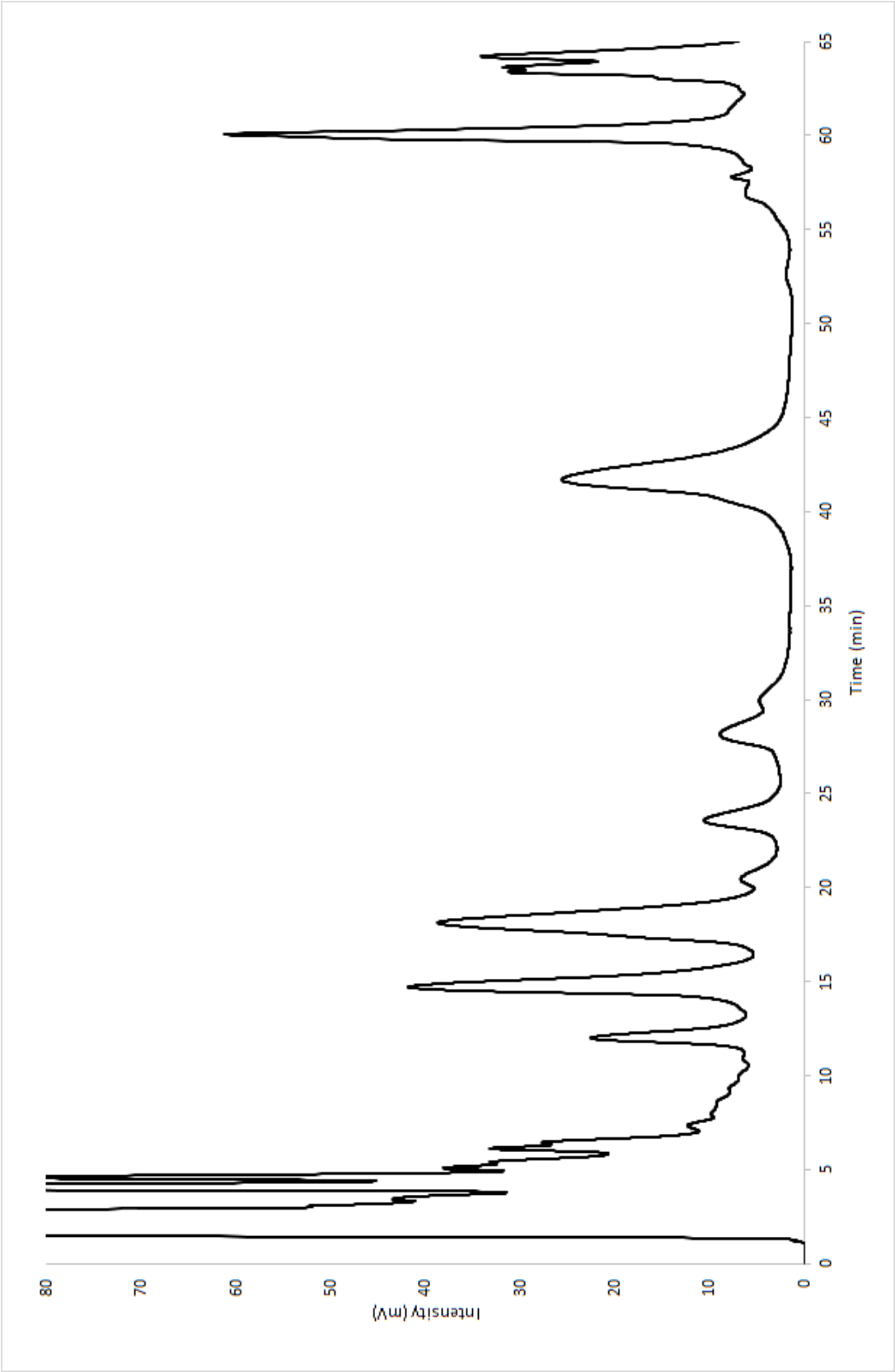


NMR Chromatogram of fraction 4 from 7002 *A. stipitatum* EtOAc bulb extract

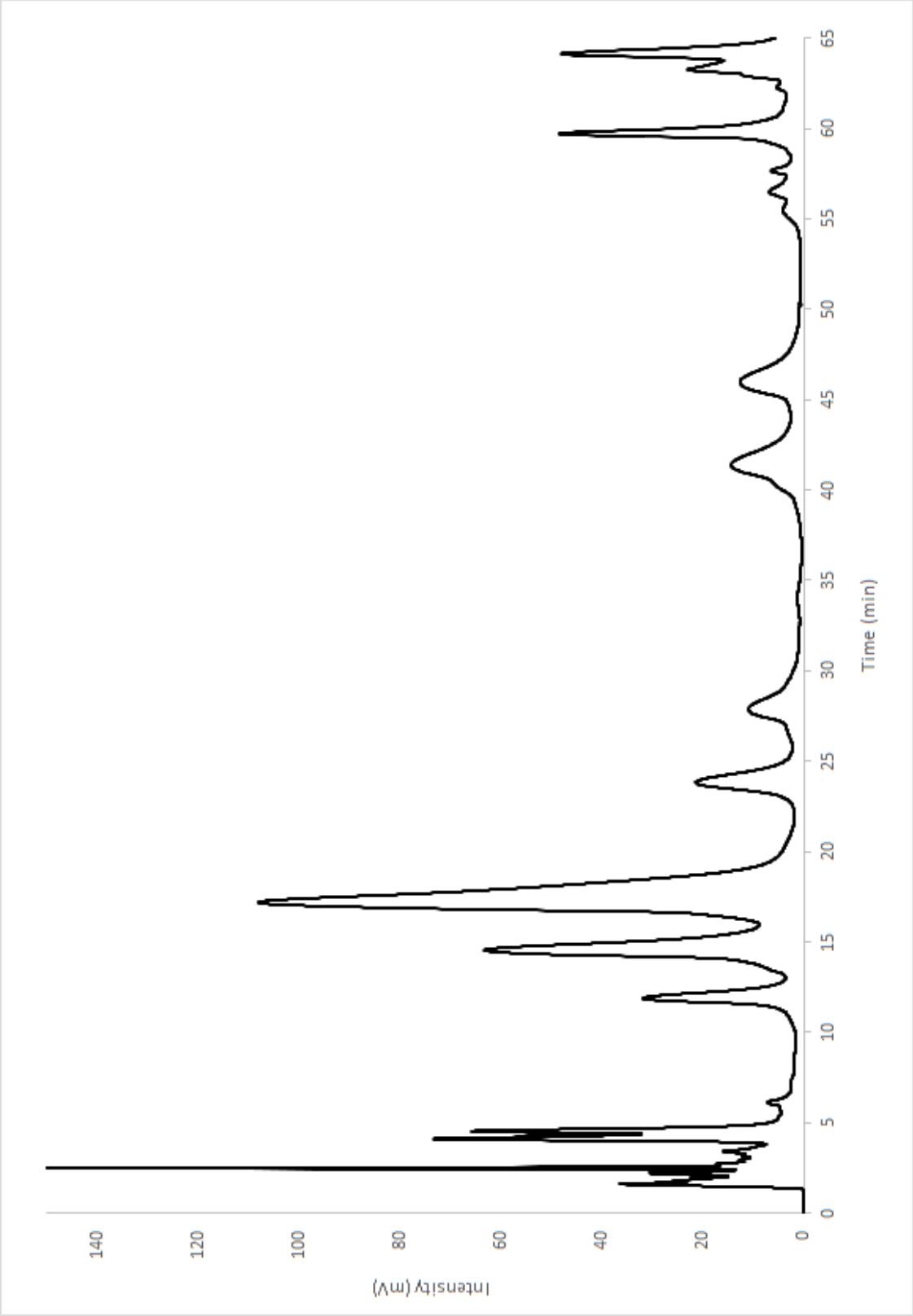
(2-[(methylthio)methyldithio]pyridine N-oxide)



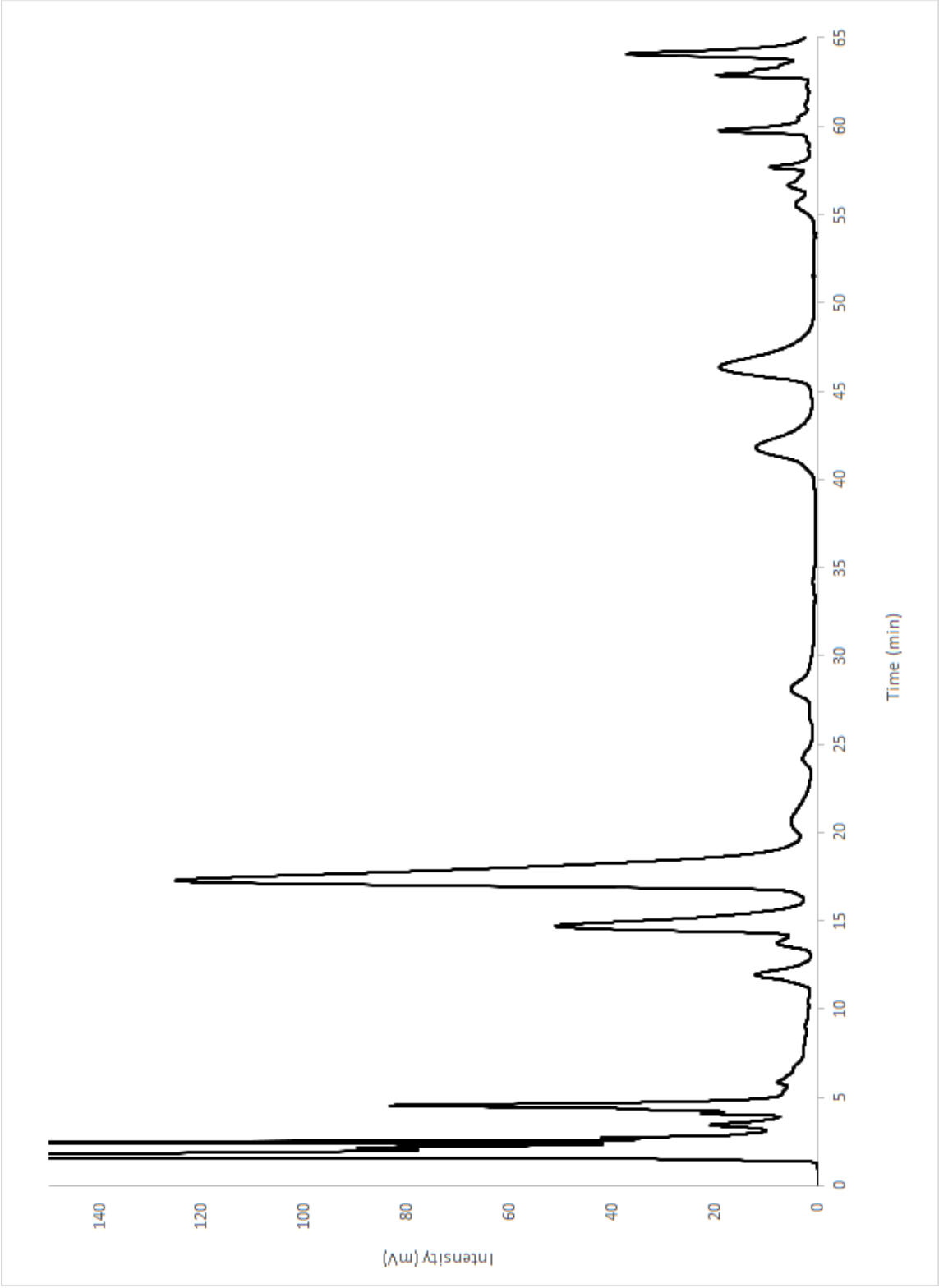
HPLC Chromatogram of 1198 *A. keusgenii* flower extract with OPA derivatization



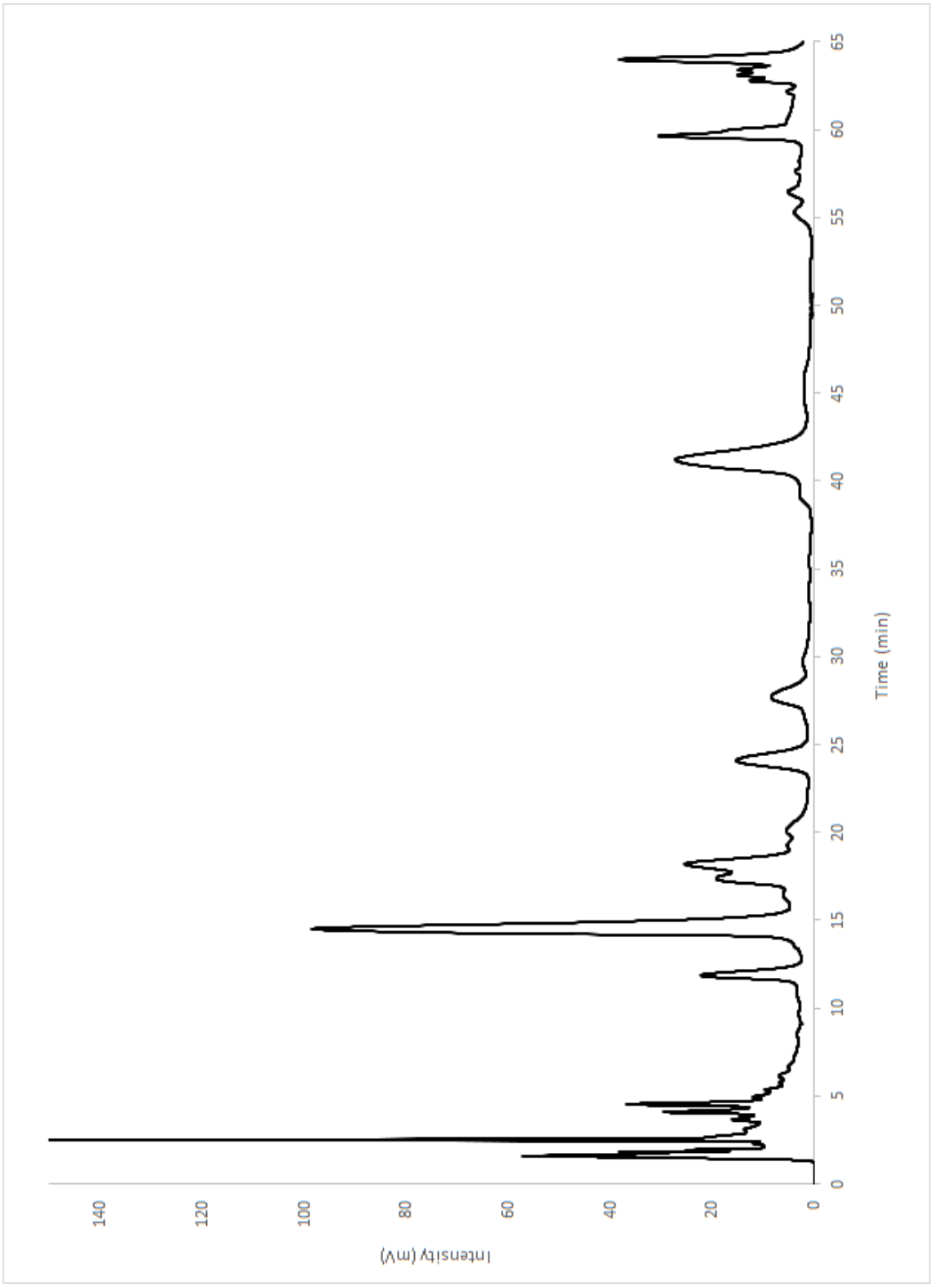
HPLC Chromatogram of 1222 *A. jesdianum* flower extract with OPA derivatization



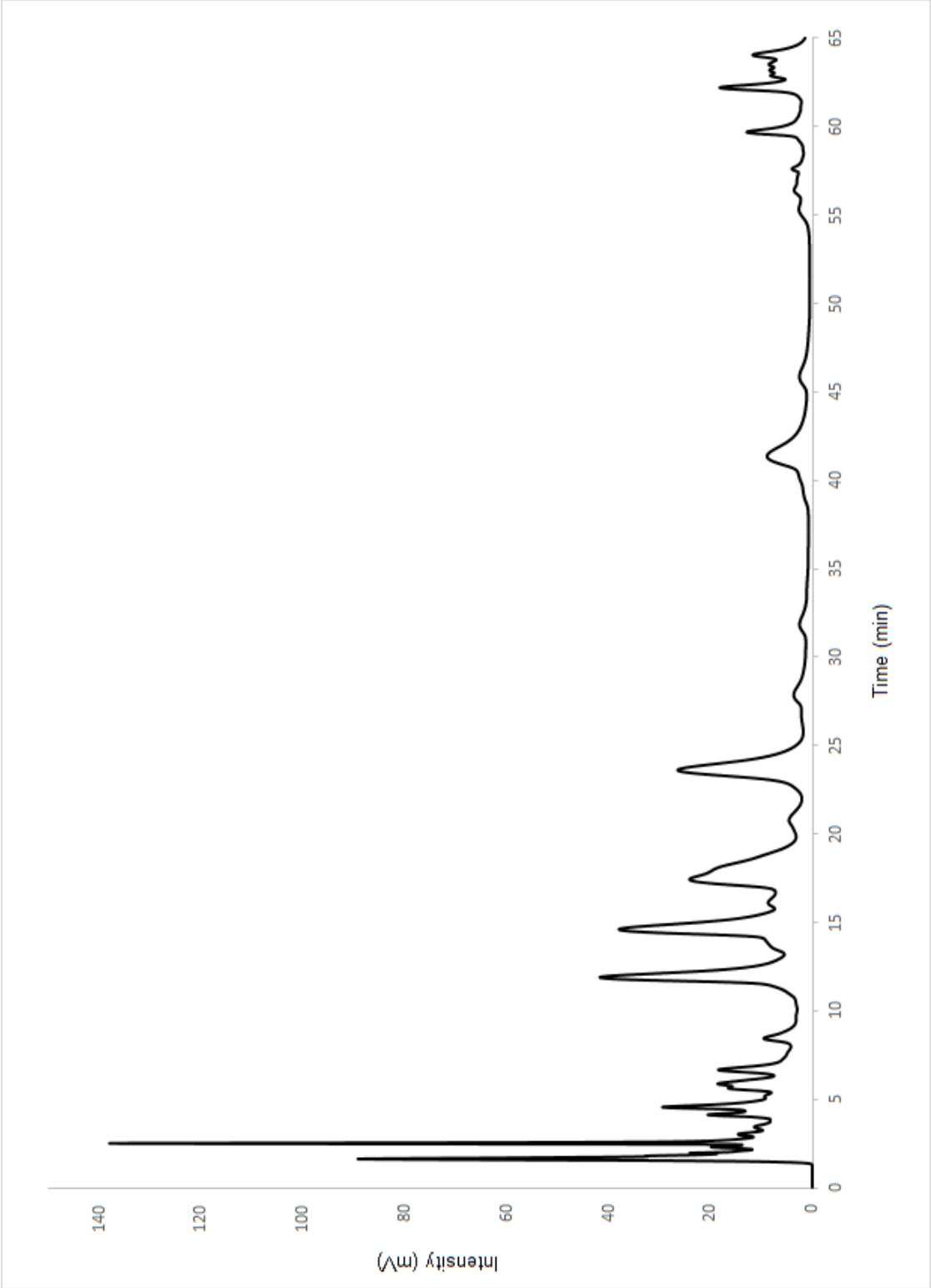
HPLC Chromatogram of 1653 *A. hollandicum* flower extract with OPA derivatization



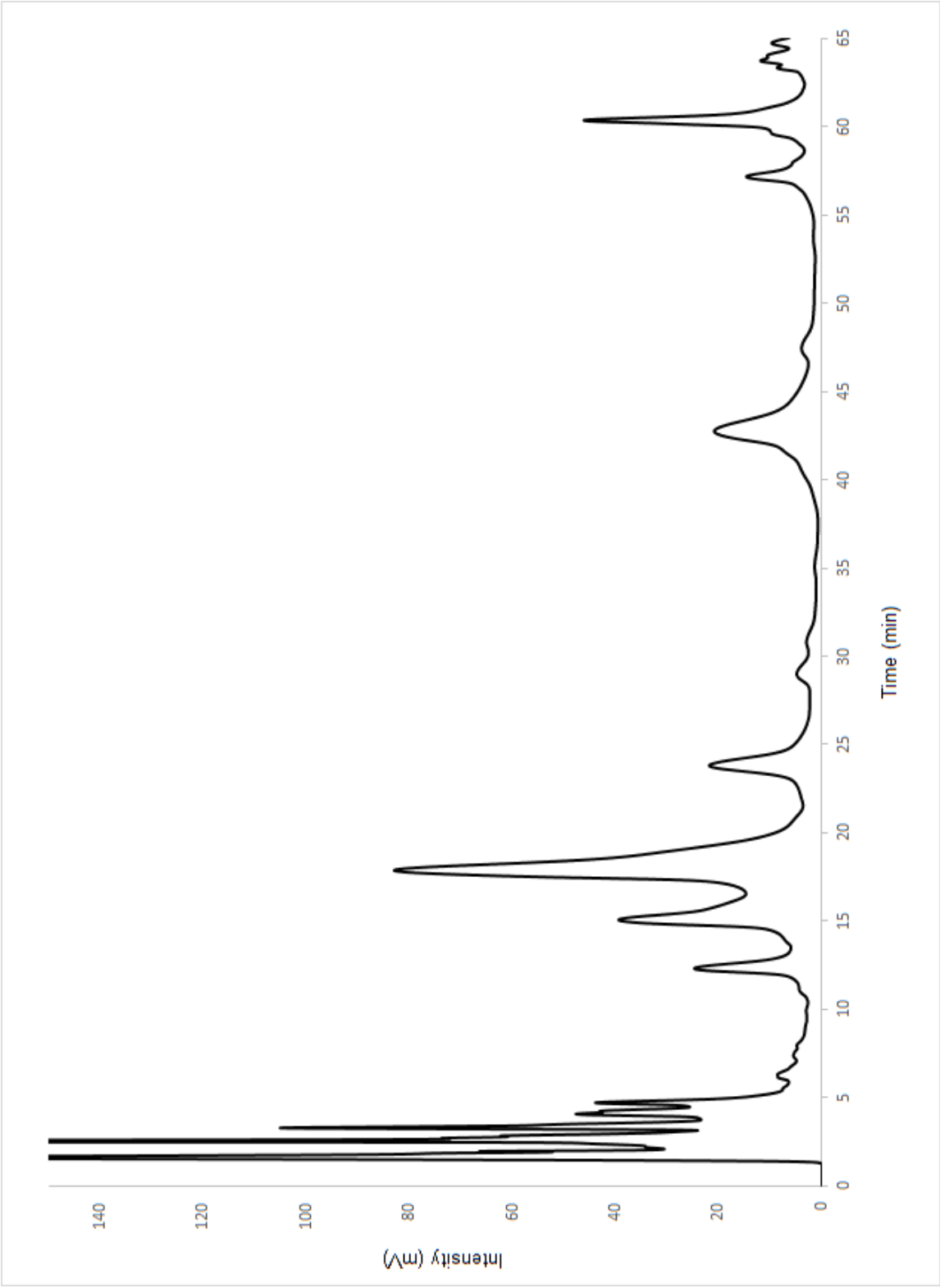
HPLC Chromatogram of 4280 *A. gypsaceum* flower extract with OPA derivatization



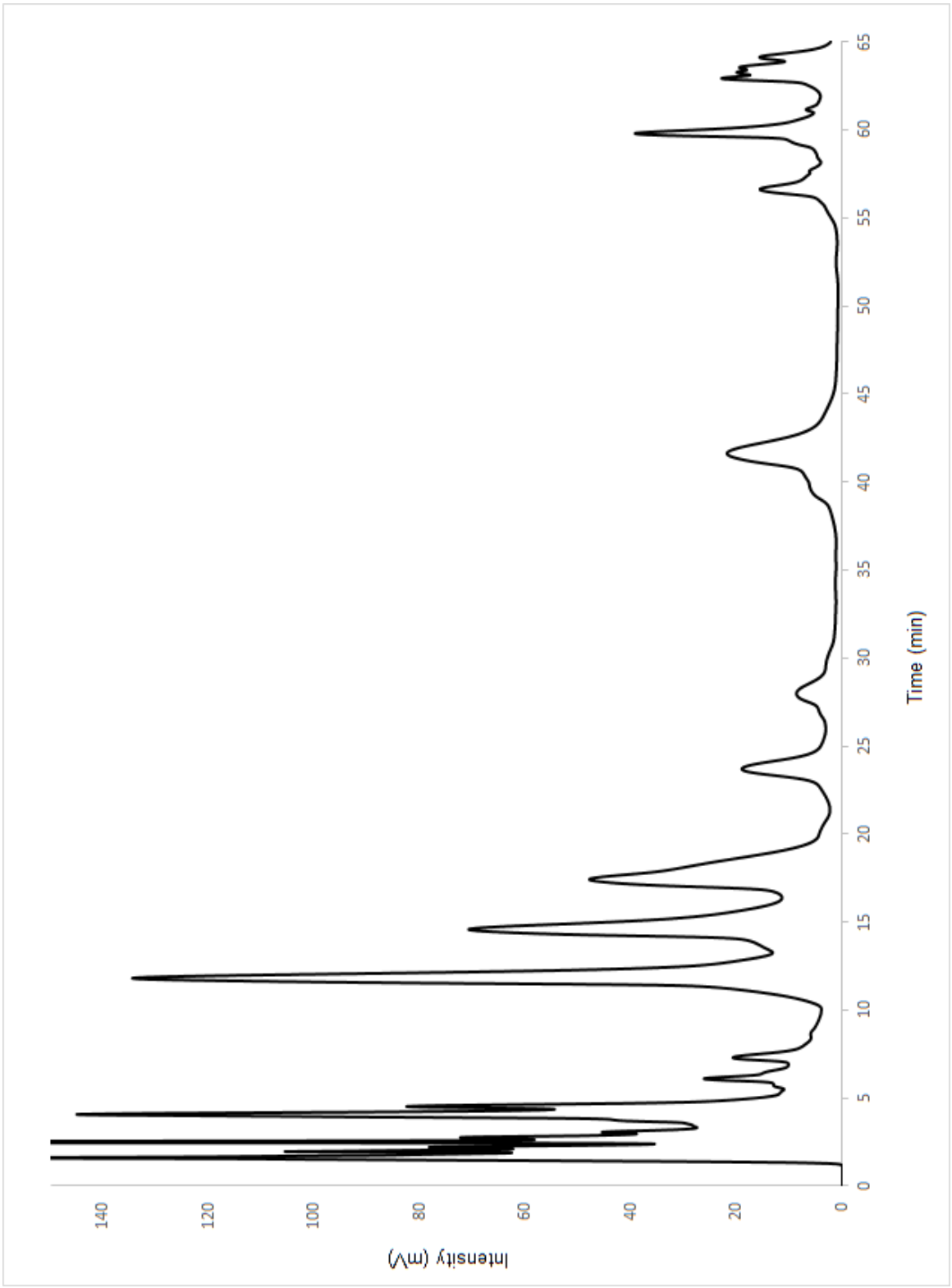
HPLC Chromatogram of 4282 *A. protensum* flower extract with OPA derivatization



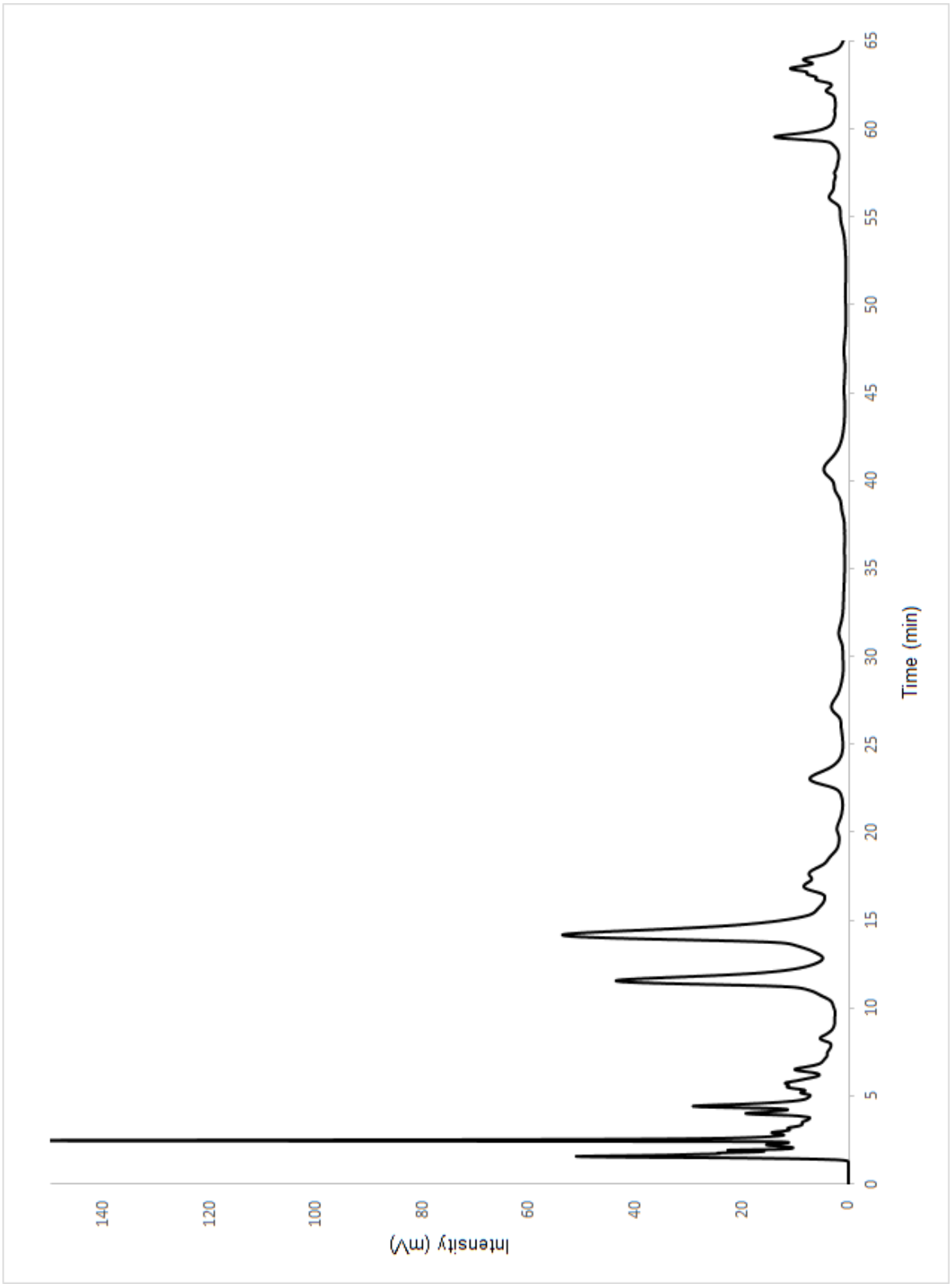
HPLC Chromatogram of 4283 *A. giganteum* flower extract with OPA derivatization



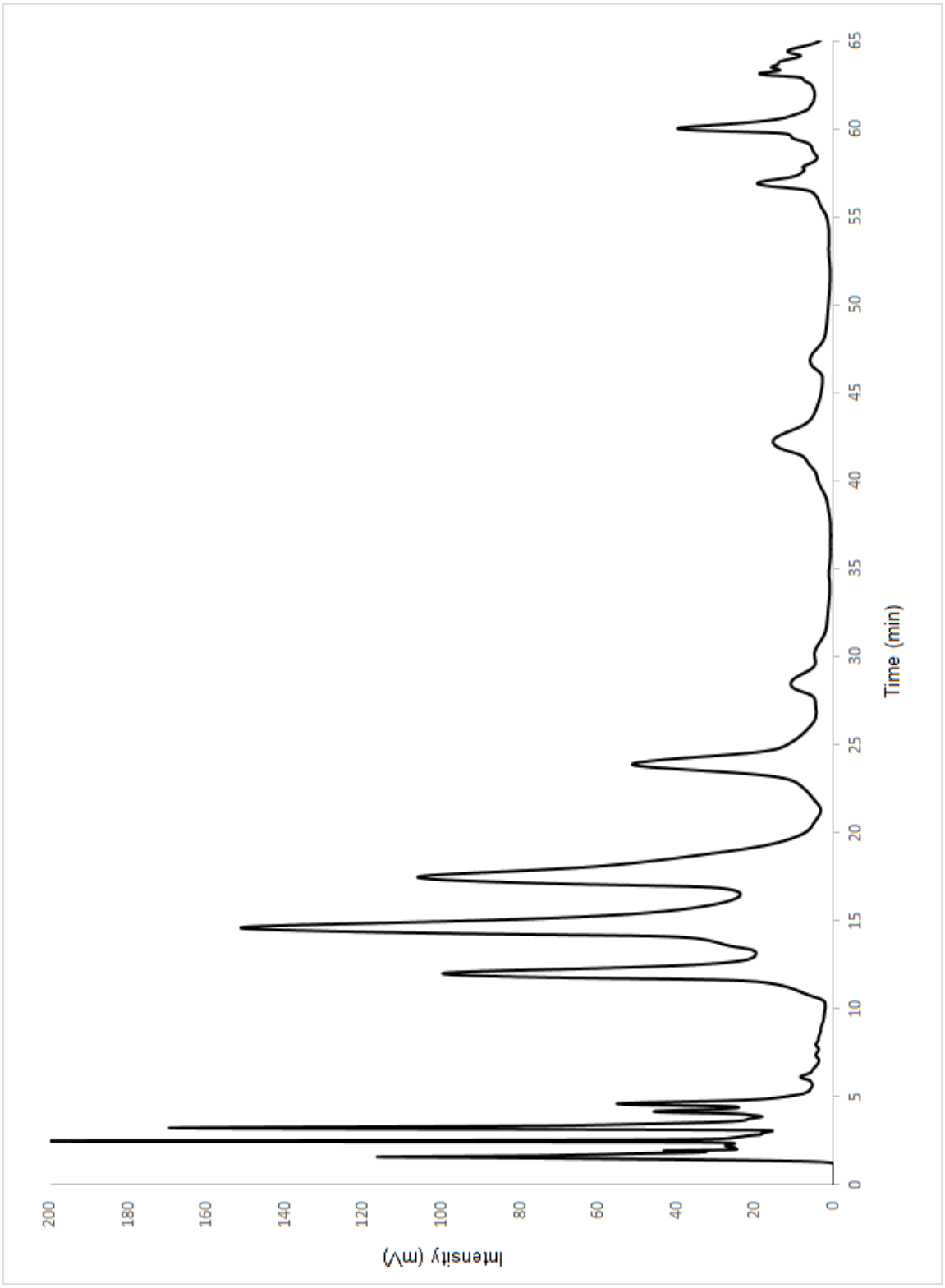
HPLC Chromatogram of 4287 *A. suworowii* flower extract with OPA derivatization



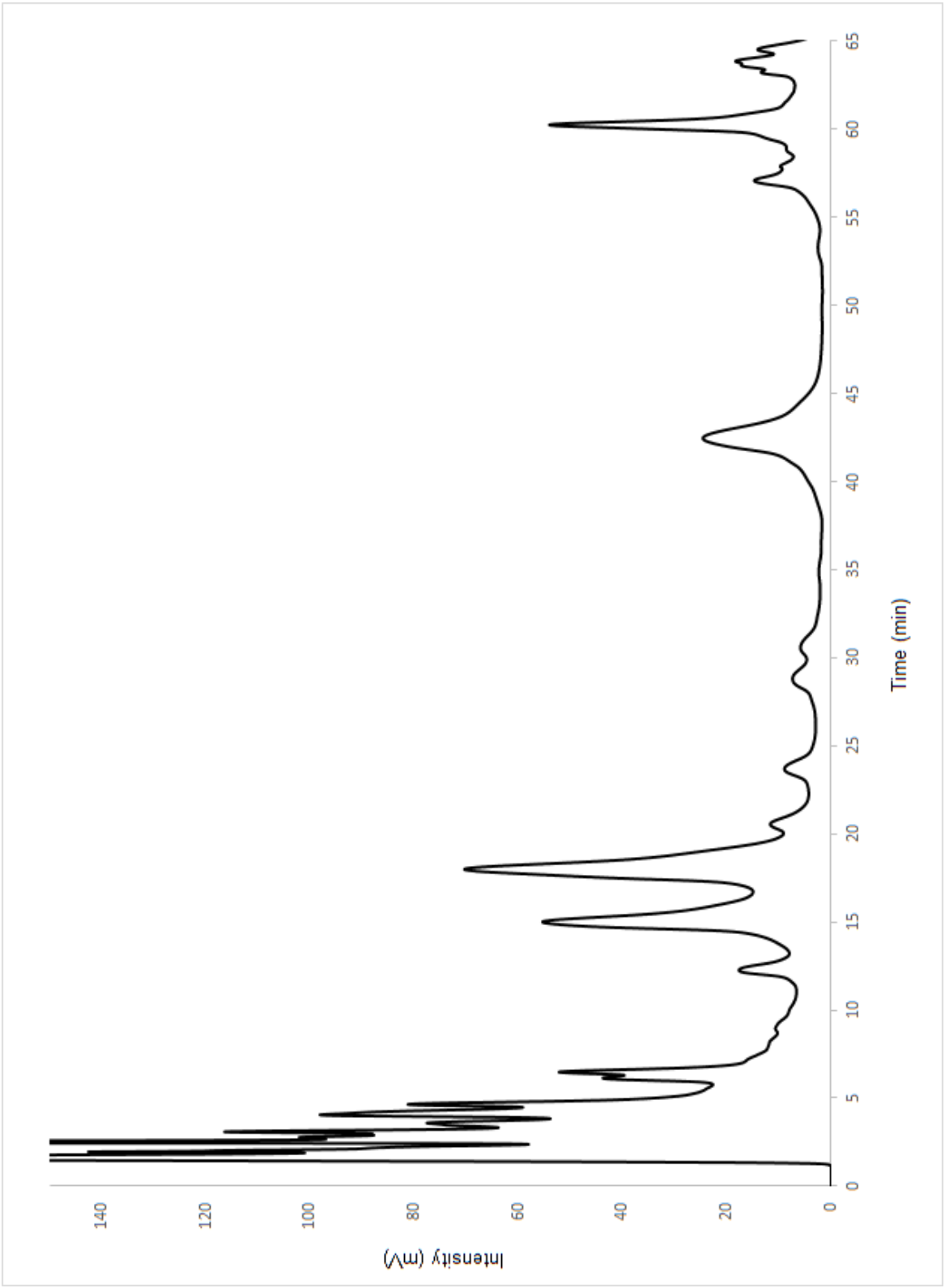
HPLC Chromatogram of 4291 *A. protensum* flower extract with OPA derivatization



HPLC Chromatogram of 4293 *A. rosenorum* flower extract with OPA derivatization



HPLC Chromatogram of 4296 *A. suworowii* flower extract with OPA derivatization



List of Publications

Posters with Abstracts

Emil Jivishov, Joerg Hänze, Rainer Hofmann, Michael Keusgen

Cytotoxic effects of extracts from *Allium* species against bladder cancer cells

2nd European Conference on Natural Products, September 2015, Frankfurt, Germany

Emil Jivishov, Steffen Neuman, Michael Keusgen

Qualitative and quantitative analysis of cysteine sulfoxides in flowers of some *Allium* species

DPhG Annual Meeting 2014, Frankfurt, Germany

Oral Presentations

Emil Jivishov, Jörg Hänze, Rainer Hofmann, Michael Keusgen.
Mechanism of action of whole extract and bioactive compounds from *A. stipitatum* from Afghanistan against T24 bladder cancer cells.
Workshop „Evaluation of Natural Resources in Afghanistan “,
Rauischholzhausen/Marburg, Germany, November 2015,

Emil Jivishov, Jörg Hänze, Rainer Hofmann, Michael Keusgen.
Evaluation of bioactivity of extracts from some *Allium* species against cancer cells
Workshop „Evaluation of Natural Resources in Afghanistan “,
Rauischholzhausen/Marburg, Germany, December 2014

Publications

Emil Jivishov, Jörg Hänze, Pietro Di Fazio, Rainer Hofmann, M.S. Faqiri, Michael Keusgen, **Screening of *Allium* Species for anticancer effects reveal compounds that induce the antioxidant signaling pathway**

In preparation

Curriculum Vitae

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11/2012-12/2016	Doctorate program at Institute of Pharmaceutical Chemistry, Philipps-Universität Marburg, Germany
09/2004-07/2007	Master's degree, Graduate School of Health Sciences, Pharmacognosy, Anadolu University, Eskisehir, Turkey
09/1998-07/2004	Bachelor's degree, Faculty of Pharmacy, Anadolu University, Eskisehir, Turkey
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01.2012-present	Lecturer - Qafqaz University, Baku, Azerbaijan
06/2011-12/2011	Dean of Academics – Harmony School of Ingenuity, Houston, TX, USA
08/2008-06/2011	Teacher, Dean of Academics – Dove Science Academy, Oklahoma City, OK, USA
11/2007-08/2008	Teacher- School of Science and Technology, San Antonio, TX, USA
07/2006-07/2007	Research assistant- TUBITAK (Research council of Turkey) funded research project "Production of Thymoquinone and related bioactive metabolites from *p*-Cymene by means of biocatalysts" (Project No: 106T117), Eskisehir, TURKEY,