Nematicidal, Phytotoxic and Brine Shrimp Lethality Activity of Some *Allium* Species and Their Bioactive Sulfur Compounds

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Sevda Jivishova

aus **Baku, Aserbaidschan**

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Erstgutachter: **Prof. Dr. Michael Keusgen**

Zweitgutachter: **Prof. Dr. Shuming Li**

Eingereicht am **.....**

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

If I have seen further than others, it is by standing upon the shoulders of giants.

Isaac Newton

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List of Abbreviations

A.	*Allium*
A. salina	*Artemia salina*
A. thaliana	*Arabidopsis thaliana*
M.	*Meloidogyne*
LF	lachrymatory factor
HPLC	High performance liquid chromatography
HR-MS	High resolution mass spectroscopy
1H NMR, ^{1}H-NMR	Proton nuclear magnetic resonance
IPK	The Leibniz Institute of Plant Genetics and Crop Plant Research at Gatersleben (Germany)
Tax / Acc No	Taxonimic identifier/ Accession number
g	Gram
mg	Milligram
µm	Microgram
PBS	Phosphate buffer saline
ml	Milliliter
°C	Centigrade degree
mg/ml	Milligram per milliliter
µl	Microliter
%	Percent
LC50	Concentration at which 50% of the nematode population was dead
LC100	Concentration at which 50% of the nematode population was dead

DMSO	Dimethyl sulfoxide
MES	2-(N-morpholino)-ethanesulfonic acid
MeOH	Methanol
PDA	Photodiode array detector
mm	Millimeter
UV	Ultraviolet
NaCl	Sodium chloride
N_2	Nitrogen gas
H_2O	Water
min	Minute
mL/min	Milliliter per minute
J2	Second-stage juveniles
h	hour
TOF	Time of flight
ESI	Electrospray ionization
m/z	Mass to charge ratio
V	Volt
EtOAc	Ethyl acetate
LC50/FW	Concentration at which 50% of the nematode population was dead per fresh weight of plant material
LC50/EW	Concentration at which 50% of the nematode population was dead per weight of extract
SD	Standard deviation
p	statistics value probability of error

nm	nanometer
J	coupling constant
HPLC/MS	High performance liquid chromatography coupled with mass spectroscopy
GSH	Glutathione
AChE	acetylcholinesterase
GST	glutathione S-transferase
H.	*Heterodera*

Summary

As human population of the Earth grows, so are the needs to provide enough food for the humans and cattle. Agriculture plays major role in everyday food supply. As with human parasites and pathogens there are various pests which affect the yield of agricultural products. Nematodes are parasitic microscopic worms which infest various crops via various mechanisms. Root-knot nematodes are considered among the most difficult ones to treat. They penetrate the roots, feed on nutrient supplies of the plant and eventually multiply there. There are several nematode control methods and actually none of the methods can be considered to be ideal, as they may have high costs or be highly hazardous to human and environmental health safety. One of the control methods is treatment with chemicals which can also be associated with human health and environmental safety concerns. Methyl bromide is one of multipurpose pesticides which was once widely used as a nematicide and banned in 2005 due to the mentioned concerns. *Allium* plants have been widely used in folk medicine since ancient times. As there are more than 750 species worldwide and majority of them are not researched for their nematicidal activity it was decided to investigate several species using their bulbs and flowers.

Bulbs and flowers of *Allium*s collected from Central Asia, Middle East and from local collection in IPK-Gatersleben in Germany were extracted using ethyl acetate. Local culture of root-knot nematodes, *Meloidogyne incognita*, was maintained by infesting tomato (*Solanum lycopersicum*) plants. The extracts were subjected to nematicidal assay against a universal root-knot nematode, *Meloidogyne incognita*. The most promising nematicidal extracts were investigated for their phytotoxic and brine shrimp lethality activities. Four most nematicidal plant extracts were investigated for their compounds responsible for the nematicidal effect. Each extract was separated in fractions and fractions were tested for the nematicidal activity. Structures of compounds in bioactive fractions were elucidated by high resolution mass spectroscopy and NMR spectroscopy analysis with further comparison and confirmation of the data with the ones previously reported in literature. As a result, it was found that, in *A. ampeloprasum* allicin is the most nematicidal compound and in *A. stipitatum*, two compounds, 2-

(methyldithio)pyridine-N-oxide and 2-[(methylthiomethyl)dithio]pyridine-N-oxide were responsible for the nematicidal activity.

Zusammenfassung

Das stetige Wachstum der Erdbevölkerung bedingt auch immer weiter zunehmende Anforderungen an die Versorgung von Mensch und Tier mit Nahrungsmitteln. Die Landwirtschaft trägt hierbei die Hauptrolle. Wie bei humanen Parasiten oder anderen Pathogenen gibt es auch bei Nutzpflanzen verschiedenste Schädlinge und Krankheiten, die großen Einfluss auf die Ernte haben. Ein Beispiel hierfür sind Nematoden; dabei handelt es sich um mikroskopisch kleine Würmer, die als Parasiten verschiedenste Nutzpflanzen befallen können. Wurzelgallennematoden werde zu den am schwierigsten zu behandelnden Nematoden gezählt; sie dringen in die Wurzeln der Wirtspflanze ein, nutzen die dort vorhandenen Nährstoffe und vermehren sich. Bis dato existieren mehrere Methoden, um Nematoden-Befall zu behandeln; jedoch ist keine dieser Methoden ideal, da sie entweder teuer oder eine stark toxische Wirkung auf Mensch und Umwelt haben. Viele chemische Substanzen, die theoretisch zur Behandlung eines Nematodenbefalls geeignet sind, sind umweltgefährdend und haben eine ungenügende Sicherheit. Eine dieser Chemikalien ist Brommethan, welches als Schädlingsbekämpfungsmittel aufgrund seiner neurotoxischen, reproduktionsschädigenden und umweltgefährdenden Eigenschaften 2005 verboten wurde. Als Alternative bieten sich Naturstoffe an. Spezies der Gattung *Allium* werden in der Volksmedizin seit dem Altertum verwendet. Von den mehr als 750 Spezies der Gattung, die es weltweit gibt, wurden nur die wenigsten auf nematozide Eigenschaften untersucht. In dieser Arbeit wurden von einigen ausgewählten Spezies Extrakte der Zwiebel und der Blüte hinsichtlich ihrer nematoziden Wirkungen erforscht. Die *Allium*-Proben wurden in Zentralasien und dem Mittleren Osten wild gesammelt, oder kamen aus der Sammlung des IPK-Gatersleben in Deutschland. Die Zwiebeln und Blüten wurden mittels Ethylacetat extrahiert. Eine Kultur von Nematoden (*Meloidogyne incognita*) wurde durch Infektion von Tomatenpflanzen (*Solanum lycopersicum*) erhalten. Die Ethylacetatextrakte wurden hinsichtlich ihrer Aktivität gegen *Meloidogyne incognita*, als typische Wurzelgallennematode, getestet. Die Extrakte mit der stärksten nematoziden Wirkung wurden daraufhin auf ihre Phytotoxizität sowie ihrer Toxizität gegenüber *Artemia salina* L. getestet. Es wurden die Wirkstoffe der vier Extrakte untersucht die den stärksten nematoziden Effekt erzielten. Dafür wurden die einzelnen

Extrakte fraktioniert und jede dieser Fraktionen auf Aktivität geprüft. Die in diesem Prozess für die nematozide Aktivität verantwortlichen Verbindungen wurden mittels Massenspektrometrie und NMR-Spektroskopie im Vergleich zu Literaturangaben bestimmt. Es zeigte sich, dass in *A. ampeloprasum* Allicin die wirksamste Verbindung darstellt. In *A. stipitatum* wurden zwei Verbindungen mit nematozider Eigenschaft gefunden; dabei handelt es sich um 2-(Methyldithio)pyridin-N-oxid und [(Methylthiomethyl)dithio]pyridin-N-oxid.

1. INTRODUCTION

Food safety and increasing human population is the global challenge in the coming years. For instance, crop yield loss due to nematodes is estimated at a value of 157 billion US dollars globally. Another sum of about US$ 500 million is spent annually on nematodes control [1].

Nematodes are roundworms that occur worldwide in all environments. Based on estimation, they are accepted to be second the most biodiverse species in the world. They are important members of the food chain that benefit agriculture by contributing to decomposition of organic matter. Some species are plant or animal parasites. More than 2,000 of the 20,000 identified nematode species are parasitic to plants. The majority of plant-parasitic nematodes are free-living species that feed ectoparasitically on roots and are so tiny that can be seen only under the microscope [2,3].

1.1. Nematode infection strategy

The most damaging nematodes in the world have a sedentary endoparasitic life style. The two main nematodes in this group are the cyst nematodes (*Heterodera* and *Globodera*) and the root-knot nematodes (*Meloidogyne*) [4].

One of the stages at which sedentary nematodes invade roots is known as second-stage juveniles (J2). The J2 of root-knot nematodes will usually penetrate in the region of elongation close to the meristematic zone. By continuous head rubbing they will weaken the thin walls of epidermal and subepidermal cells, then invade and migrate towards the root tip between cortical cells without damaging them. After destroying meristematic cells in apex of the root they will orient towards the region of root differentiation to reach the vascular cylinder in order to find a competent plant cell for the induction of a multinuclear feeding cell complex [5]. Induction of giant cells in the host root vasculature stops the migration and several progenitor cells are selected around the nematode’s head for giant cell initiation. A cluster of multinuclear giant cells is a result of cell enlargement and repeated rounds of mitosis without cytokinesis.

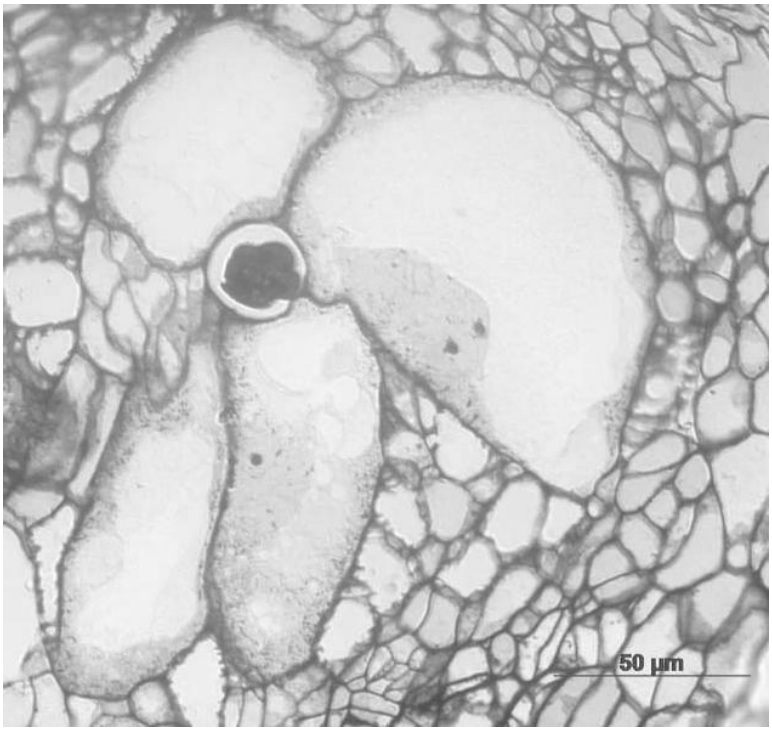

Figure 1.1. *Meloidogyne chitwoodi* feeding site in *Arabidopsis* roots. *Meloidogyne* spp. transform plant cells into giant cells, which make up the nematode's feeding site and serve as sole source of nutrition. Giant cells are multinucleate, show increased metabolic activity, and act as nutrient sinks [6].

1.2. Symptoms of injuries by root-knot nematodes

Symptoms of injuries of root-knot nematodes are similar to the ones caused by excess of fertilizer or root rot. They include poor growth, low vigor, yellowing or bronzing of the foliage, loss of leaves, stem dieback, failure to respond to fertilizer because of root damage and eventually death. The cause of plant decline can be determined by laboratory examination of the soil and some small fibrous roots [7].

Root galls or knots are the most recognizable root symptoms of any plant parasitic nematodes. Visible swellings called galls or knots are formed as a result of root tissue growth and expansion around infection points [8]. Root knots may contain egg laying females. Developed females will release eggs on the root surface in a protective,

gelatinous matrix [9]. Fungi and bacteria which cause root rots, wilt, and other plant diseases often infect nematode-damaged roots earlier and more severely than uninjured roots. Some viruses can also be transmitted by nematodes.

Figure 1.2. Root-knot nematode galls of *Meloidogyne* on carrot. (https://smartsite.ucdavis.edu/access/content/user/00002950/courses/slides/fromCD/1939/30B.GIF).

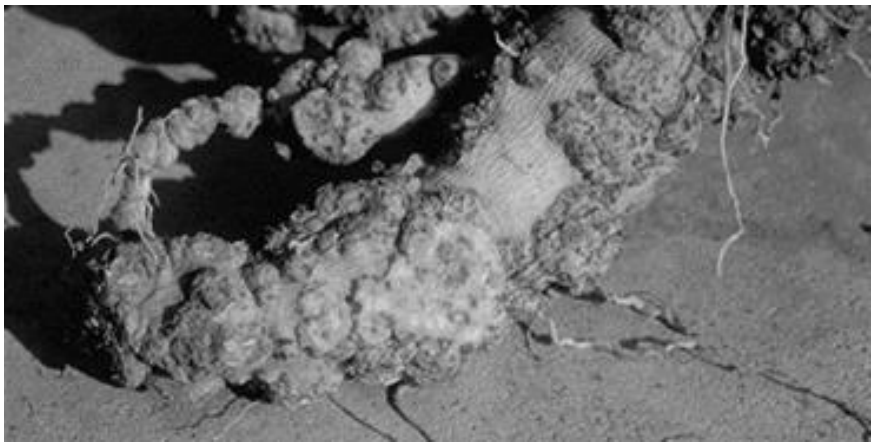

Figure 1.3. Root-knot nematode galls of *Meloidogyne* on dahlia. (https://smartsite.ucdavis.edu/access/content/user/00002950/courses/slides/fromCD/1939/46.GIF).

Figure 1.4. Root-knot nematode galls of *Meloidogyne incognita* on tomato (photo by S. Jivishova).

1.3. Genus: Meloidogyne

Among the cosmopolitan nematode species, *Meloidogyne* spp, root-knot nematodes, are endoparasitic species wide-spread throughout the world and usually have a wide range of host plants, which include crops and potatoes [10]. For developing countries this problem is of great importance [11].

Root-knot nematodes were first officially reported in 1855 by Reverend M. J. Berkeley (clergyman), who observed them causing damage on cucumbers in greenhouse in England [12]. About 20 years later, C. Jobert observed diseased coffee trees in the Province of Rio de Janeiro, Brazil, and found fibrous roots with numerous galls. He mentioned hatching of nematodes from eggs, their escape from the roots to the soil. In 1887, Göldi, investigated same problem and described *Meloidogyne exigua*,

as the cause of the disease and as the type species of a new genus. The name *Meloidogyne* is of Greek origin, meaning "apple-shaped female" [13]. Eventually, Chitwood (1949), re-erected genus *Meloidogyne* and redescribed previously known species as well as described the new species *M. hapla* and a new variety, *M. incognita* var. *acrita* [14].

According to A. Elling (2013), approximately 100 valid species of *Meloidogyne* have been recognized, although the majority of past research focused mainly on small number of species, the so-called ‘major’ species *M. arenaria*, *M. hapla*, *M. incognita*, and *M. javanica* [6].

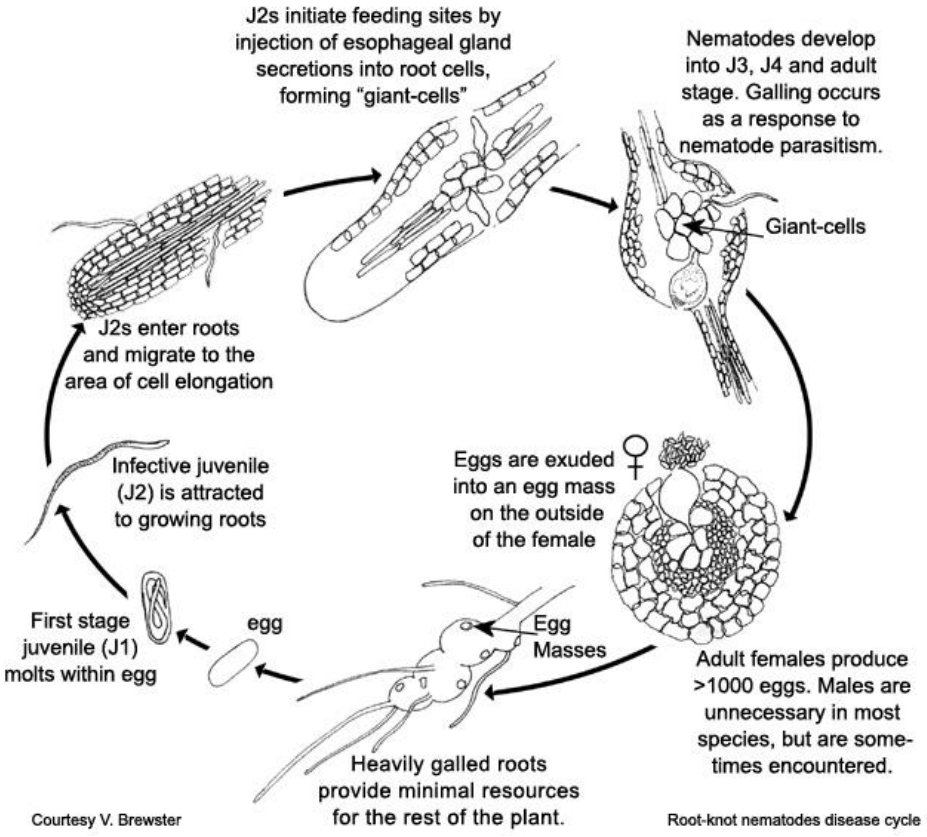

Figure 1.5. Root-knot nematode disease cycle [15].

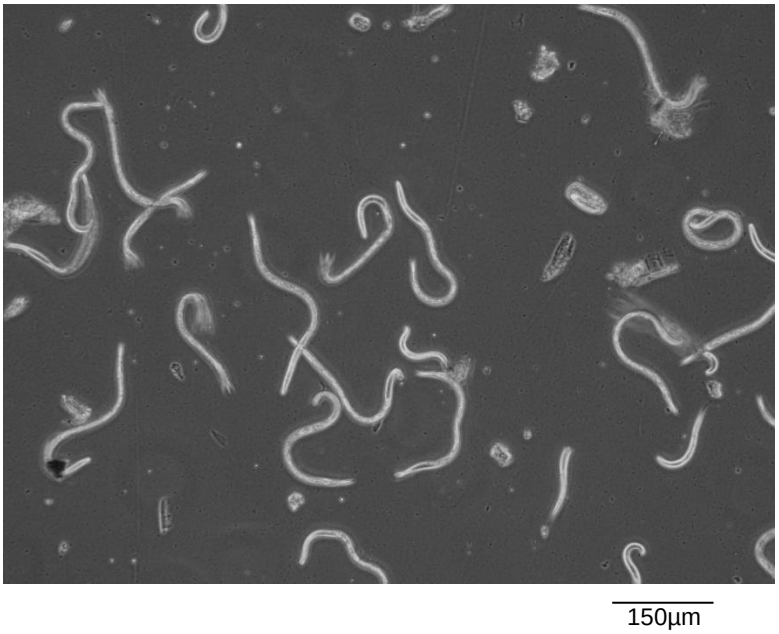

Figure 1.6. Alive and moving *Meloidogyne incognita* nematodes under the microscope (x20, photo by S. Jivishova).

1.4. Nematode control

One of the challenges associated with development of effective chemical controls for the plant-parasitic nematodes is that they spend their lives confined to the soil or within plant roots, delivery of a chemical to the immediate surroundings of a nematode is difficult. Several agronomically important nematicides (e.g., ethylene dibromide and dibromochloropropane) have been banned due to environmental or human health safety hazards [11]. Development of efficient and nonhazardous to human and environmental health nematicides is of great importance.

1.4.1. Cultural controls

The use of cultural control methods to manage root-knot nematodes is the most environmentally sustainable and potentially most successful method for limiting root-knot nematode damage.

Careful planning is required for crop rotation to be effective, since root-knot nematodes have very large host ranges, thus crops unsuitable for nematode infection, growth, or reproduction must be introduced into the rotation sequence [16,17].

Another cultural control strategy is the use of cover crops such as sudangrass and marigolds. They are known for being poor or nonhosts to *M. hapla*, and cover crop would be grown in the same field as the main crop between harvesting and planting. When incorporated as a green manure sudangrass, was effective in reducing reproduction of *M. hapla* and, therefore, its damage to lettuce plants. Marigold was more effective when was actively grown, rather than incorporated in the soil as a manure. It is believed to be due to the living plant root exudates which have nematicidal activity [18–20].

Crop rotation may be preferred for nematode management, as it provides for diversity in time and space; however, rotation may be of limited value when several parasitic species of nematodes or species with broad host ranges are present.

Presence of suitable crops and lands are decision factors for growers to use crop rotation for nematode management [21–23].

While flooding effectively kills soil nematodes, it is cost effective only if it is a natural process. Polyethylene film used in solarization is costly when used for large-scale, but could be economically accessible for use on small areas [24].

It can be suggested that, while cultural control methods are valuable tools, they require certain conditions, careful consideration, planning and economic investment before successful implementation can be achieved.

1.4.2. Biological control

It is possible to utilize antagonists of nematodes as nematode control and management measures. Several bacteria and fungi are known for their predatory activities against nematodes. To maintain a population of antagonistic microorganisms,

addition of organic matter, such as manure, to soil, a practice known since ancient times, is used. Manure treatment will not only provide required nutrients to the plants but also will stimulate bacterial activity, which may be nematicidal [25,26]. Presence of a permanent layer of plant residues on the soil surface is also an important feature in sustainable farming system, as it has high C:N (carbon:nitrogen) ratio which will help to maintain a population of nematode-trapping fungi. Presence of litter layer also is an ideal habitat for mites and hexapods *Collembola*, some of which are predacious on nematodes [27,28].

1.4.3. Chemical control

Development of effective nematicidal fumigants in middle of past century, diminished importance of research on organic amendments as nematode management and control tool [25]. Root-knot nematodes are very difficult to manage because they are soilborne pathogens with a wide host range. Because root-knot nematodes live in the soil, chemical control requires applications of large amounts of chemicals with specialized equipment [15].

The multipurpose soil fumigant, methyl bromide, was banned a decade ago [29], due to potential serious adverse effects to the human and environment health and ozone layer [30].

Utilization of chemical control in developing world is being costly, and in widespread use of compounds, such as Aldicarb – a carbamate, which is one of the most toxic and environmentally hazardous pesticides, involves additional concerns as also being a human neurotoxin [31].

One study, performed in the UK, tested a less hazardous fumigant, 1,3-dichloropropene, in various combinations with other granular nematicides aldicarb (Temik® 10G), fosthiazate (Nemathorin® 10G) and oxamyl (Vydate® 10G) in two field experiments and have demonstrated that they have been effective in reducing potato cyst nematode populations, reducing yield losses and leading to economic yield benefits when compare to the cost of the chemicals [32].

1.5. Phytochemicals as nematicides

Most plant-parasitic nematodes reside in the soil or within plant roots, and this makes work of nematicides hard. For a nematicide to be effective, it needs to penetrate usually thick layer of soil, roots and nematode cuticle, which is impermeable to many organic molecules. Application of nematicides in large amounts would help to overcome these barriers, but this will raise concern of presence of residues in vegetables produced for fresh consumption. Accumulation of the residues in human organism as a result of continuous consumption may lead to adverse health effects or death. Due to serious risks to human health and environment safety European Legislation and US Environmental Protection Agency have deeply restricted the use of pesticides on agricultural crops [33–35].

These concerns and restrictions have led to a quest of alternative control measures for the management of plant parasitic nematodes [36]. Many researchers are trying to find effective natural products to replace synthetic pesticides [37]. Secondary metabolites produced by plants and microorganisms are known to be toxic to agricultural pests for quite long time. This fact and the view that naturally-produced compounds are inherently more toxicologically and environmentally benign than are synthetic chemicals, elevates the interest in utilization of natural products as pesticides [38].

Quite recently a commercial nematicidal product, NEMguard® (ECOSpray), formulated with compounds from garlic, registered for use on carrots and parsnips [39].

1.6. Sulfur chemistry of Allium species

Plants from genus *Allium* have been used by humans since ancient times as food and medicine. Nonvolatile sulfur compounds known as cysteine sulfoxides are being responsible for the characteristic smell and taste of these plants. There are several cysteine sulfoxides known up to date (Figure 1.7). When the plant tissue is disrupted, odorless cysteine sulfoxides, such as (+)-S-(2-propenyl)-L-cysteine sulfoxide (alliin) or

(+)-*S*-(1-propenyl)-L-cysteine sulfoxide (isoalliin) react with an enzyme alliinase and will yield alk(en)yl thiosulfinates [40].

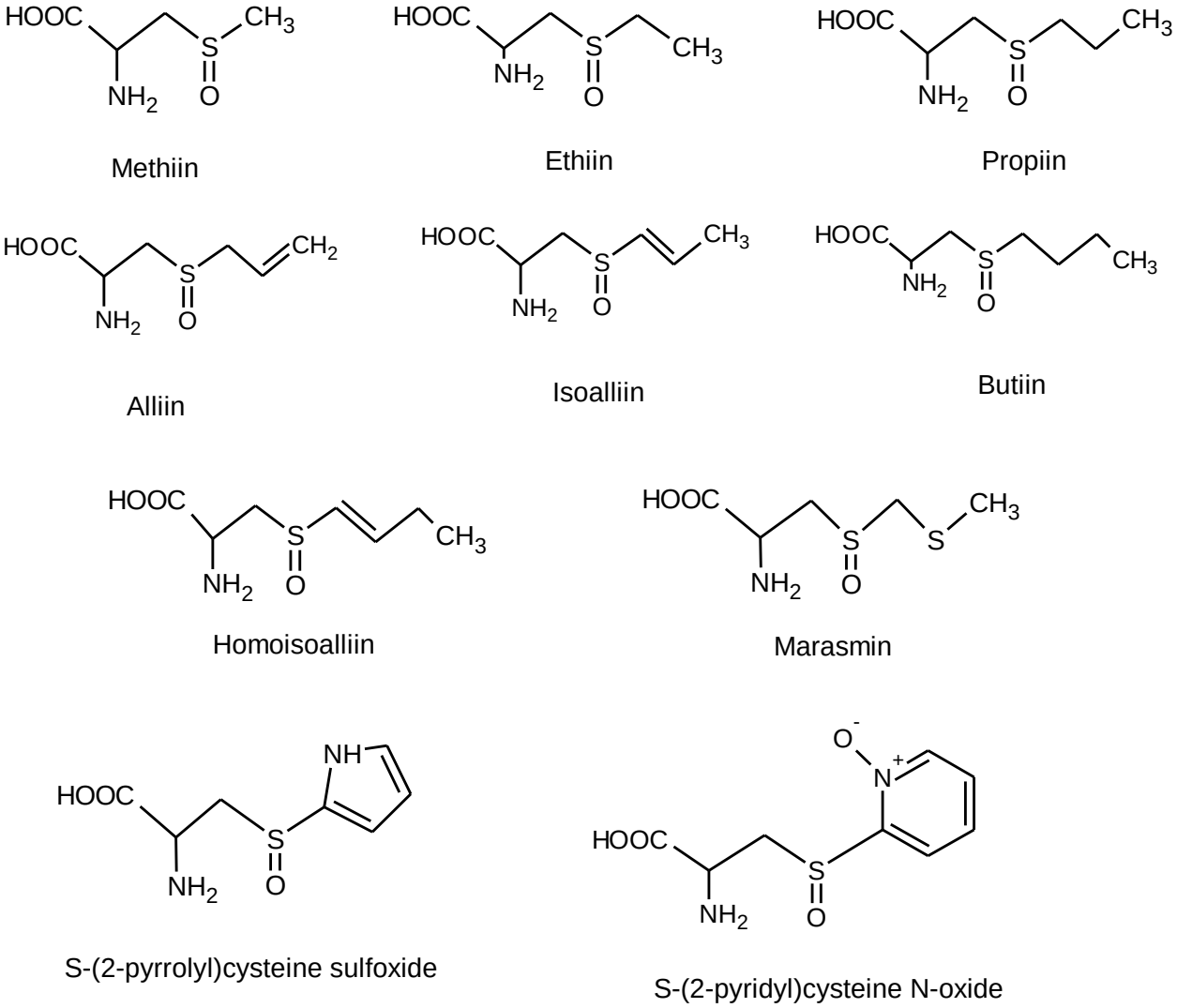

Figure 1.7. Cysteine sulfoxides found in *Allium* plants.

It has been shown that the activity of alliinase may be different in various species and will be selective to cysteine sulfoxides [41,42]. Sulfenic acids are immediate products of cysteine sulfoxide cleavage catalysed by alliinase. In garlic, 2-propenesulfenic acid gives allicin, a thiosulfinate with antibiotic properties, while in onion I-propenesulfenic acid rearranges to the sulfine (2)-propanethial S-oxide, the lachrymatory factor (LF) of onion [43]. Allicin is the compound which gives garlic its

specific odor and the LF is the one which is responsible for teary eyes when the onion is cut.

Onion and garlic, main representatives of genus *Allium*, have been extensively researched for their biological activities. They have been found to bear antibacterial, antifungal, antiviral, preventing atherosclerosis and cardiovascular diseases, anticancer and antimutagenic, anti-hypertensive, antithrombotic, immunomodulatory, etc. effects [44].

While cysteine sulfoxides are non-volatile compounds, the thiosulfinates, the products of their cleavage by alliinase are volatile, give *Allium* plant pungency and related to the biological activities. It is also known that these compounds are unstable at room and higher temperatures and give rise to transformation products [43,45].

In garlic, alliin is a major cysteine sulfoxide, and it a precursor of allicin. Allicin is highly unstable and eventually will transform into ajoene (Figure 1.8). Methiin can be present both in garlic and onion, thus products of methiin cleavage will result in products present in both plants. In onion, alliin is replaced by isoalliin and when cleaved it will yield lachrymatory factor, cepaenes and zwibelanes [46,47] (Figure 1.9).

Several aromatic sulfur compounds isolated from *A. stipitatum* have been reported by O'Donnel in 2009 [48]. Compounds 2-(methyldithio) pyridine-N-oxide and 2-[(methylthiomethyl)dithio] pyridine-N-oxide found to have some cytotoxic effect against cancer cells as well as moderate antibacterial activity.

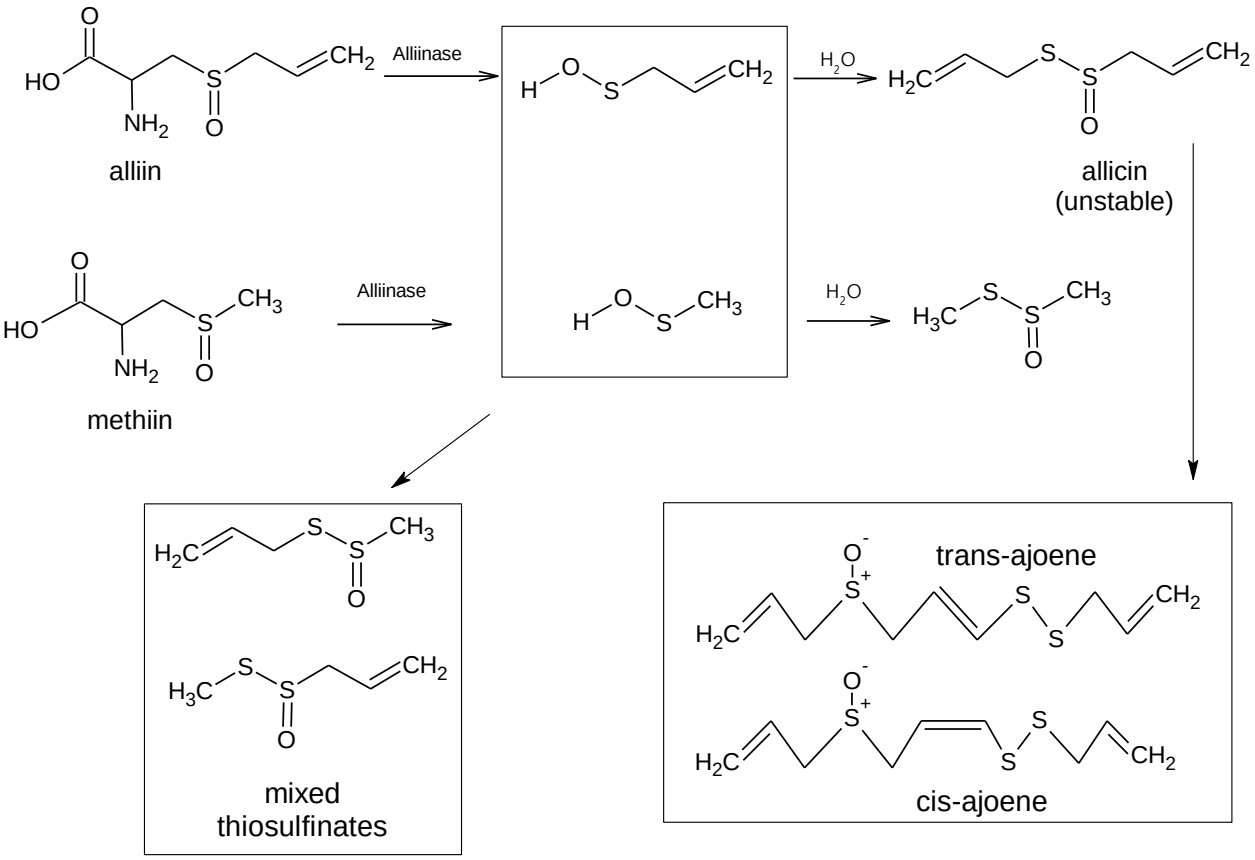

Figure 1.8. Biosynthetic pathway of thiosulfinates in garlic.

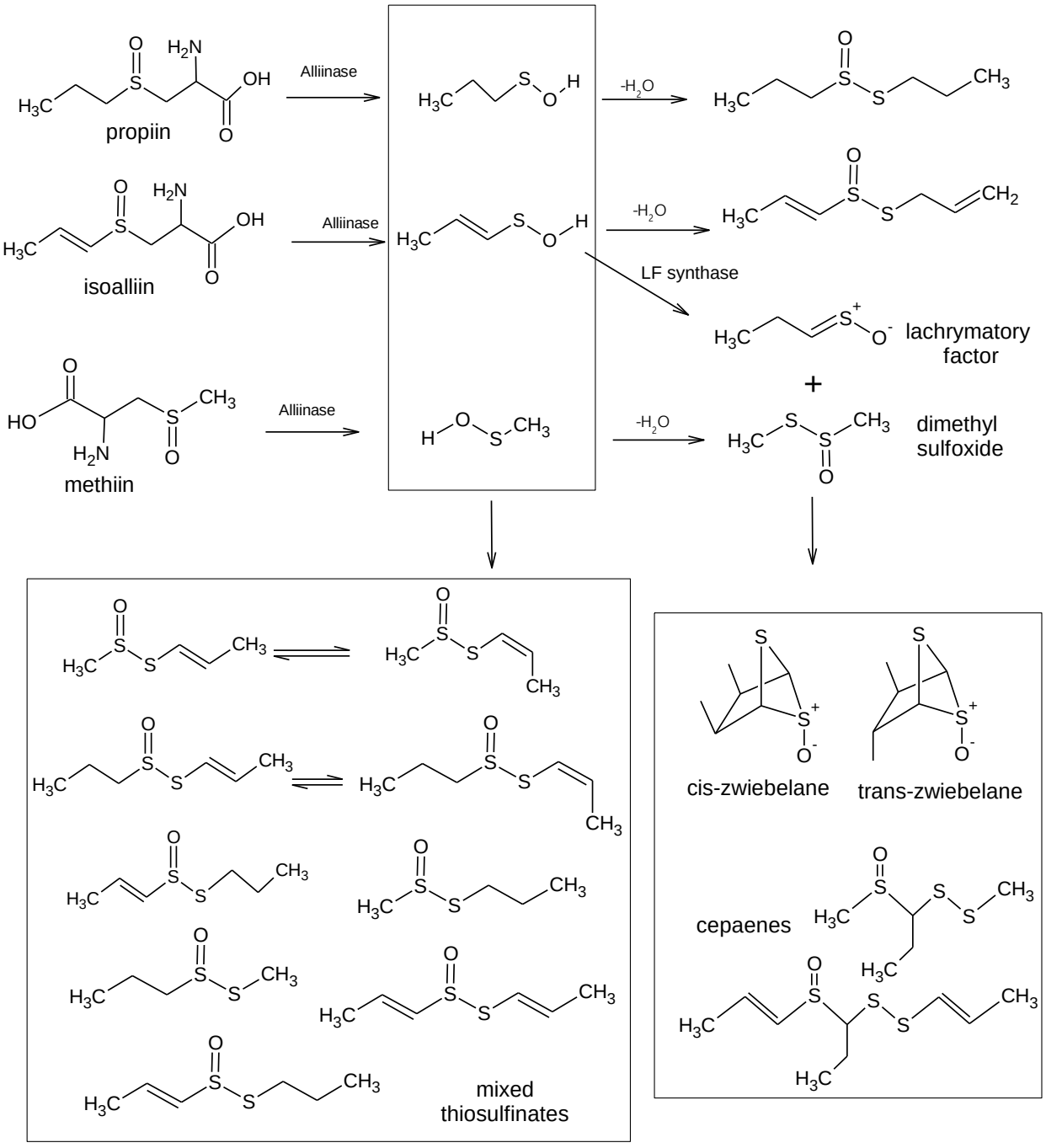

Figure 1.9. Biosynthetic pathway of thiosulfinates in onion.

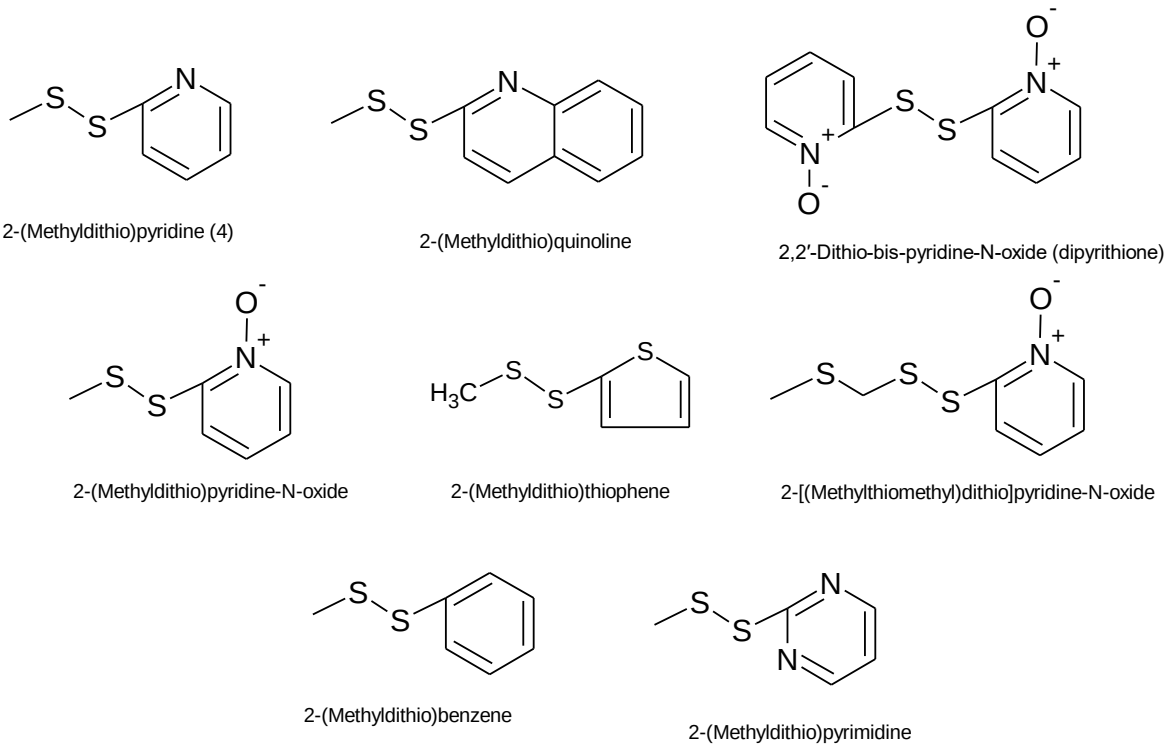

Figure 1.10 Aromatic sulfur compounds isolated from *A. stipitatum* by O'Donnel (2009).

1.7. Phytotoxicity assay

Plant damage due to application of pesticides to plants is known as phytotoxicity [49]. Therefore, even if a pesticide candidate has significant activity against plant pathogens, it is essential to assess its damaging potential it may have on the plants. Phytotoxicity is an important issue and may be of important influence on decision about application of a pesticide, as in case of fumigants (dazomet, 1,3-dichloropropene, and metham sodium or potassium), which are applied several weeks in advance of sowing or transplant due to their toxicity to the plants [50]. United States Environmental Protection Agency (US EPA) defines the phytotoxicity method as a "screening test used to predict the potential impact of chemicals on seed germination and early seedling growth" [51]. Root-knot nematode *M. incognita* is a higher plant pathogen, and a nematicide is intended to be applied to the higher plants, thus, test plants involved in the phytotoxicity method should represent this condition. Tomato (*Solanum lycopersicum*), lettuce (*Lactuca sativa*) and

cress (*Lepidium sativum*) are plant species suggested by OECD, US EPA and US FDA to be used as test subjects for this purpose [51–53].

Under phytotoxicity method, three tests - germination rate, root length (elongation) and shoot length - are employed. The inhibition of root growth is one of the most rapid responses to toxic concentrations of a heavy metal and has been frequently used in many tolerance tests [54]. Authors of a study, which tested phytotoxicity of organic and inorganic toxicants on lettuce, millet and cucumber seeds using root elongation method, suggested that lettuce and millet root elongation tests can be valuable in environmental toxicity tests for organic and inorganic pollutants. Moreover, together, they were recommended for toxicity testing of unknown, complex hazardous substances [52].

Root elongation method utilizing lettuce (*Lactuca sativa* L., cv buttercrunch) was used with two different approaches to determine phytotoxicity of some heavy metals and organic substances [55]. In the study, in first approach the seeds were germinated in dark on an inclined filter paper with one end immersed in a test solution and in the second approach the seeds were germinated directly in an aerated container with a nutrient solution and day-night cycle. Authors of the study claim that the second approach with aeration, nutrient solution and lighting cycle overall offers more sensitivity.

A study by Wang *et.al.* [56], evaluated suitability of germination rate and root elongation of *Cucumis sativus* (cucumber) as an indicator to evaluate the phytotoxicity of phenol, aniline and their 11 halogen substitutes. Authors found that, by utilizing *C. sativus* in the germination rate and root elongation methods during short exposure time it is possible to achieve excellent stability and reproducibility of germination and root growth, regular dose-response relations for all test compounds and comparably high sensitivity.

Macías *et al.*(2000) evaluated 22 commercial varieties of eight plant species [four dicotyledons *L. sativa* L. (lettuce, *Asteraceae*), *Daucus carota* L. (carrot, *Apiaceae*), *Lepidium sativum* L. (cress, *Brassicaceae*), *Lycopersicon esculentum* L. (tomato, *Solanaceae*); and four monocotyledons: *Allium cepa* L. (onion, *Amaryllidaceae*), *Hordeum vulgare* L. (barley, *Poaceae*), *Triticum aestivum* L. (wheat, *Poaceae*), and *Zea*

mays L. (corn, *Poaceae*] as models for weeds and crops during a search for a standard bioassay of phytotoxicity for allelochemicals [57]. In this comprehensive study, nine commercial varieties selected as standard target species were tested with standard commercial herbicides to ensure their sensitivity to phytotoxic compounds. Authors of the study argue that commercial crop seeds have advantages of being more genetically homogeneous, germinating more uniformly, and being readily available, therefore will help to maintain the reproducibility of the bioassay. When determining standard growth conditions with germination levels in the desirable range of 60-80%, while pH was not a determining factor in promoting germination authors suggest pH=6, as generally the optimal pH for plant growth with a possibility to its modification according to stability of tested allelochemicals. Authors claim that pH modifications will not cause significant differences on seed germination. As growth solution volume was found to have strong influence on the growth, authors suggested to consider 0.2 ml/seed for dicotyledons and while suggesting varying volumes for monocotyledons based on the species. The latter study protocol was successfully employed in subsequent experiments [58,59].

1.8. Brine shrimp lethality assay

Brine shrimp are crustaceans that inhabit salty waters around the world, both inland and on the coast [60]. Newly hatched or born live brine shrimp larvae, called a nauplius (plural: nauplii (NAW-plee-eye)). The rate of development through series of 14 or 17 stages of their life cycle (Figure 1.11) is affected by salinity, water temperature, and food availability. Brine shrimp feed on algae [61,62].

Pesticides also may exhibit toxicity to living organisms; therefore, it is crucial to evaluate level of the toxicity of pesticides. Due to issues related to constant maintenance, breeding and rearing of culture organisms for previously present bioassays, Michael *et.al.*, instituted a screening of test organisms in order to resolve related problems [63]. In the study, a brine shrimp, *Artemia salina*, was used as one of test organisms to evaluate toxicity of several insecticides. Authors claim that *A. salina* was superior to others for this purpose.

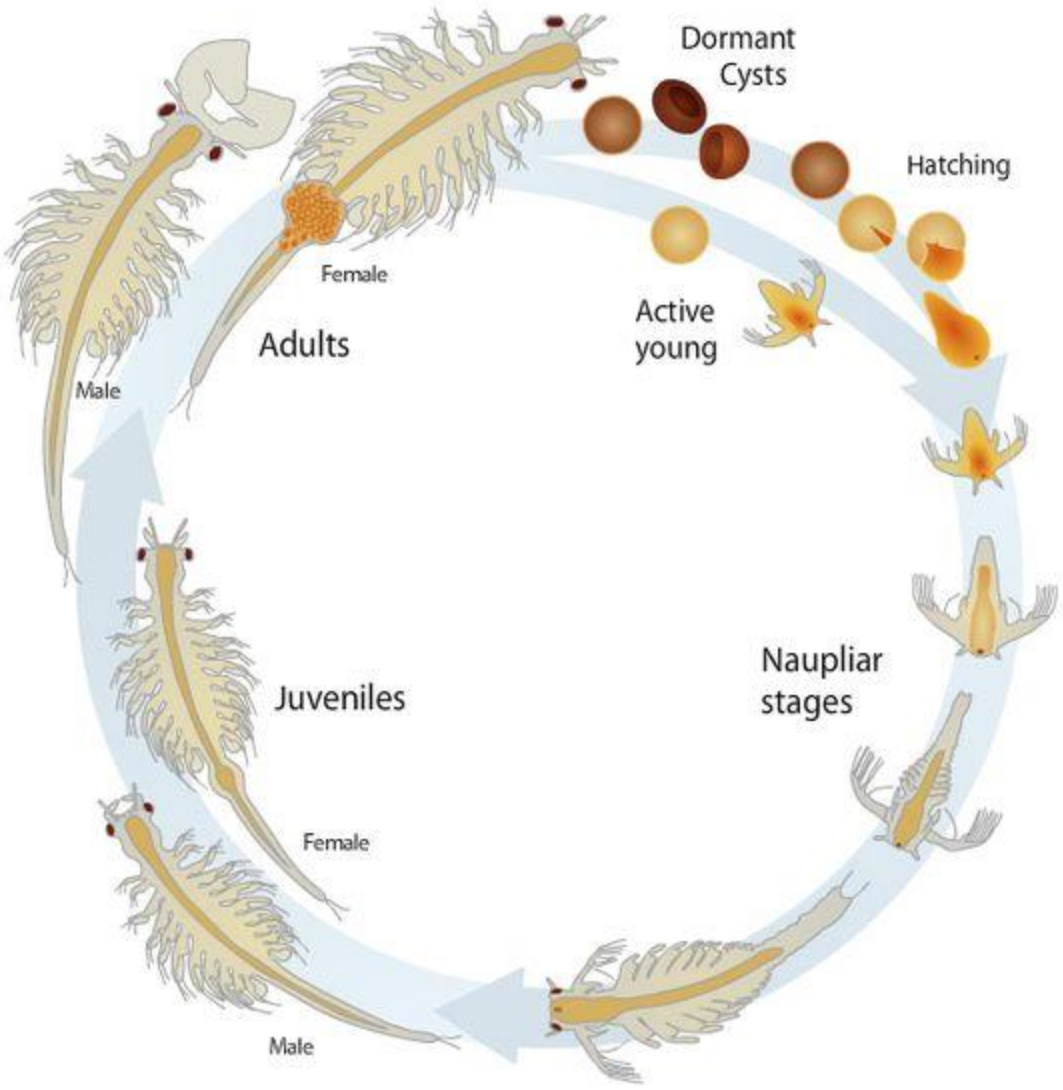

Figure 1.11. Brine shrimp life cycle [61]

Eventually, brine shrimp has gained popularity as a test organism and has been used in numerous assays for evaluation of toxicity of copper and mercury [64–66], sewage and industrial wastes effluents [67], fungal toxins [68], oil dispersant compounds [69], and biodegradability of synthetic organic compounds [70]. Amidst rising popularity of the brine shrimp bioassay, Sorgeloos et. al. [71] and Vanhaecke et.al [72] provided critical analysis and evaluation of the assay and proposed series of standard criteria to ensure validity and reproducibility of the brine shrimp toxicity method. Different variations of the assay were also proposed later, such as microplate assay for cytotoxicity [73] and using

different species of *Artemia* [74], as well as utilization in testing toxicity of pesticides [75], cyanobacteria toxins [76] and dental materials [77]. Carballo *et al.* [78] compared two brine shrimps assays used in evaluation bioactivity of extracts of marine invertebrates and macroalgae. Moreover, to assess the sensitivity of both shrimp assays to detect cytotoxic activity, same extracts were also assayed for cytotoxicity against human lung carcinoma and colon carcinoma cell lines.

Brine shrimp toxicity assay has been used in assessment of botanicals as well. Authors of the first study to test plant metabolites using *A. salina* bioassay [79], suggested to consider principally utilizing the bioassay to monitor fractionation when screening for cytotoxic plant metabolites. The authors described brine shrimp bioassay as rapid (24 hours following introduction of shrimp), inexpensive, not requiring aseptic conditions, special equipment or training, which makes it simple to set up. Easy utilization of large number of continuously available organisms provides ample data for statistical purposes.

In another study, authors claim to adopt main four “bench top” bioassays to aid “drug discovery” work with plant metabolites, with brine shrimp bioassay being one of the four [80]. With reference to their 15-year utilization of the bioassays, authors claim the bioassays to be adaptable to the purpose of standardization or quality control of bioactive components in such heterogeneous botanicals.

1.9. Objectives of this investigation

1.9.1. Screening ethyl acetate extracts of Allium species for nematicidal activity

Utilization of phytochemicals in agriculture has great potential [81]. Considering that genus *Allium* L. has more than 700 species, it is possible to think of this genus as a chest of compounds with possible bioactivities. The most common species of genus *Allium* are garlic and common onion. In Middle Asia, *Allium* species of the subgenus *Melanocrommyum*, with more than 200 species known, are used extensively in food and folk medicine [82].

There have been several nematicidal studies involving a few *Allium* species, such as *A. grayi* and *A. fistulosum var. caespitosum* [83], *A. sativum* [84,85], *A. cepa* [86–88], *A. tuberosum* [89]. These all are promising facts that species of genus *Allium* may bear potential of nematicidal activity.

In our present study 69 ethyl acetate extracts of different *Allium* species, including bulbs and flowers, were screened for nematicidal activity against root-knot nematodes *M. incognita*. To provide continuous supply of nematodes, a local culture of the nematodes was maintained by raising tomato plants and infesting their roots with J2 stage nematodes.

1.9.2. Screening of Allium extracts for phytotoxicity and environmental safety

Allium extracts, which exhibited strongest nematicidal activity, were evaluated for their phytotoxicity and environmental safety. To determine phytotoxicity of the extracts, tomato, lettuce and cress seeds were used as indicators. Environmental impact of same extracts was also evaluated using brine shrimp lethality assay.

1.9.3. Bioactivity guided fractionation of nematicidal Allium species

Promising candidates were subjected to bioactivity guided fractionation using preparative HPLC. Structures of compounds in isolated fractions were determined using HR-MS and 1H NMR techniques. HPLC chromatograms of known compound were also compared to the ones of isolated compounds.

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 Emil Jivishov with title “Investigations on Wild Allium Species. Part I: Cysteine Sulfoxides of Flowers. Part 2: Anticancer Activity of Bulb Extracts”. The list of same chromatograms is as follows: “Preparative HPLC Chromatogram of 7002 *A. stipitatum*”, “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* -(2-(methyldithio)pyridine N-oxide)", "NMR Chromatogram of fraction 4 from 7002 *A. stipitatum* -(2-[(methylthio)methyldithio]pyridine N-oxide)". At the time of writing, the thesis of Emil Jivishov is not published, therefore reference above is provided. Any other identical chromatographic and spectroscopic data found in this thesis and the thesis of Emil Jivishov is requested to be considered as a result of mutual work and agreement and not as a plagiarism.

2. MATERIALS AND METHODS

2.1. Chemicals

Table 2.1. Chemicals used for nematicidal assay.

Chemicals	Source
Lannate 20 L	DuPont (France)
Phosphate buffer saline tablets (PBS)	Sigma Aldrich
Ethyl acetate puriss. p.a.	Sigma Aldrich
Magnesium sulphate, anhydrous	Sigma Aldrich
Sodium hypochlorite (10%)	Sigma Aldrich
Triton X-100	Sigma Aldrich
Sodium chloride	Fluka
Aldrithiol-2	Sigma Aldrich
Dipyrithione	Sigma Aldrich

Table 2.2. Chemicals used for phytotoxicity assay.

Chemicals	Source
Dimethyl sulfoxide (DMSO), ≥99,5 %	Sigma Aldrich
MES hydrate, BioPerformance Certified, suitable for cell culture, ≥99.5%	Sigma Aldrich
Sodium chloride	Sigma Aldrich
Aldrithiol-2	Sigma Aldrich
Methanol analytical reagent grade	Sigma Aldrich
Lannate 20 L	DuPont (France)
Triton X-100	Sigma Aldrich

Table 2.3. Chemicals used for brine shrimp lethality assay.

Chemicals	Source
Dimethyl sulfoxide (DMSO)	Fluka
Methanol puriss. p.a.	Sigma Aldrich
Lannate 20 L	DuPont (France)
Aldrithiol-2	Sigma Aldrich
Triton X-100	Sigma Aldrich

2.2. Plant material

Plant material was obtained from the living plant collection at IPK Gatersleben, Germany, unless otherwise specified, in years 2013 and 2014. *Allium stipitatum* (Iran) was bought from local grocery store (sliced and dried) from Iran. *A. sativum* (garlic) was bought from local grocery store in Marburg, Germany and was grown in Germany. *A. stipitatum* (7002) bulbs and *A. turcomanicum* (7014) flowers were collected from Afghanistan by Prof. Dr. M. Keusgen during expedition in 2013.

Table 2.4. List of 69 *Allium* plants and their parts used to prepare EtOAc extracts.

Tax / Acc No	**Plant**	**Part**	**Fresh weight (g)**	**Extract Weight (mg)**
1025	*A. ampeloprasum* L.	bulb	10.9841	8.5
5313	*A. paniculatum* L.	bulb	10.3044	4.1
0985	*A. paniculatum* L. subsp. fuscum (Waldst. & Kit.) Arcang	bulb	10.3847	5.4
2996	*A. platyspathum* Schrenk	bulb	10.2037	9.3
7002	*A. stipitatum* Regel	bulb	9.4665	6.8
0703	*A. moly* L.	bulb	10.5616	5.5
5263	*A. stipitatum* Regel	bulb	12.8205	4.6

5301	*A. rupestre* Steven	bulb	8.244	7.6
5475-IPK 2013	*A. stipitatum* Regel	bulb	11.6656	5.6
1732	*A. rupestre* Steven	bulb	10.4237	6.5
2023	*A. canadense* L.	bulb	9.1481	4.8
3513	*A. angulosum* L.	bulb	10.1319	10.0
2584	*A. douglasii* Hook.	bulb	9.438	6.0
2390	*A. pallens* L.	bulb	7.0536	4.4
2672	*A. maximowiczii* Regel	bulb	10.2297	13.7
1513	*A. lusitanicum* Lam.	bulb	10.2472	34.3
0469	*A. flavum* L.	bulb	8.008	4.8
3193	*A. rubens* Schrad. ex Willd.	bulb	10.2452	9.8
0192	*A. schoenoprasum* L.	bulb	10.2658	7.3
3061	*A. campanulatum* S. Watson	bulb	10. 5071	6.6
3470	*A. denudatum* F. Delaroche	bulb	10.4315	14.4
3187	*A. obliquum* L.	bulb	11.4696	30.3
0515	*A. multibulbosum* Jacq.	bulb	10.361	6.1
5335	*A. sibthorpianum* Schult. & Schult.f.	bulb	10.2035	5.7
5295	*A. rotundum* L.	bulb	10.1024	8.5
0126	*A. rotundum* L.	bulb	9.0125	4.0
6158	*A. rosenorum* R. M. Fritsch	bulb	10.0618	5.7
3183	*A. strictum* Schrad.	bulb	10.8319	7.0
1482	*A. tuberosum* Rottler ex Spreng.	bulb	10.8508	6.6
3548	*A. senescens* L.	bulb	10.3763	9.8
5410	*A. ramosum* L.	bulb	10.1525	4.1
1017	*A. atropurpureum* Waldst&Kit	bulb	10.5692	5.4
5011 – Kyrgyzstan (2013)	*A. fedschenkoanum* Regel	bulb	7.0334	6.6
5015 – Kyrgyzstan (2013)	*A. oreoprasum* Schrenk (river)	bulb	10.5368	5.2
5021 – Kyrgyzstan (2013)	*A. oreoprasum* Schrenk (valley)	bulb	10.314	5.6
5022 – Kyrgyzstan (2013)	*A. oreoprasum* Schrenk	bulb	7.2164	5.9
5023 – Kyrgyzstan (2013)	*A. talassicum* Regel	bulb	10.4884	11.7
6178	*A. pskemense* B. Fedtsch.	bulb	15.1228	21.6
3558	*A. victorialis* L.	bulb	10.2771	4.0
5193	*A. cornutum* Clementi	bulb	9.9377	32.7
5738	*A. stellerianum* Willd.	bulb	10.1624	9.0
1642	*A. spirale* Willd.	bulb	10.1246	11.4
10001 – Finland (2014)	*A. schoenoprasum* L.	leaves	7.615	4.6

10001 – Finland (2014)	*A. schoenoprasum* L.	bulb	5.911	3.4
3200	*A. altaicum* Pall.	bulb	10.7233	21.5
3208	*A. nutans* L.	bulb	10.29	20.1
5309	*A. rotundum subsp. rotundum*	bulb	10.0806	7.3
5316	*A. pictistamineum* O. Schwarz	bulb	10.3633	5.4
0068	*A. cernuum* Roth	bulb	10.4658	8.3
2800	*A. hollandicum* R.M. Fritsch	bulb	9.2186	3.0
0070	*A. vineale* L.	bulb	4.1418	2.6
2256	*A. rosenorum* R. M. Fritsch	bulb	9.6116	4.8
0779	*A. karataviense* Regel	bulb	6.7739	6.3
0564	*A. cernuum* Roth	bulb	5.8696	22.3
3954	*A. umbilicatum* Boiss.	bulb	5.9754	4.9
1178	*A. aflatunense* Regel	bulb	5.2183	3.2
3246	*A. stipitatum* Regel	bulb	5.9745	5.4
IRAN	*A. stipitatum* Regel	bulb	11.1317	17.6
2802	*A. hollandicum* R.M. Fritsch	bulb	5.4916	6.8
0882	*A. oreophilum* C.A. Mey.	bulb	5.1827	5.5
1653	*A. zebdanense* Boiss. & Noë	bulb	5.1103	8.2
5372	*A. sphaerocephalon* L.	bulb	4.7818	7.7
Tegut	*A. sativum* L.	bulb	14.157	9.9
3246	*A. stipitatum* Regel	flowers	9.6007	15.7
1631	*A. hollandicum* R.M. Fritsch	flowers	5.6207	9.3
7014	*A. turcomanicum* Regel	flowers	9.1159	21.6
5451	*A. nevskianum* Vved. ex Wendelbo	flowers	6.121	9.9
1222	*A. jesdianum* Boiss. & Buhse subsp*. angustitepalum* (Wendelbo) F.O. Khass. & R.M. Fritsch	flowers	5.477	6.0
2218	*A. macleanii* Baker	flowers	5.3723	13.6

* All plants are from IPK (2014) unless otherwise indicated.

2.3. Nematodes

Tomato roots infested with *Meloidogyne incognita* (Kofoid & White) Chitwood nematodes were provided by Julius Kühn Institute (Münster, Germany). To extract nematode eggs, firstly, roots were thoroughly washed to get rid of soil. Roots were cut into small pieces and put in a jar. Enough NaOCl (1%) solution was added to cover the root pieces. The jar was shaken vigorously to ensure dissolution of gelatinous membrane surrounding eggs (Figure 2.1).

Figure 2.1. Extraction of tomato roots (Photo by S. Jivishova).

Sieves of 400 mesh, 100 mesh and 25 µm aperture were stacked on top of each other in order of decreasing pore size. The mixture was transferred to the top sieve and each of the sieves was rinsed thoroughly with water until bleach smell was gone (Figure 2.2). The *M. incognita* eggs were collected from bottom sieve and transferred into a glass beaker (Figure 2.3).

Figure 2.2. Washing cut root pieces with nematode galls to remove bleach (Photo by S. Jivishova).

Figure 2.3. Collecting nematode eggs from the sieves (Photo by S. Jivishova).

Clean basket wires with Kimwipe paper layers were placed atop of Petri dishes. Nematode eggs were transferred from beaker by pouring carefully over the basket wires with paper. Enough water was added in the Petri dishes, so that the bottom of the wire basket touched the surface of the water. The lid was put atop of the Petri dishes to prevent evaporation (Figure 2.4). Water content was checked daily and was added

based on the need. Petri dishes were covered with aluminium folio to provide natural conditions of darkness. Second day juveniles were collected every other day in a beaker. Part of the juveniles was used to infest and maintain a local stock of tomato plants.

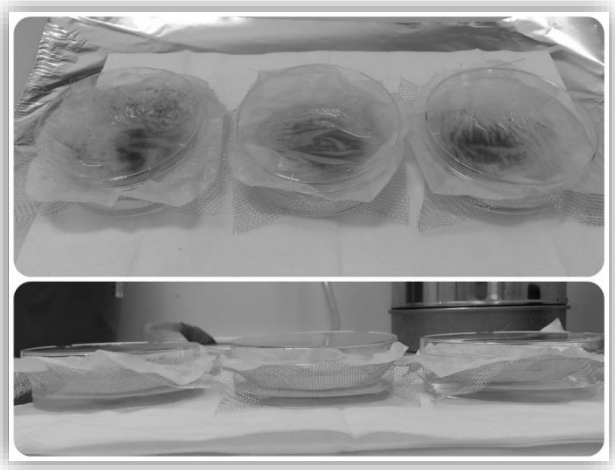

Figure 2.4. Hatching J2 stage juveniles in Petri dishes (Photo by S. Jivishova).

2.4. Preparation of Allium extracts

Around 5-10 g of plant material (flowers and bulbs) were weighed and crushed in mortar containing sea sand. Enough phosphate buffer saline of pH 7.4 (PBS) was added in mortar to provide optimum conditions for alliinase enzyme reaction. Mixture was stored for 1 hour to complete transformation of cysteine sulfoxides into volatile sulfur compounds. After 1 hour, the contents of mortar were transferred into a flask and subjected to liquid-liquid extraction 4 times with total of 500 ml of ethyl acetate as follows. Initially, 200 ml of ethyl acetate were added to the material. After 15 minutes of extraction by carefully rotating the flask, the organic phase was separated and transferred into another flask. Same procedures were repeated three times more using 100 ml of ethyl acetate each time and the organic phases were combined together. Combined organic phase was dried over enough magnesium sulfate and filtered through filter paper to get rid of particles. Ethyl acetate was evaporated from the extract under reduced pressure until about 60 ml of the extract was left in the flask. Water bath temperature of the rotary evaporator was kept around 27 °C. The final residue was

transferred into 20 ml glass vials and carefully evaporated further under nitrogen gas until preferably oily residue left. Vials with extracts were stored at -20 °C until application in assays.

2.5. In vitro nematicidal assay

Allium extracts were dissolved in 1% aqueous Triton-X. Extracts were diluted in 2-fold series with maximum concentration of 2.5 mg/ml in first well with a total of 8 doses. Final concentrations in wells were 2.500 mg/ml, 1.2500 mg/ml, 0.6250 mg/ml, 0.3125 mg/ml, 0.1563 mg/ml, 0.0781 mg/ml, 0.0391 mg/ml, 0.0195 mg/ml and final volume in wells was 200 µl.

Lannate 20 L (Dupont) was used as a positive control with maximum concentration of 0.05% in first well. Serial two-fold dilutions were made to obtain 8 concentrations. About 30 of J2 nematodes were added in each plate well. All treatments were replicated 3 times. Mortality was determined by counting nematodes after 48 h under a microscope (Figure 2.5). Lowest concentration of *Allium* extracts which killed 50% and 100% of nematode population in the nematicidal screening assay were calculated as their respective LC50 and LC100 values using GraphPad Prism (v6) software.

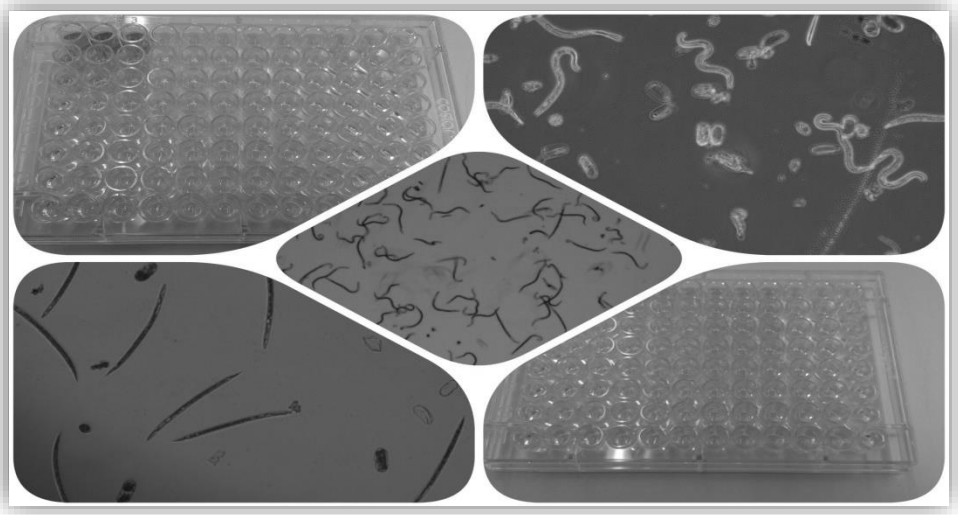

Figure 2.5. Microwell plate based nematicidal assay (view under the microscope) (Photo by S. Jivishova).

2.6. In vitro phytotoxicity assay

Phytotoxicity assay was performed based on process described by Imatomi *et al*. [59]. For this assay, cress (*Lepidium sativum*, *"*Gartenkresse Einfache"), tomato (*Solanum lycopersicum,* Stabtomate "Moneymaker") and lettuce (*Lactuca sativa,* Kopfsalat "Victoria") seeds were used. The seeds were purchased from local store (OBI, Marburg, Germany; producer company: "N.L. Chrestensen", Erfurt, Germany) and sterilized using 10% sodium chloride solution (Figure 2.6).

Figure 2.6. Sterilization of testing material and preparing phytotoxicity test (Photo by S. Jivishova).

Lowest concentration of *Allium* extracts which killed 100% of nematodes in the nematicidal screening assay were calculated as their respective LC100 values. The stock *Allium* extracts were diluted to obtain solutions with concentrations of their respective LC100 values. For this, *Allium* extracts first were dissolved in 1% dimethyl sulfoxide (DMSO) and then diluted with a mixture of 10 millimolar 2-(N-morpholino)-ethanesulfonic acid and 1 millimolar of sodium hydroxide (MES buffer solution, pH =

5.6). Lannate 20 L was used as a positive control. Filter papers were placed inside Petri dishes (3/plant/extract) and were moistened with test material solution. Ten seeds of the test plants were placed in each of Petri dishes. Three replicates were allocated for each seed-test subject combination. Lids were placed on top of the dishes to prevent evaporation (Figure 2.7). Toxicity was calculated after 4 days for cress and after 5 days for tomato and lettuce. Room temperature maintained at 23-25 ºC.

Figure 2.7. Tested material in Petri dishes (Photo by S. Jivishova).

Tested Materials Used in Phytotoxicity Assay

- *Allium* extracts in DMSO+MES Buffer (LC100 concentration)
- Aldrithiol-2 in MeOH+MES Buffer (LC100 concentration)
- MES Buffer
- Lannate + MES Buffer (LC100 concentration)
- MeOH + MES Buffer (0.01% positive control for Aldrithiol)
- DMSO + MES Buffer (0.1% positive control for *Allium* extracts)
- Triton X + MES Buffer (0.25%)

2.7. Brine shrimp lethality assay

To determine cytotoxicity of test materials brine shrimp lethality assay was used. Dried eggs of brine shrimp (*Artemia salina)* were purchased from local store (OBI, Marburg, Germany). To hatch, 10 grams of eggs of *Artemia salina* were placed in artificial seawater (35 grams of sodium chloride dissolved in 1 liter of tap water) and were kept for 48 hours under constant light source and water aeration (Figure 2.8).

Figure 2.8. Hatching nauplii of *Artemia salina* (Photo by S. Jivishova).

About 15-20 of hatched larvae (nauplii) were transferred into each well of a multiwell plate in triplicates. Extracts were dissolved in 1%DMSO+seawater mixture and added into wells to obtain concentration of their respective LC100 values. Lannate 20 L, dissolved in seawater was used as a positive control at final concentration of its respective LC100 value (Figure 2.8). Brine shrimp lethality was calculated after 24 hours. Brine shrimps were counted as dead when they did not move and precipitated to the bottom of the well.

Figure 2.8. Brine shrimps in microwell plates (view under the microscope. Photo by S. Jivishova).

2.7.1. Tested materials used in brine shrimp lethality assay

- *Allium* extracts in DMSO+Brine (LC100 concentrations)
- Aldrithiol-2 in MeOH+Brine (LC100 concentration)
- Lannate + Brine (LC100 concentration)
- MeOH + Brine (0.01% positive control for Aldrithiol)
- DMSO + Brine (0.4% positive control for *Allium* extracts)
- Triton X + Brine (0.25%)

2.8. HPLC-MS

2.8.1. Preparative HPLC of A. stipitatum, A. ampeloprasum, A. paniculatum, A. platyspathum

30 grams of bulbs of *A. stipitatum, A. ampeloprasum, A. paniculatum*, *A. platyspathum* were extracted with ethyl acetate (1000 mL) using procedure described above. Prior to injection, the extract of *A. stipitatum* was dissolved in 40% methanol solution and extracts of *A. ampeloprasum, A. paniculatum* and *A. platyspathum* were

dissolved in 45% methanol solution to obtain final extract concentration of 90 mg/ml. The resulting extract solution was first filtered through 0.45 µm pore size syringe filter following with 0.2 µm pore size syringe filter. 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 program in Table 2.5 was used for *A. stipitatum* and gradient program in Table 2.6 was used for *A. ampeloprasum, A. paniculatum, A. platyspathum*.

Initial number of collected fractions for *A. ampeloprasum* was five, for *A. stipitatum* was six, for *A. paniculatum* was five and for *A. platyspathum* was five. To ensure extraction of the sulfur compounds from methanol-water azeotrope and to prevent mixing it with ethyl acetate, a salting out method was applied. For this, saturated sodium chloride solution (NaCl solution) was prepared. For about 50 ml of collected fraction about 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 evaporated under reduced pressure until near dryness with further evaporation using N_2 gas. The residues were stored at -20 ºC until further processing.

Table 2.5. The gradient preparative HPLC program used to isolate fractions of *A. stipitatum*.

Time (min)	Flow rate (mL/min)	MeOH %	H_2O %
0	7.0	45	55
2.00	7.0	45	55
30.00	7.0	95	5

Table 2.6. The gradient preparative HPLC program used to isolate fractions of *A. ampeloprasum, A. paniculatum*, and *A. platyspathum.*

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

2.8.2. Nematicidal assay of preparative HPLC fractions from Allium species

Fractions of *Allium* plants were dissolved in 1% aqueous Triton-X and diluted in two-fold series with maximum concentration of 2.5 mg/ml in first well with total of 8 concentrations. Lannate 20 L was used as a positive control with initial concentration of 0.05% and diluted in two-fold series to obtain 8 concentrations. About 30 of J2 nematodes were added in each plate well. All treatments were replicated 3 times. Mortality was determined by counting dead nematodes after 48 h using microscope.

2.8.3. High resolution mass spectroscopy (HR-MS) analysis of nematicidal fractions of A. stipitatum and A. ampeloprasum

Two fractions of *A. ampeloprasum* and two fractions of *A. stipitatum*, which were detected to be active against the nematodes, were analyzed by means of HR-MS (micrOTOF-Q III, Bruker, USA). The acquisition parameters for analysis are provided in the Table 2.7.

Table 2.7. Acquisition parameters for HR-MS analysis

Source Type	ESI
Focus	Not active
Scan Begin	50 *m/z*
Scan End	1500 *m/z*
Ion Polarity	Positive
Capillary	4500 V
End Plate Offset	-500 V
Collision Cell RF	180.0 Vpp
Nebulizer	2.5 Bar
Dry Heater	200 °C
Dry Gas	60 l/min

2.8.4. Nuclear magnetic resonance (NMR) spectroscopic analysis of nematicidal fractions of A. stipitatum and A. ampeloprasum.

In order to determine their structures, two nematicidal fractions of *A. ampeloprasum* and two nematicidal fractions of *A. stipitatum* were analyzed by means of ^{1}H-NMR spectroscopy (JEOL-ECA 500 NMR-Spectrometer, Jeol, Tokyo, Japan).

2.9. Planting tomatoes

Tomato plants were used in experiments to maintain nematode culture. Tomato seeds (“Moneymaker”) were purchased from local OBI store in Marburg, Germany. Seeds were planted in paper containers with soil (Compo Sana® Anzucht- und Kräutererde, OBI, Marburg, Germany) and watered regularly (Figure 2.9).

Figure 2.9. Tomato seedlings in paper containers (Photo by S. Jivishova).

After seedlings have germinated they were transplanted into bigger containers filled with good quality, well moistened potting mix (Figure 2.10).

Figure 2.10. Tomato plants in pots (Photo by S. Jivishova).

When tomato plants were 16-20 weeks old, the *M. incognita* nematodes at J2 stage were transferred into roots through the holes opened in the soil close to the stems.

Figure 2.11. Tomato plants with roots that are ready for extraction nematodes.

The plants were grown using 12h day-night regime. Fluorescent light with UV output and color temperature of 6000K was used to promote the growth of the tomato plants (Figure 2.11). Plants were taken care for 12 weeks to ensure development of nematode galls on the roots (Figure 2.12).

Figure 2.12. Tomato root with nematode galls on it (Photo by S. Jivishova).

3. RESULTS

3.1. Screening of Allium extracts for nematicidal effect

Being a plant pathogen, a root-knot nematode *Meloidogyne incognita* is responsible for serious crop damage worldwide. Pesticides used to fight the pest may pose serious risks to the environment, animal and human health. Therefore, it is crucial to discover new “green” nematicidal solutions. Recently, a UK company, ECOSpray, presented a pesticide NEMguard®, based on refined garlic (*Allium sativum*) extract. *Allium* species are rich in cysteine sulfoxides which are converted into volatile sulfur compounds when their cells are damaged. Formed volatile sulfur compounds previously reported to have biological and pesticidal activity and related information presented in the Introduction section of this work. Up to date, only a few species have been evaluated for their nematicidal activity. Considering the garlic based commercial nematicide and insufficient research regarding nematicidal potential of the genus *Allium*, it was decided to screen for and evaluate nematicidal, phytotoxic and brine shrimp lethality activity of *Allium* species, which were not studied before. The target nematode species was root-knot plant pathogen *M. incognita* cultivated in tomato plant roots grown in-house for this purpose.

For this purpose, initially, bulb or flower of an *Allium* species was ground in a pestle and left intact for 1 hour to promote synthesis of volatile sulfur compounds from cysteine sulfoxides. Afterwards, the mixture was subjected to liquid-liquid extraction with portions of ethyl acetate. Organic phases were collected, dried over magnesium sulfate and evaporated under reduced pressure until some oily residue left. After further careful evaporation with a nitrogen gas, the extracts were stored at -20 ºC for further utilization. Nematode eggs were extracted from infested roots of tomato plants and left for hatching. Every other day nematodes hatched from the eggs were collected and used in nematicidal assay to be treated with *Allium* extracts. After 48 hours of treatment with *Allium* extracts, the nematodes were counted using a microscope. Concentration at which 50% of the nematode population was dead (LC50) was calculated for each tested subject. Out of 69 test subjects screened in this study (Table 1), 9 had nematicidal activity with LC50<0.5mg/ml and were classified as “highly effective”. Test subjects with

LC50 values between 0.5-1.6 mg/ml were considered as “mildly effective”. Test subjects with LC50 values higher than 1.6 mg/ml were treated as “ineffective”. The extracts with highest nematicidal activity (“highly effective” subjects) were evaluated for their phytotoxic and brine shrimp lethality activity. Finally, four most promising extracts were subjected to bioactivity guided fractionation to find and identify volatile sulfur compounds with nematicidal activity. All tests were performed in triplicates (n=3) and standard deviations were calculated.

3.1.1. “Highly effective” test subjects

While Lannate killed 50% of nematodes at a concentration of 0.52 mg/ml, extracts of *A. sativum* (garlic) had LC50=0.11 mg/ml, *A. ampeloprasum* had LC50=0.086 mg/ml, *A. paniculatum* had LC50=0.14 mg/ml, *A. platyspathum* had LC50=0.24 mg/ml, *A. stipitatum*’s (Iran) LC50 was 0.28 mg/ml, *A. zebdanense’s* LC50 was 0.28 mg/ml, *A. aflatunense’s* LC50 was 0.29 mg/ml and *A. moly’s* LC50 was 0.30 mg/ml. Pure compound Aldrithiol-2 also had LC50 of 0.47 mg/ml (Figure 3.1).

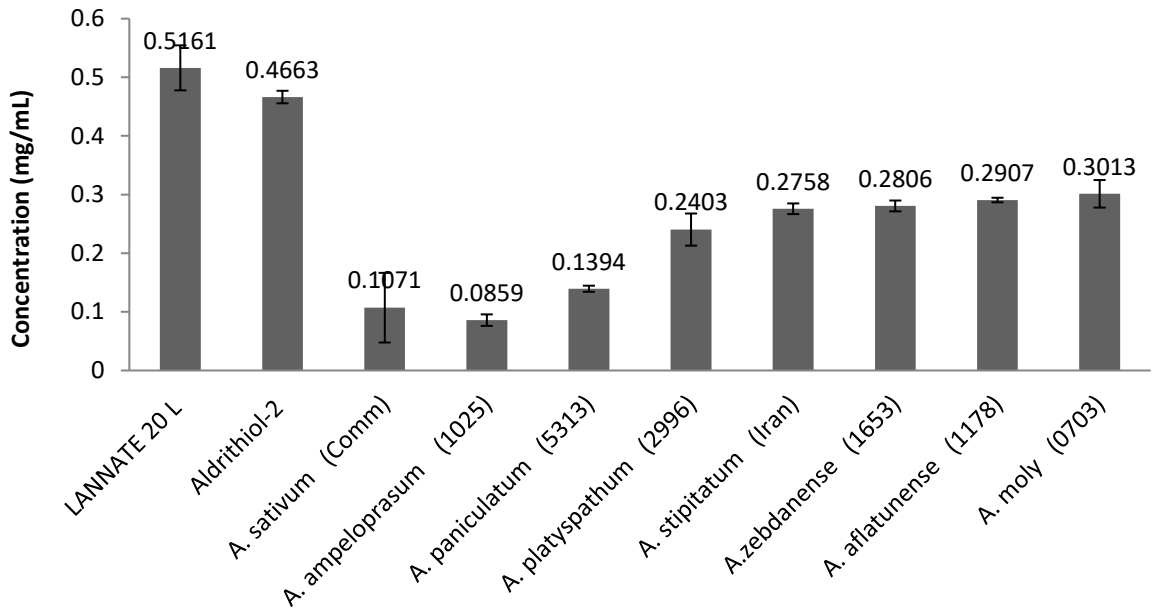

Figure 3.1. Concentrations (mg/ml) of *Allium* EtOAc extracts which killed 50% of *M. incognita* population in the wells. Lannate 20 L was used as positive control. Standard deviations presented as error bars (n=3).

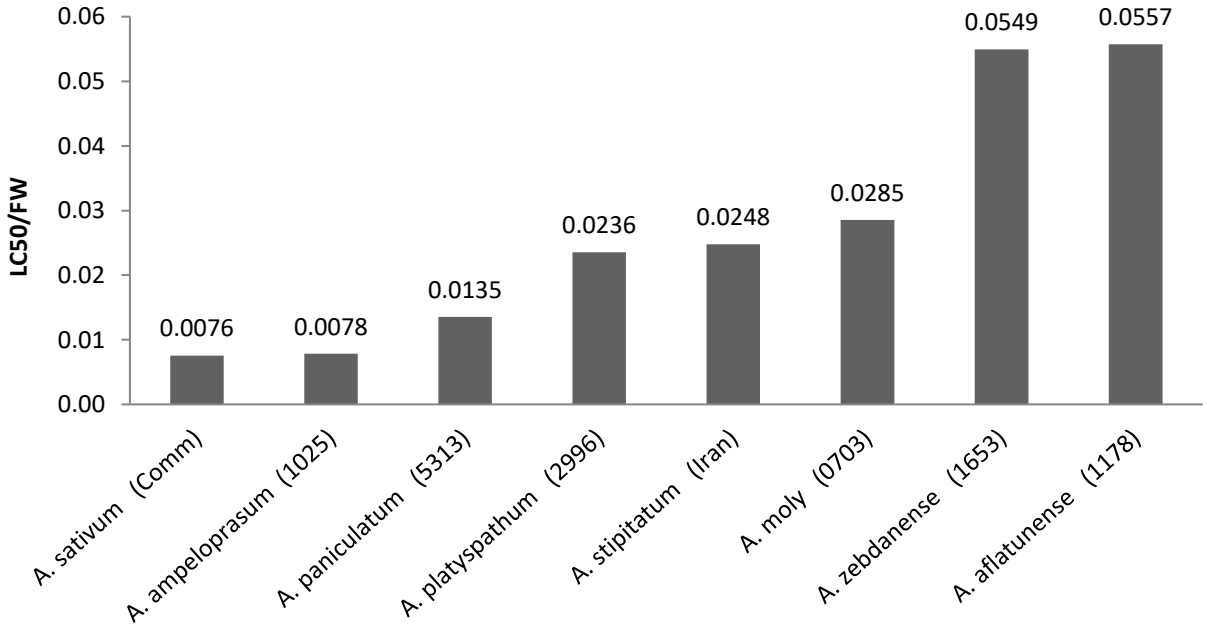

Figure 3.2. Ratios of LC50 values of extracts to their respective fresh material weights (LC50/FW).

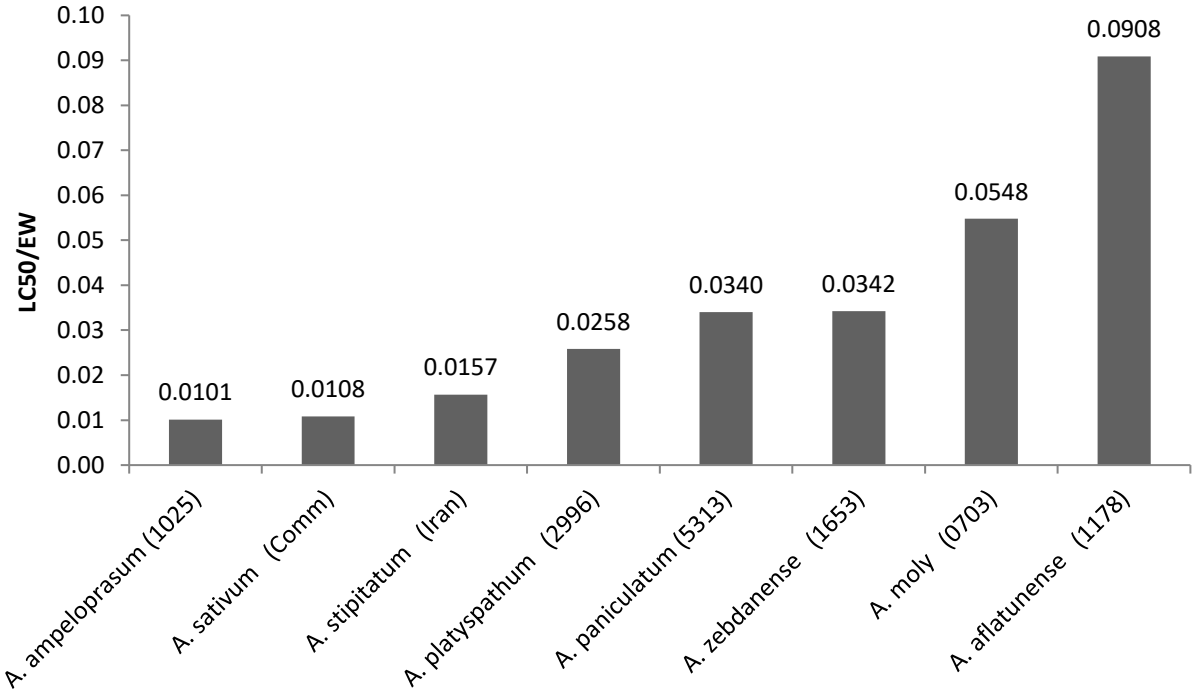

Figure 3.3. Ratios of LC50 values of extracts to their respective extract weights (LC50/EW).

The weight of a biological material will affect the quantity of extracted compounds. Various *Allium* species have bulbs which differ in size, a property affecting amount of water and thus the weight of a plant material. All plants were dried under same conditions and for extraction purposes the intention was to use around 10 grams of plant material whenever enough material was available. It was decided to calculate ratios of LC50 values of extracts to their material weights (LC50/FW, Figure 3.2) and of LC50 values of extracts to their extract weights (LC50/EW, Figure 3.3). The purpose of this approach was to offset the difference in plant material weights due to varying water content and to obtain fair comparison of the nematicidal activity among the extracts. As a result of both calculations, slightly different order of nematicidal activity of the extracts observed. When grouped based on their LC50/FW ratios, *A. ampeloprasum* and *A. sativum* appear to have almost same ration values of 0.0078 and 0.0076, respectively. Same similarity in the activity of *A. ampeloprasum* and *A. sativum* extracts is observed in the calculation of LC50/EW ratios and the values are 0.0101 and 0.0108, respectively

(Figure 3.3). The order of the extracts slightly changes in each calculation scenario. For example, on the list of LC50/EW ratios (Figure 3.3), *A. paniculatum*, placed third on LC50/FW list (Figure 3.2), swaps its place with *A. stipitatum* (IRAN), which is placed fifth on LC50/FW list; as a result, *A. stipitatum* climbs to third place on LC50/EW list and *A. paniculatum* drops to fifth on LC50/EW list. Extract of *A. zebdanense* swaps it seventh place (LC50/FW) with sixth place of *A. moly* (LC50/FW) and they become sixth and seventh, respectively, on the list of LC50/EW ratios. The order number and ratio value of *A. platyspathum* on both lists remained same. While the order number of *A. aflatunense* did not change on both lists, the ratio values on LC50/FW and LC50/EW lists were different with 0.0557 and 0.0908, respectively.

3.1.2. "Mildly effective" test subjects

Only six *Allium* extracts were found to have moderate nematicidal effect. Extracts of *A. stipitatum* (5263), *A. rupestre*, *A. stipitatum* (5475), *A. canadense*, *A. angulosum* had LC50 values between 0.53mg/ml-1.56 mg/ml and were classified as "mildly effective" against *M. incognita* nematodes and listed in Table 3.1. Standard deviations are calculated based on n=3.

Table 3.1. List of *Allium* extracts with LC50 values between 0.53mg/ml-1.60 mg/ml. SD=standard deviation.

TAX/ACC No	MATERIAL	FRESH WEIGHT (g)	LC50 (mg/mL)	SD
5263	*A. stipitatum*	12.82	0.5332	0.0181
5301	*A. rupestre*	8.24	0.5649	0.0197
5475	*A. stipitatum*	11.66	0.6007	0.0430
1732	*A. rupestre*	10.42	1.3387	0.0353
2023	*A. canadense*	9.15	1.5089	0.1275
3513	*A. angulosum*	10.13	1.5558	0.0718

3.1.3. "Ineffective" test subjects

Vast majority of the tested *Allium* species showed very low or no nematicidal effect within tested concentration range. Dipyrithione (Figure 1.10), was previously reported to be present in *A. stipitatum* and to be highly active against the three *Staphylococcus aureus* strains [48]. It was also reported to be found in the extract of basidiomycete mushroom of the genus *Cortinarius*, although suggested to be rather an artifact of the extraction procedure than a true metabolite of the species [90]. Authors of the study also report that dipyrithione had significant antimicrobial activity and cytotoxicity.

Dipyrithione was purchased and included in nematicidal screening assay. As a result, it had nematicidal activity with LC50>1.6 mg/ml. Since it is a pure compound, the activity was accepted to be low. For this reason, dipyrithione was included on the list of "ineffective" test subjects and presented in Table 3.2 with other *Allium* extracts with LC50 values>2.5 mg/ml.

Table 3.2. List of test subjects with LC50 values >1.6 mg/ml.

TAX/ACC No	MATERIAL	FRESH WEIGHT (g)	LC50 (mg/mL)
	Dipyrithione		>1.6
2584	*A. douglasii*	9.44	>2.5
2390	*A. pallens*	7.05	>2.5
2672	*A. maximowiczii*	10.23	>2.5
1513	*A. lusitanicum*	10.25	>2.5
0469	*A. flavum*	8.01	>2.5
3193	*A. rubens*	10.25	>2.5
0192	*A. schoenoprasum*	10.27	>2.5
3061	*A. campanulatum*	10.51	>2.5

3470	*A. denudatum*	10.43	>2.5
3187	*A. obliquum*	11.47	>2.5
0515	*A. multibulbulosum*	10.36	>2.5
5335	*A. sibthorpianum*	10.20	>2.5
3558	*A. victorialis*	10.28	>2.5
5193	*A. cornutum*	9.94	>2.5
5738	*A. stellerianum*	10.16	>2.5
1642	*A. spirale*	10.12	>2.5
10001	*A. schoenoprasum (leaves)*	7.62	>2.5
10001	*A. schoenoprasum (bulbs)*	5.91	>2.5
3200	*A. altaicum*	10.72	>2.5
3208	*A. nutans*	10.29	>2.5
5309	*A. rotundum subsp. rotundum*	10.08	>2.5
5316	*A. pictistamineum*	10.36	>2.5
0068	*A. cernuum*	10.47	>2.5
2800	*A. hollandicum*	9.22	>2.5
0070	*A. vineale*	4.14	>2.5
2256	*A. rosenorum*	9.61	>2.5
5295	*A. rotundum*	10.10	>2.5
126	*A. rotundum*	9.01	>2.5
6158	*A. rosenorum*	10.06	>2.5
3183	*A. strictum*	10.83	>2.5
1482	*A. tuberosum*	10.85	>2.5
3548	*A. senescens*	10.38	>2.5
5410	*A. ramosum*	10.15	>2.5
1017	*A. atropurpureum*	10.57	>2.5

5011	*A. fedschenkoanum*	7.03	>2.5
5015	*A. oreoprasum* (river)	10.54	>2.5
5021	*A. oreoprasum* (valley)	10.31	>2.5
5022	*A. sp. sect. reticulatobulbosum*	7.22	>2.5
5023	*A. aff. talassicum*	10.49	>2.5
6178	*A. pskemense*	15.12	>2.5

3.2. Phytotoxicity assay

Several *Allium* extracts which were accepted as “highly effective” were further assessed for their ecological risk using phytotoxicity assay. Table 3.3 shows *Allium* extracts, Lannate 20 and Aldrithiol-2 with their calculated nematicidal LC100 values which were used in the assay. Extracts of 1025 *A. ampeloprasum*, *A. sativum* (commercial), 5313 *A. paniculatum*, 2996 *A. platyspathum*, 1178 *A. aflatunense*, 0703 *A. moly* were calculated to have LC100 values between 0.4867-0.5966 mg/ml, and extracts of *A. stipitatum* (Iran) and 1653 *A. zebdanense* had very close values of 0.9866 mg/ml and 0.9882 mg/ml, respectively. The seeds of test plants in the phytotoxicity assay were treated with *Allium* extracts at concentrations presented in Table 3.3. Phytotoxicity, germination rate, root length and shoot length of the test seeds treated with *Allium* extracts were measured and compared against their controls based on literature review provided in the section 1.7. The results were presented as percent (%) variation from controls.

Table 3.3. List of *Allium* extracts, Lannate 20 and Aldrithiol-2 with their corresponding calculated LC100 values used in phytotoxicity assay.

TAX/ACC No	*Allium* spp.	LC100 (mg/mL)
1025	*A. ampeloprasum*	0.5042
Commercial	*A. sativum*	0.4867
5313	*A. paniculatum*	0.4987
2996	*A. platyspathum*	0.5690
IRAN	*A. stipitatum*	0.9866
1653	*A. zebdanense*	0.9882
1178	*A. aflatunense*	0.5680
0703	*A. moly*	0.5966
	Aldrithiol	0.8778
	Lannate 20	3.8522

3.2.1. Germination rate

Tomato, lettuce and cress seeds were treated with extracts, pure compound, Aldrithiol-2, and negative controls for 48 hours. Length of germinated seedlings were measured after 48 hours. Lannate inhibited germination of tomato, lettuce and cress seeds by 46.67%, 56.67%, 96.67% showing variations of -53% and -25% from buffer control. Variation of cress seed germination was +3.34%. Pure compound, Aldrithiol-2, had variations of -100%, -81.5%, and -20% for tomato, lettuce and cress, respectively (Figure 3.3).

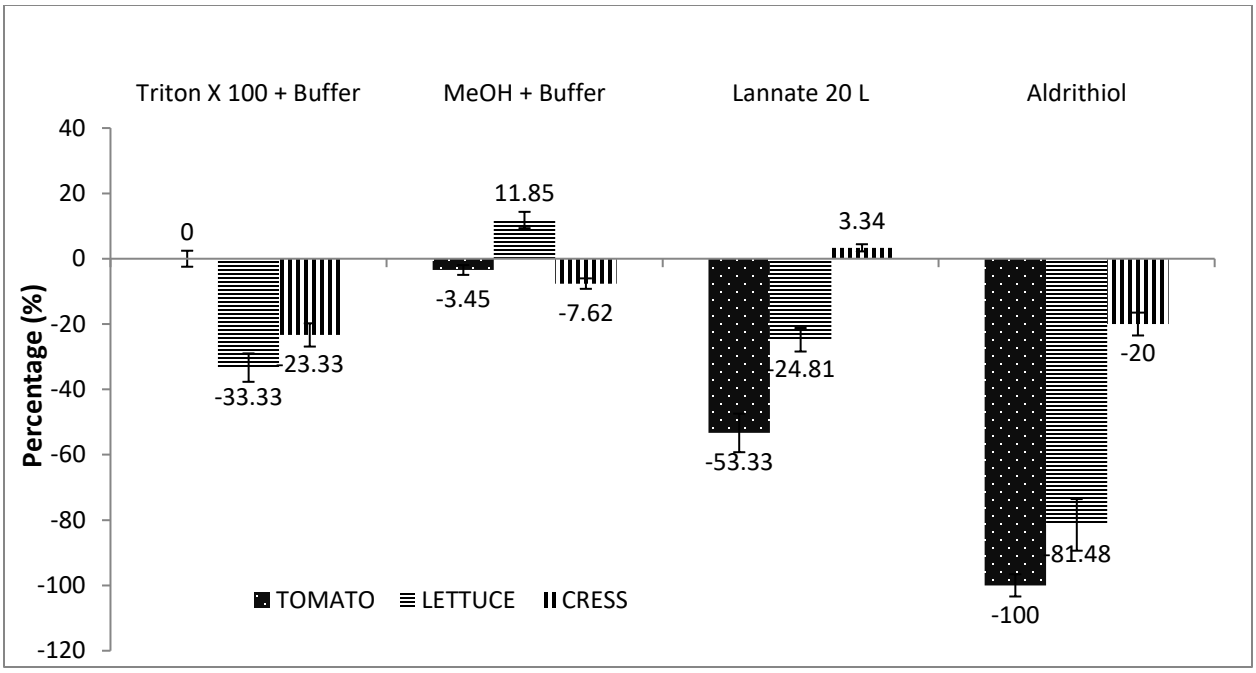

Figure 3.3. Effect of controls and pure compound Aldrithiol-2 on germination of the tomato, lettuce and cress seeds. Data shown as germinated seed percentage variation from negative control. Standard deviations are presented as error bars (n=3).

Among *Allium* extracts, *A. zebdanense* had no negative impact on germination of tomato seeds (100% germination rate). The highest inhibition upon germination of tomato seeds was exhibited by *A. stipitatum* and *A. aflatunense*, with values of -80% and -96.67% variation from control, respectively. Extract of *A. sativum* had inhibition effect on tomato seed by -16.67% and extracts of *A. ampeloprasum*, *A. paniculatum, A. moly* and *A. platyspathum* had inhibition percentages of -6.67%, -6.67%, -5.00% and -3.33%, respectively, when compared to control.

Lettuce seeds were the most inhibited by *A. sativum* extract, by -86.55%, compared to control. Extracts of *A. zebdanense*, *A. stipitatum, A. aflatunense* inhibited more than half of the lettuce seeds with variation values of -56.55%, 61.55% and 63.22%, respectively. Extract of *A. platyspatum* inhibited almost half of the lettuce seeds with -48.27% variation from control and *A. ampeloprasum*, *A. moly* and *A. paniculatum*

had least effect with values of -23.22%, -26.55% and -33.22%, respectively. Although cress seeds treated with *A. sativum*, *A. paniculatum* and *A. aflatunense* extracts had -6.67%, -3.57% and -3.33% variation respectively, a Wilcoxon test showed that results from treated seeds were not significantly different from control ($p>0.05$, two-tailed test). Extracts of *A. ampeloprasum*, *A. zebdanense*, *A. stipitatum*, *A. platyspathum* and *A. moly* had no effect at all on germination rate of cress seeds (Figure 3.4).

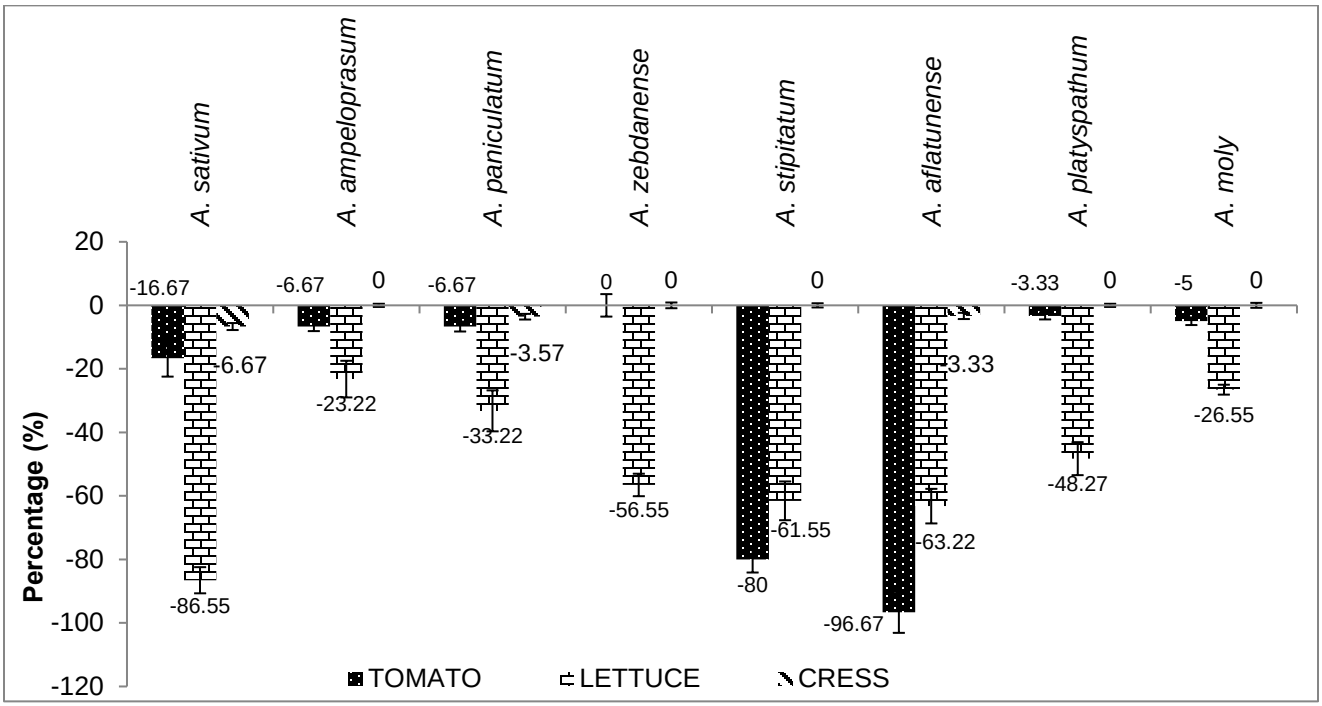

Figure 3.4. Effect of *A. sativum*, *A. ampeloprasum*, *A. paniculatum*, *A. zebdanense, A. stipitatum, A. aflatunense* and *A. platyspathum* extracts on germination of the tomato, lettuce and cress seeds. Data shown as germinated seed percentage variation from negative control. Standard deviation shown as error bars (n=3). Concentrations of the extracts are given in Table 3.3.

3.2.2. Root length

Effect of controls on the root length has shown in Figure 3.6 and effect of test subjects is shown in Figure 3.5. Positive control, Lannate completely inhibited root growth in tomato, lettuce and cress seeds with 100% inhibition.

It was observed that, all test subjects, but *A. ampeloprasum*, were highly toxic to tomato seeds and almost totally inhibited their radical development with toxicity being more than 91.15% (Figure 3.5). Extract of *A. ampeloprasum* inhibited tomato root length by 79.15%.

The lettuce seeds were also highly inhibited by the most of *Allium* extracts by almost 90% and over compared to control. Only *A. ampeloprasum* and *A. platyspathum* had root length inhibition percentages by 55.88% and 77.77%, respectively.

Most of the *Allium* extracts did not affect the cress seeds' root elongation and most of inhibition values were below 50%. Most toxic ones were extracts of *A. sativum* and *A. platyspathum* with 66.37% and 63.95%, respectively.

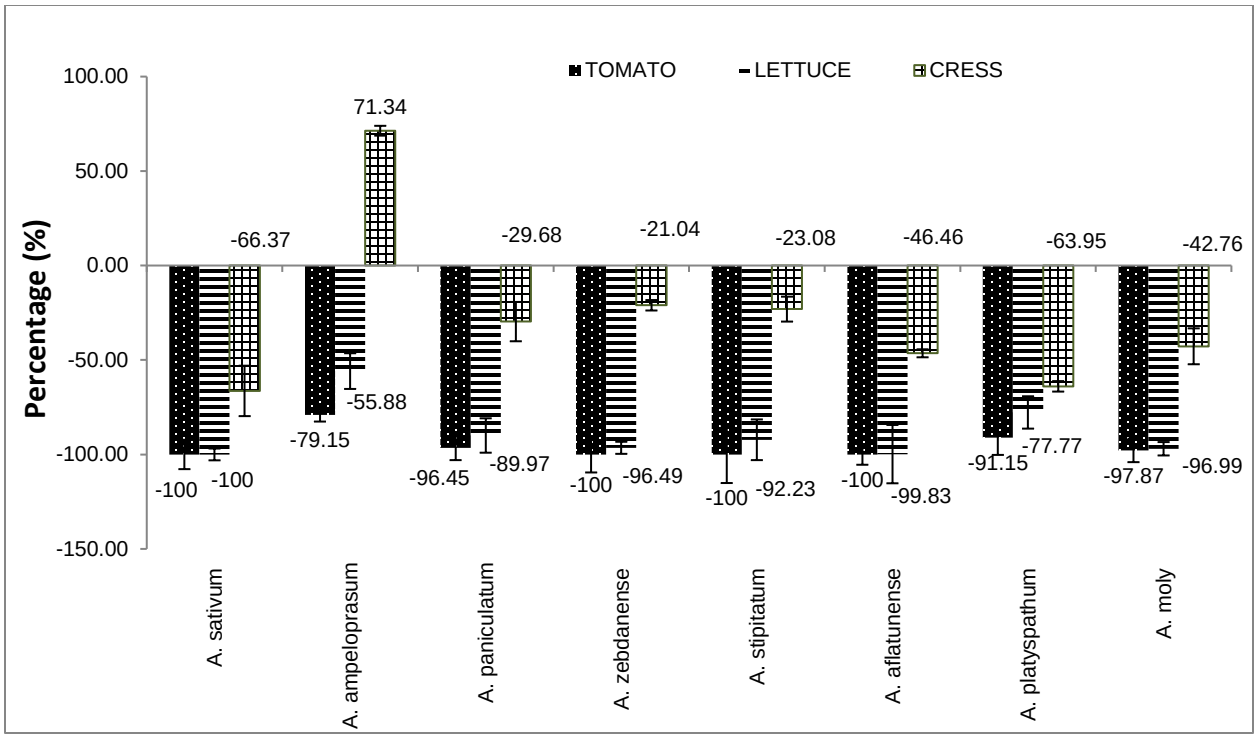

Figure 3.5. Effect of *Allium* extracts on root length of germinated tomato, lettuce and cress seeds expressed as % variation from control. Standard deviations are given as error bars (n=3). Concentrations of the extracts are given in Table 3.3.

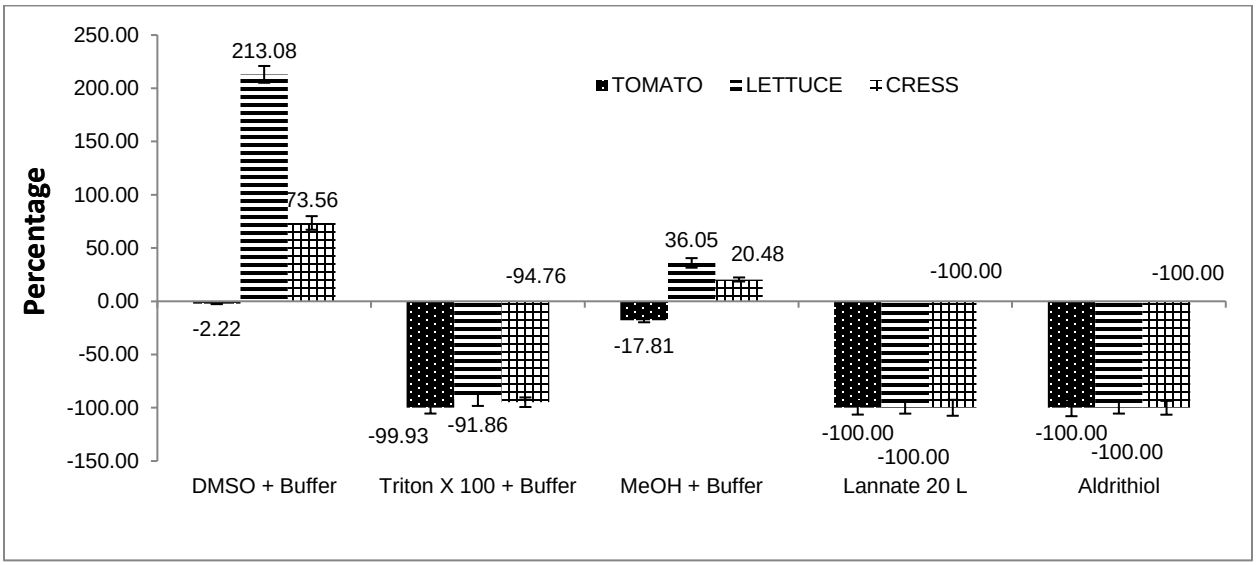

Figure 3.6. Effect of controls and Aldritiol-2 on root length of germinated tomato, lettuce and cress seeds expressed as % variation from control. Standard deviations are given as error bars (n=3). Concentrations of the extracts are given in Table 3.3.

3.2.3. Shoot length

Shoot lengths of treated tomato, lettuce and cress seeds were calculated and presented as percentage variation from control in Figure 3.7. Lannate inhibited shoot growth in all seeds with more than 87%. Extracts of *A. stipitatum* and *A. aflatunense* prevented shoot growth in tomato seed with more than 99% percentage variation from control. Extracts of *A. sativum and A. moly* inhibited the shoot length in tomato seeds by 88.80% and 81.10%, respectively. All other extracts had tomato shoot length percentage variations between -50.10% and -74.95%.

Shoot lengths of lettuce seeds treated with *A. sativum*, *A. zebdanense*, *A. stipitatum* and *A. aflatunense* were 99.12%, 80.17%, 83.32%, and 92.41%, respectively, shorter than control seeds. All other extracts had lettuce shoot length percentage variations between 51.51% and 77.43%.

Cress seeds' shoot lengths were shorter by almost 53% when treated with *A. sativum* extract. Extracts of *A. paniculatum, A. platyspathum* and *A. moly* inhibited shoot length by 15.27%, 13.83% and 21.46%, respectively. Extracts of *A. ampeloprasum* and *A. stipitatum* significantly induced shoot growth in cress seeds and percentage variations were +45.01% and 47.04%, respectively. Although cress seeds treated with *A. zebdanense* and *A. aflatunense* extracts showed 6.04% increase in shoot length, a Wilcoxon test showed that results from treated seeds were not significantly different from control ($p>0.05$, two-tailed test). Therefore, it can be suggested that both extracts had no effect on the shoot lengths.

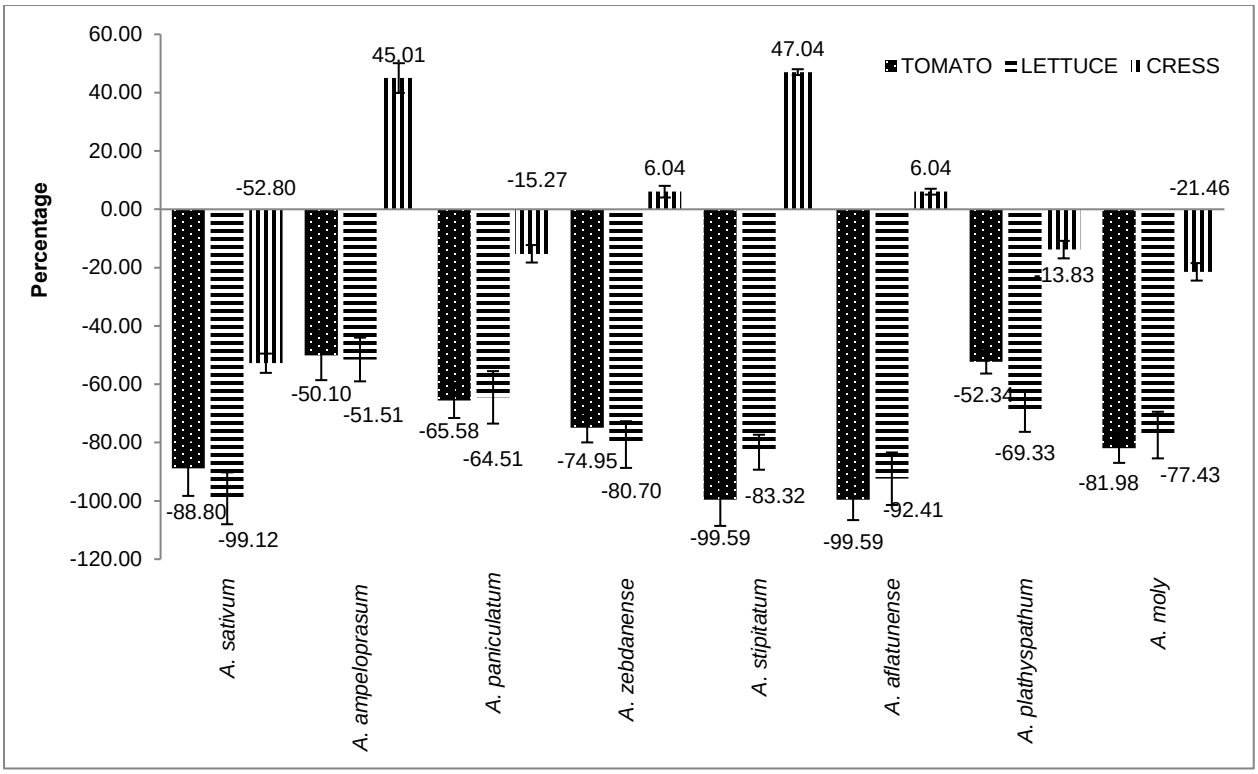

Figure 3.7. Effect of *Allium* extracts on shoot length of germinated tomato, lettuce and cress seeds expressed as % variation from control. Standard deviations are given as error bars (n=3). Concentrations of the extracts are given in Table 3.3.

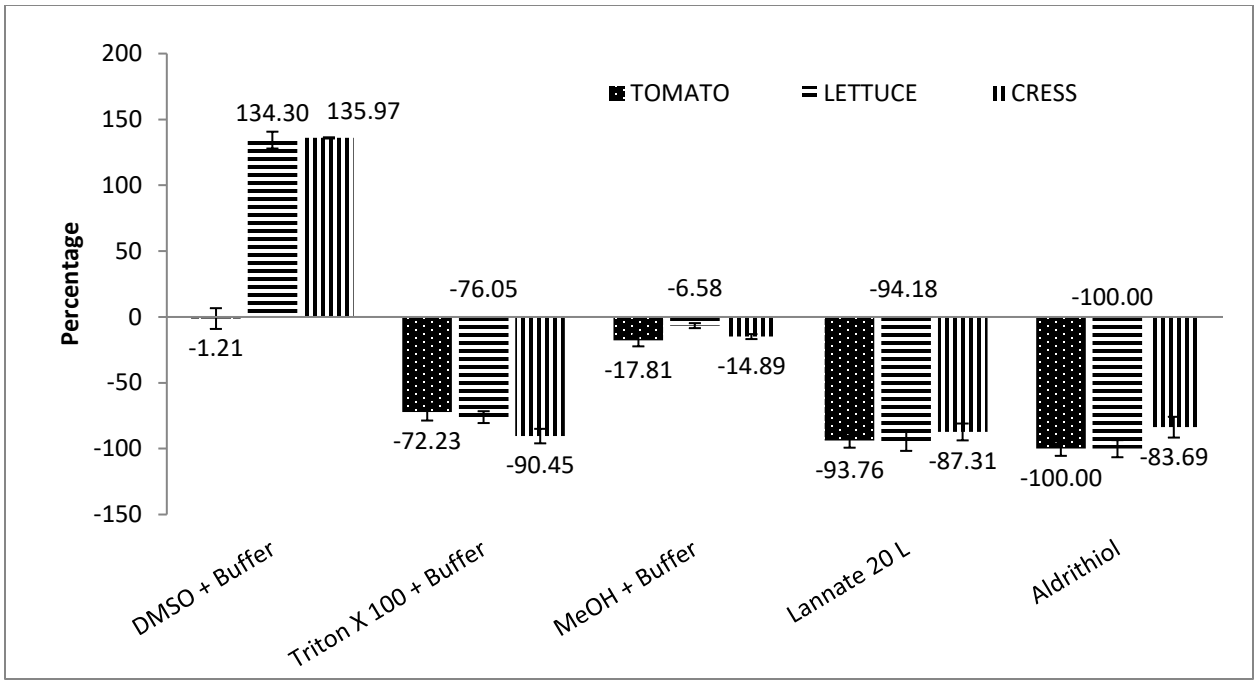

Figure 3.8. Effect of controls on shoot length of germinated tomato, lettuce and cress seeds expressed as % variation from control. Standard deviations are given as error bars (n=3). Concentrations of the extracts are given in Table 3.3.

3.2.4. Brine shrimp lethality assay

Hatched brine shrimps were transferred in microwell plates and treated with *Allium* extracts, Lannate, Aldrithiol-2, and solvents of the test subjects. Extracts of *Allium* plants, Lannate and Aldrithiol-2 were applied at their corresponding LC100 values shown in Table 3.3. Experiment was performed in triplicate (n=3) and results presented as percentage (%) of survived brine shrimps with standard deviation (Figure 3.9 and Figure 3.10).

After 48 h of contact with Lannate and *Allium* extracts, survival rates of brine shrimps treated with *A. paniculatum*, *A. zebdanense*, *A. moly, A. aflatunense, A. stipitatum, A. platyspathum, A. ampeloprasum, A. sativum,* Aldrithiol-2 and Lannate were 88.89%, 66.67%, 31.48%, 3.70%, 1.85% 0%, 0%, 0%, 0%, 83.33% respectively. DMSO solution in which extracts were dissolved (final concentration 0.05%, data not

shown) had 100% survival rate. Solvents previously used to dissolve extracts were also tested with their final concentrations. Methanol (0.01%) had 98.15%, whereas Triton X (0.25%) had lower survival rate of 44.44%.

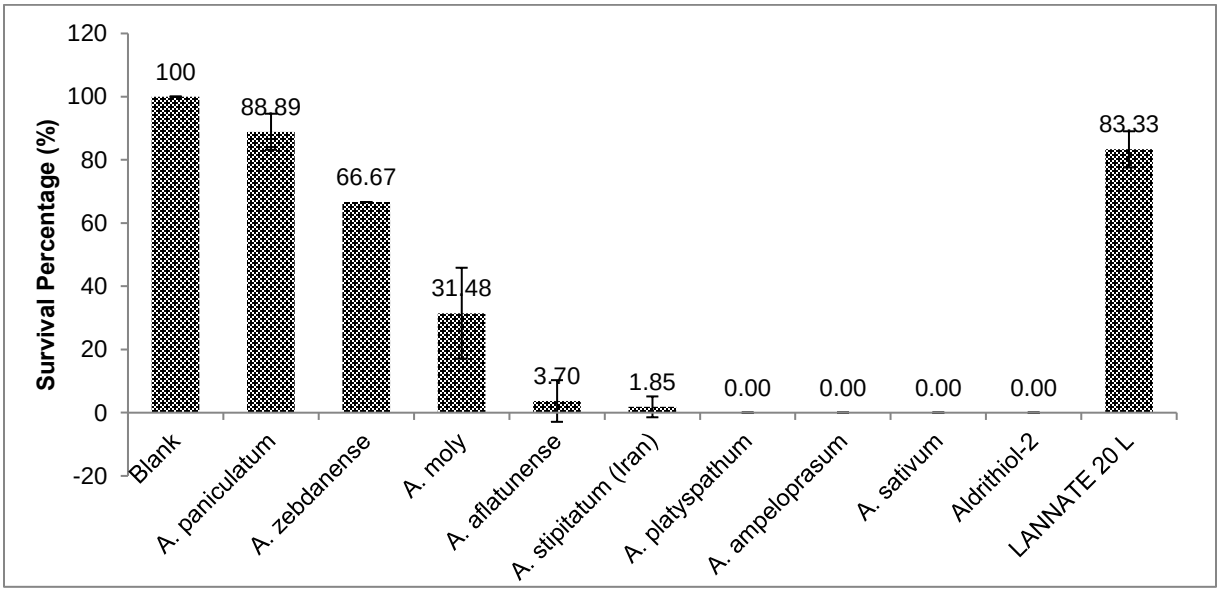

Figure 3.9. Effect of blank, *Allium* extracts, pure compound Aldrithiol-2 and Lannate on brine shrimp survival. Standard deviations are given as error bars (n=3). Concentrations of the extracts are given in Table 3.3.

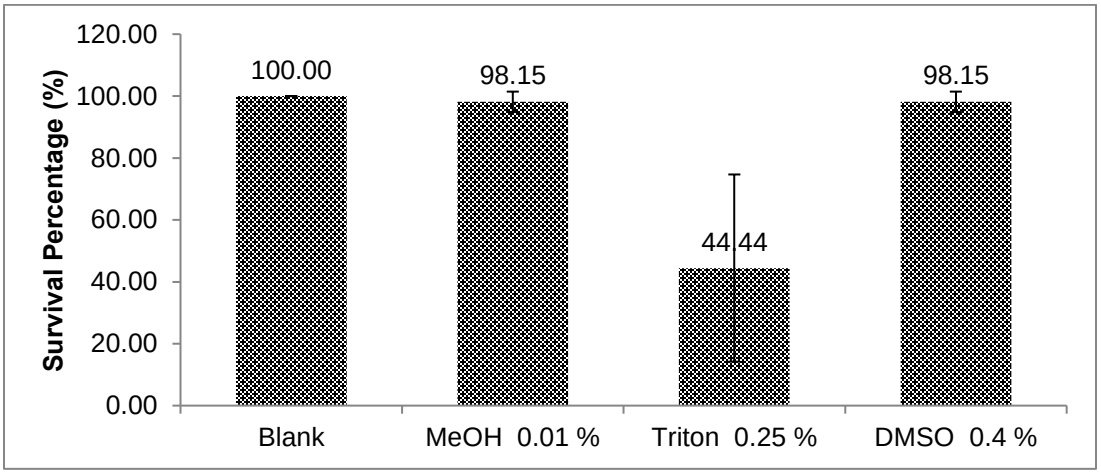

Figure 3.10. Effect of controls on brine shrimp survival. Standard deviations are given as error bars (n=3).

3.3. Isolation of fractions by means of preparative HPLC

Several *Allium* extracts exhibited nematicidal activity against root-knot nematode *M. incognita*. It is crucial to determine active compounds responsible for the bioactivity of the extracts. For this reason, compounds first need to be isolated from the extract. Preparative HPLC technique was used to isolate several fractions from *Allium* extracts. HPLC UV detector at 254 nm was used. Sulfur compounds present in *Allium* extracts usually have a chromatophore yielding good signal at 254 nm. A preliminary test run with small amount of the *Allium* extracts was performed to obtain overall picture of available peaks. One of the general approaches on active compound prediction is to target peaks which represent compounds available in greater amounts. In addition, this approach allows to isolate compounds in amounts necessary for further bioactivity and structure elucidation steps. The approach of targeting major peaks was also dictated by relatively low amounts of available plant material. Based on the preliminary HPLC runs it was decided which peaks should be isolated. It was also noticed that peaks were doubling or tripling, and it was assumed that the reason was a crack in matrix bed of the HPLC column. Four *Allium* extracts, *A. ampeloprasum*, *A. stipitatum*, *A. platyspathum* and *A. paniculatum*, which had considerable nematicidal activity were subjected for further chromatographic, spectroscopic and bioactivity tests.

3.3.1. A. ampeloprasum

The crude ethyl acetate extract of *A. ampeloprasum* appeared to be very oily, therefore it is critical to dissolve the extract in initial 45% methanol solution used in the HPLC method. Considerable amount of oily precipitation was observed. Top filtered solution of the extract was used for the HPLC fractionation. After preliminary HPLC run of *A. ampeloprasum* extract it was decided to isolate five major peaks by assuming and combining the doubling and tripling broad peaks as a single fraction. The approximate peak collection ranges are shown in Figure 3.11. The elution times of peaks are presented in Table 3.4.

Table 3.4. Time intervals of fractions of *A. ampeloprasum* extract using preparative HPLC with UV detector at 254 nm.

Peak	Elution Time (min)
Fraction 1	4.37 – 8.94
Fraction 2	13.42 – 14.80
Fraction 3	19.89 – 21.40
Fraction 4	24.30 – 25.83
Fraction 5	29.42 – 30.99

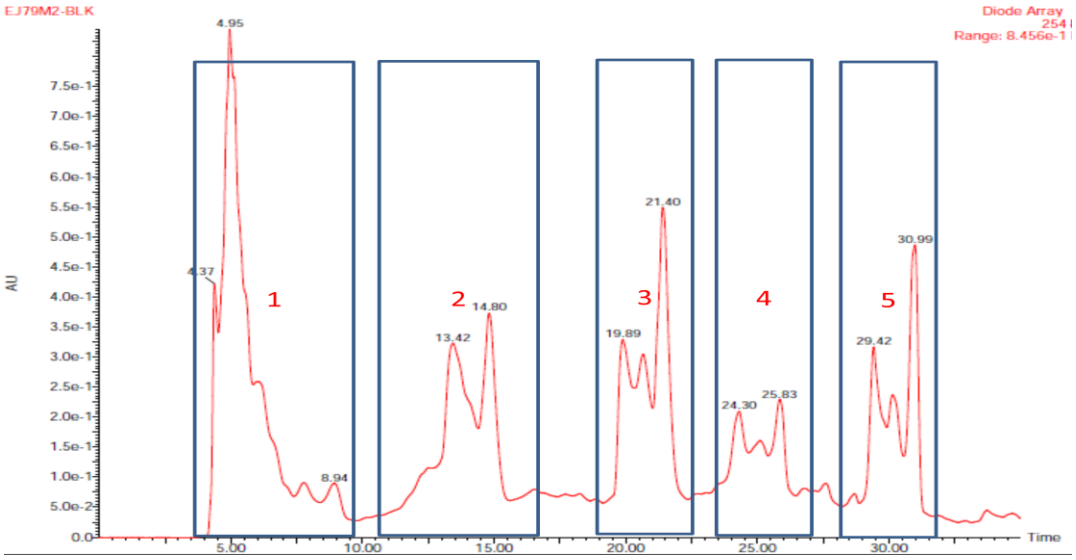

Figure 3.11. HPLC chromatogram of *A. ampeloprasum* with UV detector at 254 nm. Boxes around peaks indicate collected fractions.

3.3.2. A. stipitatum

When *A. stipitatum* extract was dissolved in initial 45% methanol solution, some oily precipitation was also observed, although not as much as it was observed in the *A. ampeloprasum* extract. In a preliminary, run two major peaks were detected along with other smaller ones. In total, five fractions were collected from *A. stipitatum* to be

tested in nematicidal assay. Due to broad peaks, collected ranges are marked by frames. Peak elution times are presented in Table 3.5.

Table 3.5. Time intervals of fractions of *A. stipitatum* extract using preparative HPLC with UV detector at 254 nm.

Peak	Elution Time (min)
Fraction 1	5.00 – 8.17
Fraction 2	10.00 – 11.28
Fraction 3	14.39 – 15.12
Fraction 4	20.18 – 21.53
Fraction 5	23.00 – 30.00

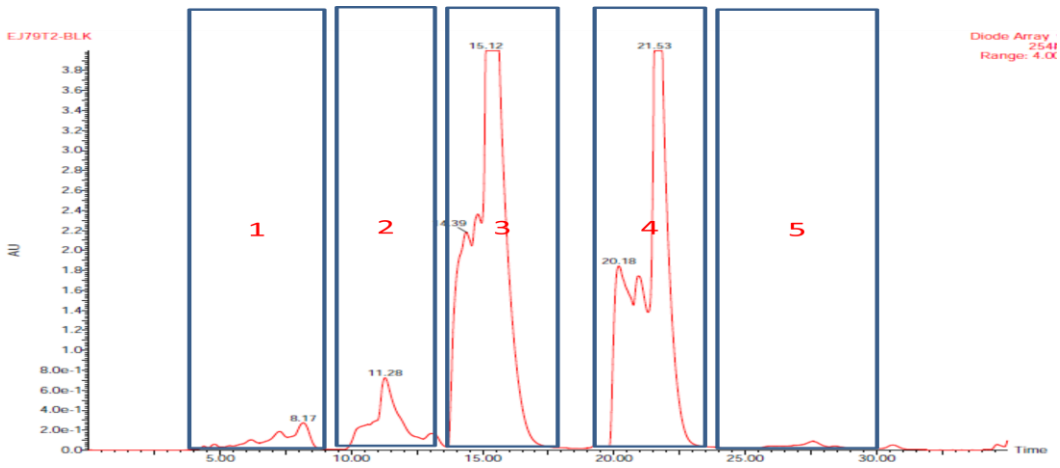

Figure 3.12. HPLC chromatogram of *A. stipitatum* with UV detector at 254 nm. Boxes around peaks indicate collected fractions.

3.3.3. A. platyspathum

As the quality of separation was not satisfying after multiple preliminary HPLC runs, it was decided to isolate fractions in collections as they are depicted in Figure 3.13. The decision was to isolate in five-minute intervals for first 4 fractions. Last fraction was collected over 10 minutes as mainly highly non-polar compounds are expected to elute during this period and they are not of high interest. Five fractions were collected

from *A. platyspathum* in two batches and combined, as the amount of extract was abundant. Time intervals of fractions are presented in Table 3.6.

Table 3.6. Time intervals of fractions of *A. ampeloprasum* extract using preparative HPLC with UV detector at 254 nm.

Peak	Elution Time (min)
Fraction 1	5.00 – 10.00
Fraction 2	10.00 – 15.00
Fraction 3	15.00 – 20.00
Fraction 4	20.00 – 25.00
Fraction 5	25.00 – 35.00

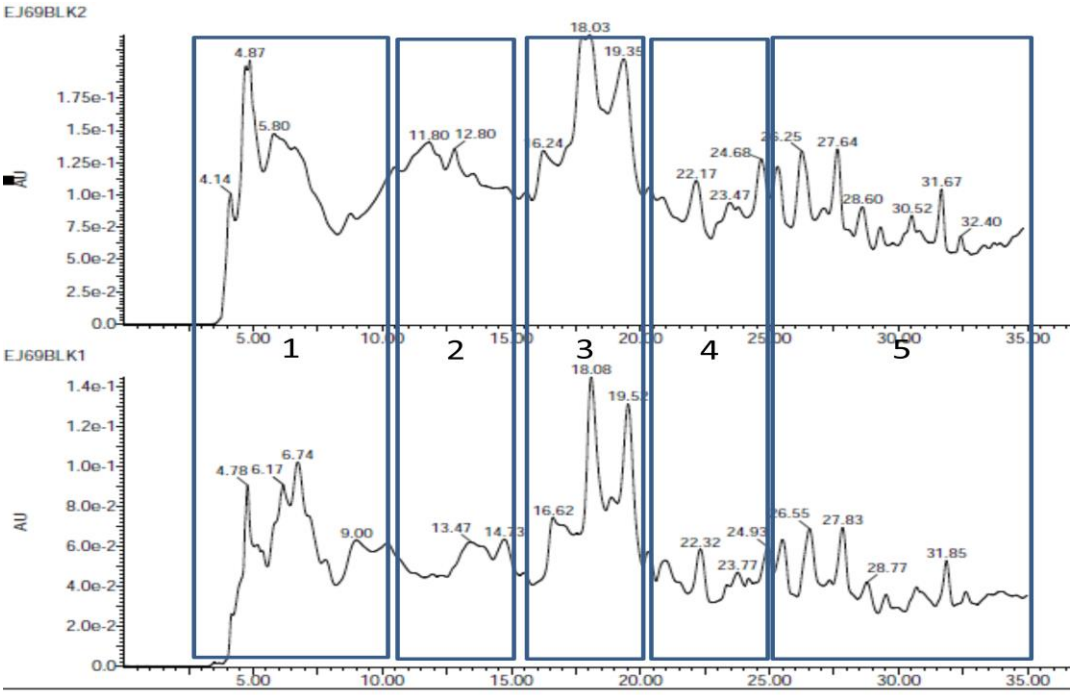

Figure 3.13. HPLC chromatogram of *A. platyspathum* with UV detector at 254 nm. Boxes around peaks indicate collected fractions. Fractions were collected over two runs of the extract.

3.3.4. A. paniculatum

Due to poor peak separation, similar strategy as with *A. platyspathum* was used when performing preparative HPLC run with *A. paniculatum* extract. Since the quality of peak separation was not satisfying after the preliminary extract elution, it was decided to isolate fractions in collections as they are depicted in Figure 3.14. After both runs it was decided to combine Fraction 2 and 3 into a single fraction, therefore finally 4 fractions were tested for their nematicidal activity. Collection time intervals are presented in Table 3.7.

Table 3.7. Time intervals of fractions of *A. paniculatum* extract using preparative HPLC with UV detector at 254 nm.

Peak	Elution Time (min)
Fraction 1	3.00 – 8.00
Fraction 2 and 3	10.00 – 14.00
Fraction 4	15.00 – 22.00
Fraction 5	22.00 – 35.00

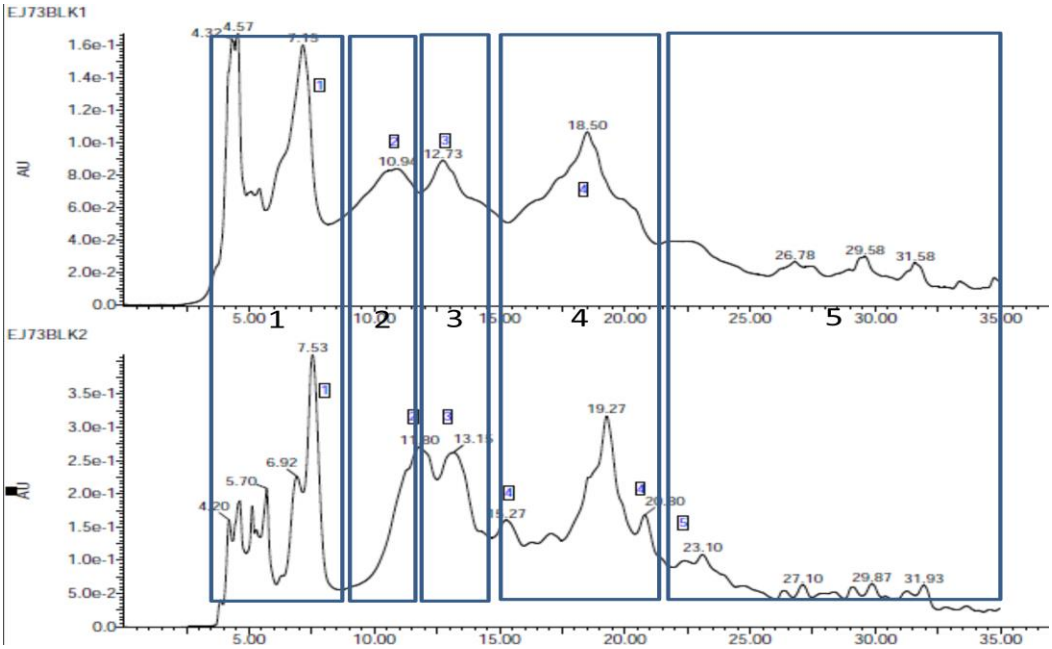

Figure 3.14. HPLC chromatogram of *A. paniculatum* with UV detector at 254 nm. Boxes around peaks indicate collected fractions. Fractions were collected over two runs of the extract.

3.4. Nematicidal effect of fractions isolated from Allium species

3.4.1. Fractions of A. ampeloprasum

Five fractions were isolated from the EtOAc extract of *A. ampeloprasum* bulb to be tested in nematicidal assay. Fractions 1, 4 and 5 showed no nematicidal activity and their LC50 values were higher than 2.5 mg/ml. Fraction 2 had low nematicidal activity with LC50 of 2.5-1.25 mg/ml. Fraction 3 had LC50 around 1.25-0.625 mg/ml. The whole extract LC50 value was calculated to be 0.15625 - 0.078125 mg/ml.

Table 3.7. Results of nematicidal activity of *A. ampeloprasum* fractions (n=3).

SAMPLE	LC50 (mg/ml)
F2	2.5-1.25
F3	1.25-0.625
Whole extract	0.15625 - 0.078125

3.4.2. Fractions of A. stipitatum

Five fractions were isolated from the EtOAc extract of *A. stipitatum* bulb to be tested in nematicidal assay. Fractions 1, 4 and 5 had no nematicidal activity and their LC50 values were higher than 2.5mg/ml. Fraction 3 had low nematicidal activity with LC50 value of 2.5-1.25 mg/ml. Fraction 4 fraction had LC50 between 0.0781-0.0391 mg/ml. The whole extract LC50 was calculated to be 0.0781-0.0391 mg/ml.

Table 3.8. Results of nematicidal activity of *A. stipitatum* fractions (n=3).

SAMPLE	LC50 (mg/ml)
F3	2.5-1.25
F4	0.0781-0.0391
Whole extract	0.0781-0.0391

3.4.3. Fractions of A. platyspathum and A. paniculatum

All five fractions of *A. platyspathum* and *A. paniculatum* had almost no nematicidal effect within tested concentration range.

3.5. Structure elucidation of active fractions from Allium species

3.5.1. Structure elucidation of nematicidal fractions from A. ampeloprasum

Two fractions, which showed nematicidal effect within tested concentration range were subjected to structure elucidation process using high resolution mass spectroscopy (HR-MS) and proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) methods.

3.5.2. Fraction 2

No satisfactory spectral and chromatographic data was collected from Fraction 2's HPLC/MS analysis. Nevertheless, it was possible to locate the peak on the chromatogram of the whole extract (Figure 3.15). Mass spectrum analysis suggested protonated adduct, $[M+H^+]$ of 136. Other adducts were suggested to be ammonia adduct, $[M+NH_4^+]$ =154, and methanol adduct, $[M+CH_3OH+H^+]$ =186. The analysis of the fraction by means of high resolution mass spectroscopy also did not provide any clear results.

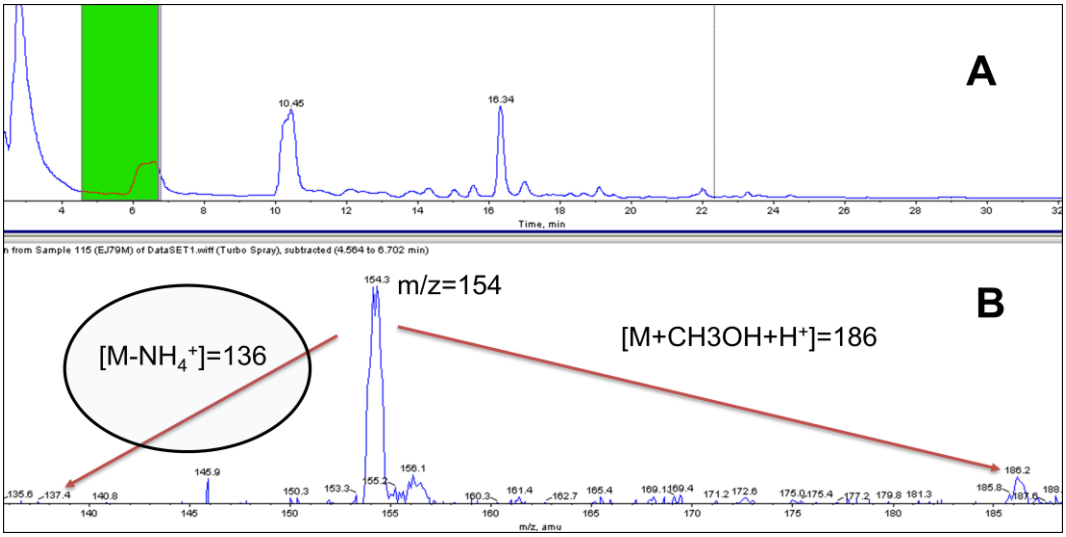

Figure 3.15. HPLC chromatogram with UV detector at 254 nm of *A. ampeloprasum* whole extract (A). Mass spectrogram of selected peak in chromatogram (B).

3.5.3. Fraction 3

No satisfactory spectral and chromatographic data was collected from Fraction 3's HPLC/MS analysis. It was possible to locate the peak on the chromatogram of the whole extract (Figure 3.16). Mass spectrum analysis suggested protonated adduct $[M+H^+]$ of 163.0. Other adduct was suggested to be ammonia adduct, $[M+NH_4^+]$ =180.2. The analysis of the fraction by means of high resolution mass spectroscopy suggested *m/z* of $[M+H^+]$ =163.0240 and formula of $C_6H_{10}OS_2$ and second *m/z* of $[M+Na^+]$ =185.0054 and formula of $C_6H_{10}NaOS_2$. By combining results above and results of comparison of the chromatographic and mass spectrum data of fraction 3 from *A. ampeloprasum* and of allicin from *A. sativum* (garlic) (Figure 3.16), it can be concluded that the fraction 3 contains compound allicin.

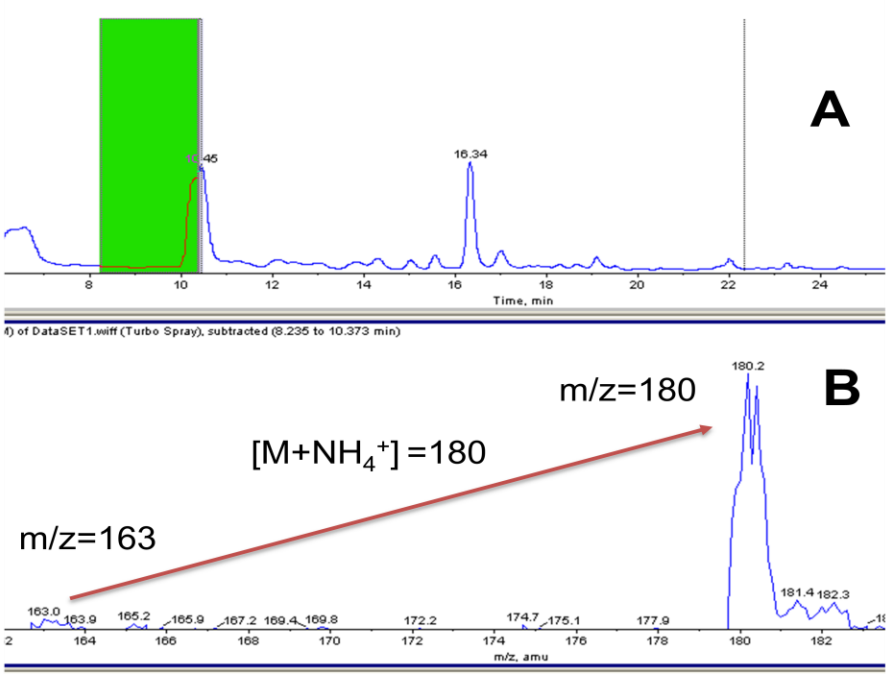

Figure 3.16. Part of HPLC chromatogram with UV detector at 254 nm of *A. ampeloprasum* whole extract (A). Mass spectrogram of selected peak in chromatogram (B).

3.5.4. Structure elucidation of nematicidal fractions from A. stipitatum

Two fractions out of four isolated from *A. stipitatum* exhibited nematicidal activity. They represented two major peaks in the chromatogram of the whole extract. Compounds found in the two nematicidal fractions were investigated further for their chemical structures using high resolution mass spectroscopy (HR-MS) and proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) methods.

3.5.5. Fraction 3

Fraction 3 of *A. stipitatum* was isolated with about 95% purity at 254 nm. Mass spectrum analysis suggested protonated adduct [M+H$^+$] of 174.3. Other suggested

adducts were [2M+H$^+$] =347.4 and methanol adduct [M+CH$_3$OH+H$^+$] =206.2 (Figure 3.17).

A high-resolution MS analysis indicated a protonated [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).

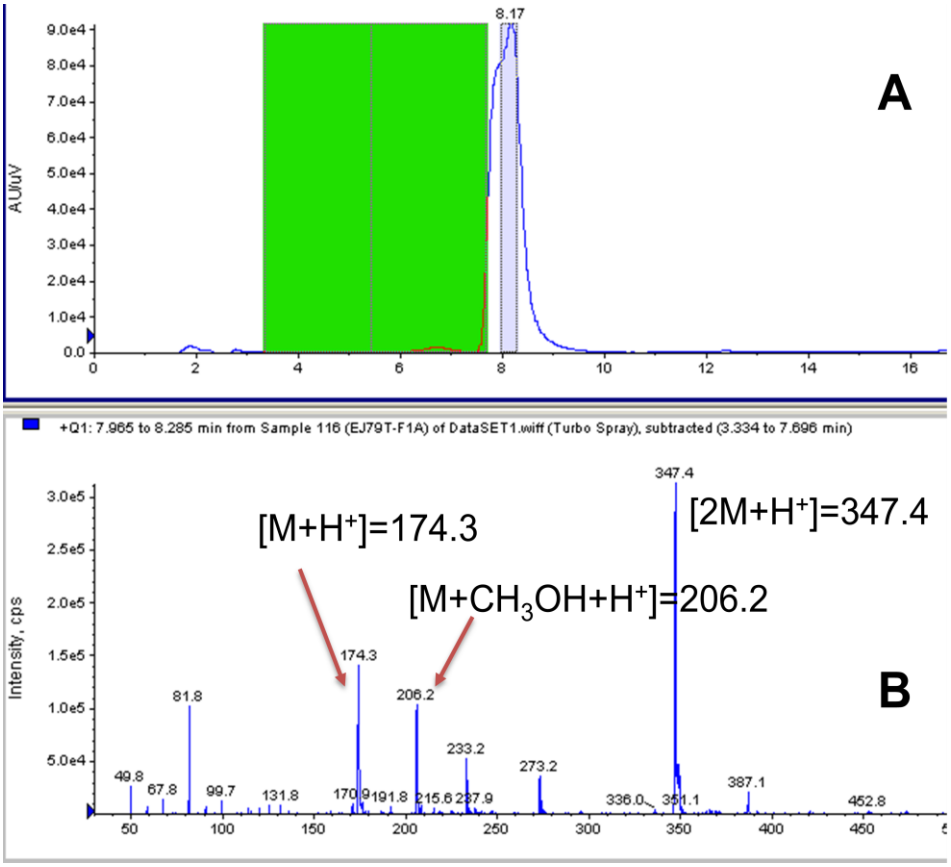

Figure 3.17. Chromatogram with main peak of fraction 3 of *A. stipitatum* (A). Mass fragments of the compound represented by the peak in chromatogram A (B).

3.5.6. Fraction 4

Fraction 4 of *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 (Figure 3.18).

A high-resolution MS analysis indicated a [M+H$^+$] ion at *m/z* 219.99 and a [M+Na$^+$] ion at *m/z* 241.98. 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).

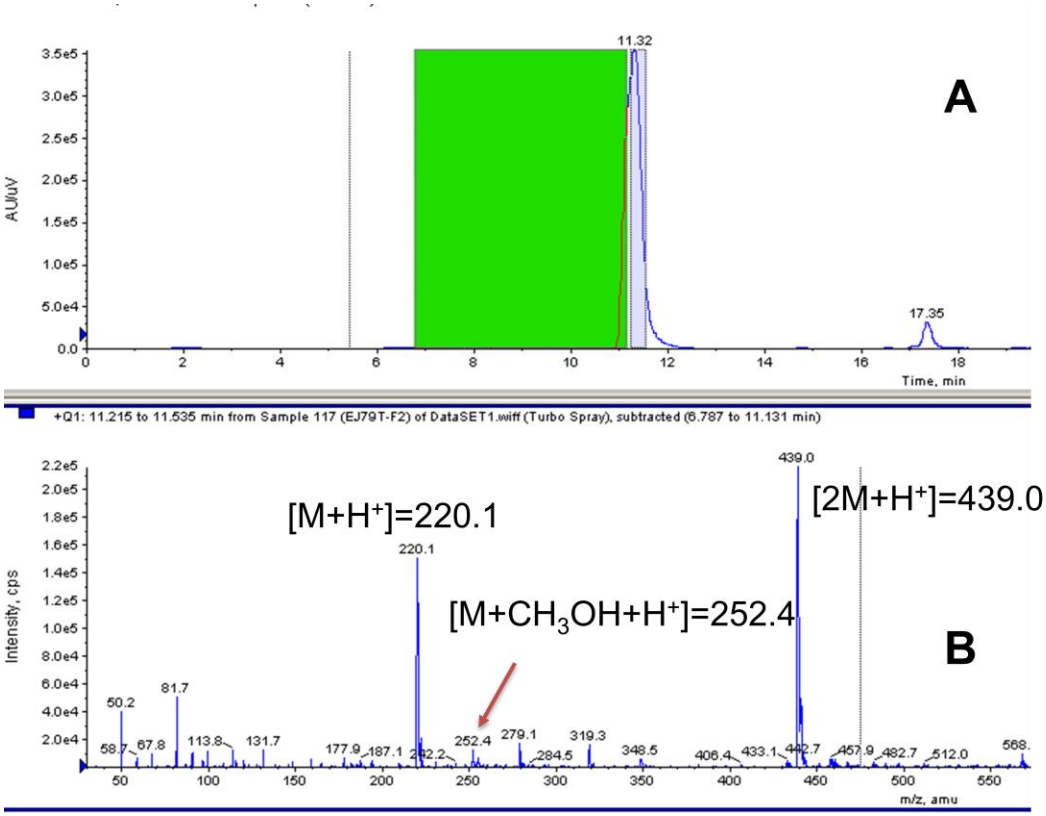

Figure 3.18. Chromatogram with main peak of fraction 4 of *A. stipitatum* (A). Mass fragments of the compound represented by the peak in chromatogram A.

4. DISCUSSION

4.1. Extraction process of plant material

Time required to obtain single *Allium* extract is usually 4-6 hours, which includes waiting time when material is immersed in phosphate buffer to ensure maximum transformation of cysteine sulfoxides into volatile sulfur compounds (Figures 1.8 and 1.9). Volatile sulfur compounds which were of high interest for the research are nonpolar compounds. Using “like dissolves like” principle, organic solvents with polarity similar to one of volatile sulfur compounds should be used for the extraction. Solvents such as, but not limited to, ethyl acetate, toluene, diethyl ether, chloroform, dichloromethane can be utilized for such purpose. All mentioned organic solvents are volatile as well and fall in polarity range of volatile sulfur compounds. Due to extensive interaction with solvents during the research period, it is critical to consider environmental safety and human health issues which may be caused by solvent disposal and vapors. Under mentioned conditions, ethyl acetate steps up as a better candidate for extraction than others. It was decided to use ethyl acetate to extract volatile sulfur compounds from ground *Allium* material. To maximize extraction rate and yield of the sulfur compounds from the plant material it is necessary to rotate the flask. This operation increases contact surface areas of the plant material and the solvent. Main obstacle which was hindering extraction with organic solvent is a presence of saponins in plant material which promoted formation of emulsion during extract. For example, extraction of *A. sativum*, *A. ampeloprasum* and *A. stipitatum* was very problematic due to fast formation of heavy and stable emulsion. Using minimum volume of the soaking buffer and greater volume of ethyl acetate and manually rotating the extraction mixture flask in a slowest way possible, although did not resolve the issue to the whole content, but provided some relief and possibility to obtain enough amounts of extract. It was important to dry organic solvent phase using drying agents such as sodium sulfate or magnesium sulfate. Although, sodium sulfate is more suitable than magnesium sulfate for drying ethyl acetate, to reduce costs, cheaper magnesium sulfate was used during production of extracts for screening purposes. Sodium sulfate, which is more expensive, was mainly used during preparative isolation of fractions of four *Allium*

extracts. Another main point of the extraction process is to carefully evaporate organic solvent under reduced pressure. Since, the goal is to obtain volatile sulfur compounds, evaporating extract until dryness will result in significant loss of target chemical compounds present in the extract. Therefore, it is recommended to stop evaporation process while there about 60 ml of the extract left in a flask. By distributing the residue into 3-5 glass vials (20 ml size) and carefully evaporating the organic solvent under slow flow of pure nitrogen gas, it is possible to obtain oily residue with negligible amounts of ethyl acetate. In some cases, the extracts were left in the working fume hood to prevent overevaporation of the extract and their weight change was controlled until stable weight of the extract was reached. The amount of the oil residue was different in each of the species, as not every *Allium* species may be rich volatile sulfur compounds. Due to difficulties of handling nematodes and very limited life span of the hatched nematodes it was difficult to use fresh *Allium* extracts. Hence, it was decided to initially finalize extraction of all available plant material and then start with nematicidal screening process. It is assumed that during the period when the extracts were stored at -20 °C, up to 6 weeks, less stable volatile sulfur compounds were converted into more stable ones.

4.2. Screening Allium extracts and pure compounds for nematicidal activity

Ethyl acetate extracts of total of 69 subjects - extracts of *Allium* bulbs, flowers and pure compounds previously reported to be found in *A. stipitatum* - initially were screened for their nematicidal activities.

Total of 10 test subjects exhibited nematicidal effect comparable to or higher than effect of Lannate. When evaluated based on their LC50 values, extracts of *A. sativum*, *A. ampeloprasum*, *A. paniculatum* were most active with values ranging between 0.086 mg/ml-0.14 mg/ml. The extract of *A. platyspathum* had LC50 value of 0.24 mg/ml. Values of *A. stipitatum* (Iran), *A. stipitatum* (7002), *A. zebdanense* and *A. aflatunense* and *A. moly* were very close to each other having values of 0.27 mg/ml and 0.28 mg/ml, 0.28 mg/ml, 0.29mg/ml and 0.30 mg/ml, respectively. It is suggested that these all

species are potential sources of nematicidal substances which also may be tested alone or in combination with each other.

Species *A. sativum* and *A. ampeloprasum* L. var. *ampeloprasum* (1025) both being representatives of section *Allium* under subgenus *Allium* are closely related. This relation explains strong garlic smell produced when both bulbs were crushed during extraction process of this study. Characteristic garlic smell caused by allicin formed when garlic bulbs are crushed. Presence of allicin was also confirmed by means of HPLC in *A. ampeloprasum* extract used in this study. Species *A. paniculatum* is related with *A. sativum* and *A. ampeloprasum* within subgenus *Allium* [91,92]. Thus, the similarity of their effects on nematodes is reasonable, as they possibly have similar compound(s) responsible for the activity. Polysulfides found in garlic and leek species have been previously investigated for their activity on nematodes. As a general rule, it was proposed that, polysulfides with certain number of sulfurs and shorter aliphatic chain will exhibit nematicidal effect. For example, dimethyl disulfide is more nematicidal than dimethyl trisulfide or dipropyl disulfide [83].

According to verbal information from Prof. Dr. Michael Keusgen and Dr. Reinard Frtisch (IPK, Gatersleben), species *A. stipitatum* and *A. aflatunense* also morphologically look similar and differ mainly in structure of leaves, and can be misidentified for each other. The similarity in chemical composition is shown in Results and other section of Discussion part.

4.3. Mechanism of action of commercial nematicides and sulfur compounds from Allium spp

Egg shells of root-knot nematodes contain vitelline, chitinous and glycolipid layers and are very resistant to harsh chemicals. Unhatched nematodes will survive much longer in the soil than hatched J2, as they are vulnerable to environmental stresses and needs to find a host as soon as possible [93]. Some host plants also found to have resistance genes to *Meloidogyne* spp. which make them less susceptible to the infection [94–98]. Volatile sulfur compounds are usually small molecules which may easier permeate

through phospholipid membranes [99]. Once the compounds pass inside the organism, they may act as by binding to thiol groups (-SH) of cysteine and glutathione (GSH) or inhibiting activity of acetylcholinesterase.

4.3.1. Mode of action of commercial nematicides

Acetylcholine is the major excitatory neurotransmitter controlling motor activities in many living things, and acetylcholinesterase (AChE) is an enzyme which hydrolyses and inactivates acetylcholine, thus allowing cholinergic neuron to return to its resting state after activation [100][101]. Therefore, AChE is essential for regulation of cholinergic transmission, and motor activities in whole.

It is known that aldicarb (Figure 4.1) is a carbamate insecticide which is the active substance of commercial pesticide Temik® [102]. It is a multifunctional pesticide which is effective against wide range of pests, but is primarily used as a nematicide. As all carbamates, aldicarb is a cholinesterase inhibitor which prevents the breakdown of acetylcholine in the synapse. It has been shown that aldicarb disrupts juvenile orientation and host recognition of a sugarbeet parasitic nematode *Heterodera schachtii*, but did not prevent root penetration [103]. Authors argued that the concentration it was used was not enough for root penetration and at higher doses carbofuran (Figure 4.3), also carbamate, reduced penetrations of *Meloidogyne incognita* and *Pratylenchus penetrans* parasitic nematodes [104].

Lannate is an insecticide, which is also reported to be used as nematicide [105–107]. The active component of this commercially available product is methomyl (Figure 4.2), which is also a carbamate [108].

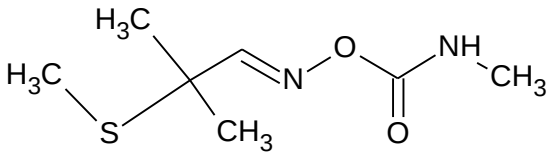

Figure 4.1. Chemical structure of aldicarb.

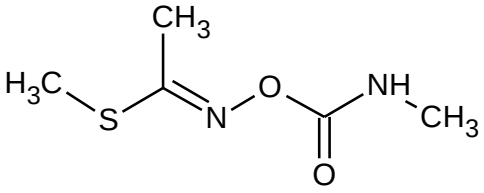

Figure 4.2. Chemical structure of methomyl.

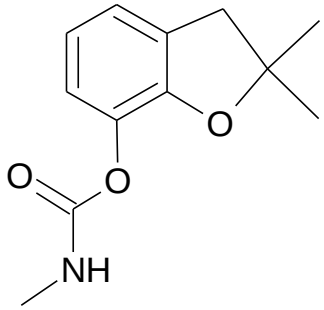

Figure 4.3. Chemical structure of carbofuran.

4.3.1. Inhibition of glutathione (GSH) or cysteine via reaction with their (-SH) thiol groups

Glutathione is a tripeptide, γ-glutamyl-cysteinyl-glycine, and de facto antioxidant tool present as part of defense mechanism in cells of most plants, animals, microorganisms and insects [109–113]. Analysis of *M. incognita* genes and proteins revealed presence of transcript of glutathione S-transferase (GST) gene *Mi-gsts-1* and protein with GST domain [114,115]. Authors of one study suggest that glutathione S-transferases are secreted during parasitism and are required for completion of the nematode life cycle in its host [114]. GST enzyme involved in detoxication and toxification mechanisms via

conjugation of reduced GSH and produced in an organism in response to oxidative stress [116]. A more recent study confirmed presence of GSH in *M. incognita* [117]. Allicin is a reactive sulfur species [118] has been known for its ability to react with thiol groups (-SH) of GSH and proteins, such as cysteine [119–121]. A more oxidized glutathione pool leads to a higher cellular redox potential. Oxidation of protein thiols can lead to changes in protein conformation, for example through disulfide bond formation, and therefore result in changes of functions of proteins [122]. Therefore, it is suggested that allicin may deplete GSH or influence enzymatic activity of cysteine in *M. incognita* and both mechanisms may be fatal for the nematode.

Extract of *A. stipitatum* was found to have compounds 2-(methyldithio)pyridine N-oxide, 2-[(methylthio)methyldithio]pyridine N-oxide and di(2-pyridyl) disulfide N,N'-dioxide (Figure 1.10). Both compounds may react with thiol group of glutathione or cysteine via thiol-disulfide interchange reaction. Thiol-disulfrde interchange is the reaction of a thiol (RSH) with a disulfide (R'SSR'), with formation of a new disulfide (RSSR') and a thiol (R'SH) derived from the original disulfide (Equation 1).

$$RSH + R'SSR' \rightarrow RSSR' + HSR' \quad \text{(Equation 1)}$$

Therefore, it is suggested that both compounds from *A. stipitatum* may act as nematicides by depleting glutathione levels or by influencing enzymatic activity of cysteine proteins in *M. incognita*.

4.3.2. Inhibition of acetylcholinesterase (AChE) enzyme

Several molecular forms of acetylcholinesterase with varying physical and chemical properties have been discovered in *M. incognita* and *M. arenaria* J2 [123,124] and AChE was suggested to be considered as a target in *Meloidogyne* control [125]. In a study by V. K. Singh and D. K. Singh [126], allicin was tested *in vivo* for its molluscicidal activity against *Lymnaea acuminata.* Authors of the study found that allicin exhibited molluscicidal action by inhibiting activities of AChE, lactic dehydrogenase and alkaline phosphatase. Another study [127] evaluated potential of pyrithione and related sulfur-

containing pyridine N-oxides from *A. stipitatum* to inhibit AChE activity. As a result, compounds 2-(methyldithio)pyridine N-oxide, 2-[(methylthio)methyldithio]pyridine N-oxide and di(2-pyridyl) disulfide N,N'-dioxide (Figure 1.10), naturally found in *A. stipitatum,* were found to be more active than other compounds tested. Based on these findings it is possible to assume, that allicin from *A. ampeloprasum* and sulfur-containing pyridine N-oxides from *A. stipitatum* exhibit their nematicidal action also by inhibiting activity of acetylcholinesterase enzyme of *M. incognita*.

4.4. Phytotoxicity effect of selected Allium extracts

4.4.1. Germination rate

After evaluation of germination rate of tomato seeds, it was found that *A. zebdanense* was least toxic to tomato seeds and 100% of the seeds germinated. Extracts of *A. ampeloprasum* (0.5042 mg/mL), *A. paniculatum* (0.4987 mg/mL), *A. moly* (0.5966 mg/mL) and *A. platyspathum* (0.5690 mg/mL) were also not so toxic to germination process of tomato seeds. The tomato seeds treated with these extracts had highest germination rate of 93.33% 93.33%, 95%, and 96.67%, respectively. Extract of *A. zebdanense* had not toxic effect at all to the seed germination and 100.00% of tomato seeds germinated.

Extract of *A. sativum* (0.4867 mg/mL) was mildly toxic to tomato seeds showing -17% variation from control and 83% germination rate.

Lannate had greater toxicity to tomato seeds with -53% variation value, inhibiting germination of almost half of the seeds.

Extracts of *A. stipitatum* (0.9866 mg/mL) and *A. aflatunense* (0.5680 mg/mL) were very toxic compared to all tested subjects with variation values of -80% and -97%, thus *A. aflatunense* having only 3% of the tomato seeds germinated. It is obvious that both extracts are very toxic to the tomato seeds. It may be speculated that compounds in the extract easily pass through seed coat, interfere with embryogenesis and prevent seed germination. Since, applied concentrations of extracts were LC100 values of

nematicidal concentrations, it may be suggested to test the extracts at lower concentrations.

Cress seeds looked to be more resistant to the toxicity and almost all the extracts had no toxic effect with 100% germination rate. Extract of *A. paniculatum* had low toxicity on cress seeds and 96.43% of the seed germinated. Cress seeds treated with Lannate looked to be stimulated and had 3.34% higher germination rate than untreated control.

Plant extracts generally were more toxic to lettuce seeds, with *A. ampeloprasum* and *A. moly* having maximum germination rate of 76.78% and 73.45% respectively. The extracts of *A. stipitatum* and *A. aflatunense* had highest toxicity to the cress seeds and only 38.45% and 36.78% of the treated seeds germinated. Lannate had mild toxicity and 75.19% of the lettuce seeds germinated.

4.4.2. Root development

After a seed has germinated development of its root is essential plant's healthy growth and development as it provides water and essential nutrients from soil. Root elongation is controlled by PLETHORA genes and their proteins [128,129]. Abundance of PLETHORA proteins decreases each time stem cells divide. When very little amount or no proteins left cells start to elongate and differentiate.

Root length in the tomato seeds was significantly inhibited by Lannate (100%) and the *Allium* extracts with values between 79.15%-100% of inhibition. Extract of *A. zebdanense* which had no toxic effect on seed germination of tomato plants had relatively least toxicity on root elongation with almost -80% variation from the control.

Cress seeds treated with *A. zebdanense* and *A. stipitatum* extracts did not yield results significantly different from each other and inhibited root length by 21% and 23% respectively. *A. paniculatum* extract inhibited the root length almost by 30%. Extract of *A. aflatunense* inhibited the root length of cress seeds by half and had 46% of inhibition,

while *A. moly* inhibited root length by almost 43%. Extracts of *A. sativum* and *A. platyspathum* were more toxic and inhibited root length by 66% and 64%, respectively. Extract of *A. aflatunense* seemed to stimulate the root development and the root length was more by 71%, compared to control. Lannate inhibited the root development in all seeds and had inhibition rate of 100%.

Surprisingly, DMSO+MES buffer solution had significant effect on root growth and roots were longer by 213% in lettuce seeds and 74% in cress seeds when compared to MES buffer alone (data not shown).

Lettuce seeds' root development was significantly inhibited by Lannate and *Allium* extracts. Lannate inhibited the root development in the seeds by 100%. Extract of *A. ampeloprasum* inhibited the root development by almost 56%, and *A. platyspathum* by almost 78%. All other *Allium* extracts had inhibition of more or equal to 90%.

It is suggested that sulfur compounds of the *Allium* extracts interfere with stem cell division. As stem cells don't divide at a regular rate, amount of PLETHORA proteins doesn't decrease which prevents initiation of cell elongation and therefore root elongation.

4.4.3. Shoot length

The shoot length of all seeds was significantly inhibited by Lannate with 94% for tomato, 94% for lettuce and 87% for cress seeds.

In tomato seeds the least toxic were *A. ampeloprasum* and *A. platyspathum*, which inhibited the shoot length by 50% and 52% significantly. The most toxic ones were *A. stipitatum* and *A. aflatunense* with inhibition rate of 99.6% for both extracts. It may be proposed that, due to relation between these two *Allium* species, possibly a common compound displays such a toxic effect upon the shoots.

In lettuce seeds, *A. sativum* and *A. aflatunense* were most toxic with 99% and 92%, respectively. Extract of *A. ampeloprasum* was least toxic to the shoot development and had 51% inhibition.

Cress seeds had their shoots' growth induced by extracts of *A. ampeloprasum* and *A. stipitatum* by 45% and 47%, respectively. Although, cress seeds treated with *A. zebdanense* and *A. aflatunense* extracts had 6% increase in shoot length, this development did not have statistically significant effect ($p>0.05$).

Solution of DMSO+MES buffer significantly promoted the shoot growth in lettuce and cress seeds by 134% and 136%, respectively, when compared to MES buffer alone (data not shown).

Root development is important factor in healthy plant growth and development. Root lengths of tomato and lettuce plants treated with *Allium* extracts were significantly reduced which may be leading factor of poor shoot elongation. As it is told in virtually every elementary textbook of plant physiology, plant hormones such as ethylene, auxin, gibberellin, and abscisic acid regulate plant tropism. It is possible to suggest that sulfur compounds from *Allium* extracts interfere either with production of any of the growth hormones or directly with the hormones.

4.4.4. Additional information of mechanism of action of some sulfur compounds in phytotoxicity assay

Given that extracts of *Alliums* were applied at their maximum calculated nematicidal concentrations, it is suggested to test the extracts at lower concentrations or better with consecutive concentrations to get clear picture of dose-dependent phytotoxicity of the extracts.

In a study [122] where phytotoxicity of allicin was evaluated against *Arabidopsis thaliana* it was found that allicin inhibited primary root elongation in a concentration-dependent manner and exposure of the seedlings to a much higher concentration of allicin (500 µM) led to their bleaching. Authors suggested that allicin, by reducing GSH

levels of the plant inhibits seed germination and primary root growth. They confirmed their hypothesis by testing mutant *A. thaliana* which only had 20% of normal GSH levels against wild type with normal GSH levels. The mutant plant was approximately 3 times more sensitive to allicin than the wild type and seed germination and primary root growth was inhibited at lower allicin concentrations. Extracts of *A. sativum* and *A. ampeloprasum* have allicin as their main component. Based on the finding above it is possible to claim that both extracts inhibit seed germination and root elongation due to allicin's reactivity with GSH. The phytotoxicity results obtained in our study correlate with aforementioned result in terms of toxicity exhibited by garlic extract. Garlic extract showed to inhibit root development of tomato and lettuce seeds by 100% and cress seed by more than 60%. While this concentration may be solely attributed to allicin alone, it may also be due to effect of combination of other sulfur compounds.

Diallyl disulfide is another compound found in *Allium sativum* extract and authors of one study [130] examined its effect on seed germination, root growth, mitotic index, and cell size in root meristem, as well as the phytohormone levels and expression profile of auxin biosynthesis genes (FZYs), auxin transport genes (SlPINs), and expansin genes (EXPs) in tomato root. They found that, while lower doses of diallyl disulfide significantly promoted root growth of tomato plant, higher doses inhibited its growth. Root growth inhibition was caused by diallyl disulfide affecting both the length and division activity of meristematic cells, however the cell width of the root meristem was not affected. After several days of treatment by diallyl disulfide at 0.41mM expression levels of EXP genes were increased which promoted the growth of the plant. In addition, diallyl disulfide affected levels of phytohormones in a dose-dependent manner. Levels of indole-3-acetic acid and zeatin riboside phytohormones were highest in samples with the highest amount of diallyl disulfide, however levels of gibberellic acid were highest in samples treated with the lowest diallyl disulfide concentration.

Compounds of *A. stipitatum* extract, 2-(methyldithio)pyridine N-oxide, 2-[(methylthio)methyldithio]pyridine N-oxide and di(2-pyridyl) disulfide N,N'-dioxide (Figure 1.10), also may exhibit phytotoxicity by reacting with glutathione in the plant cells via thiol-disulfide exchange reaction (Equation 1)

4.5. Brine shrimp lethality assay

In brine shrimp lethality assay *A. paniculatum* was least toxic to the shrimps with 89% survival rate, comparable to Lannate's of 83%. Extract of *A. sativum, A. ampeloprasum* and *A. platyspathum* were most toxic to the shrimps and none of them survived. Survival rates of brine shrimps treated with extracts of *A. aflatunense* and *A. stipitatum* were also very low and were of 3.70% and 1.9%, respectively.

Brine shrimps are known to have an internal system of detoxification. One component of such a system is glutathione S-transferase (GST; EC 2.5.1.18), a family of enzymes catalyzing the conjugation of reduced glutathione (GSH) to a wide variety of electrophilic compounds [131]. Therefore, it is possible to suggest that death of *A. salina* is the result of depletion of GSH in the organism as *Allium* sulfur compounds bind to thiol group of the GSH.

4.6. Overall toxicity of Allium extracts based on cumulative phytotoxicity and brine shrimp lethality results

Several data sets have been generated from phytotoxicity and brine shrimp lethality assays. An attempt to evaluate an overall toxicity of *Allium* extracts based on cumulative phytotoxicity and brine shrimp lethality results is presented herewith. It is suggested to average the phytotoxicity data (variation % from control) per test plant per *Allium* extract and add it to 100 (%) to obtain final number which will show overall effectiveness of an *Allium* extract on the growth of a specific test plant. It is possible to represent a growth percentage of test plant as an equation (Equation 2) below:

Equation 2:

$$Growth\ Percentage\ (\%) = 100 + \frac{Germination\ rate + Root\ length + Shoot\ length}{3}$$

Calculated growth percentage per test plant per *Allium* extract combined with brine shrimp survival percentage and nematicidal LD100 values is presented in Table 4.1.

Table 4.1. Calculated growth percentage per test plant, brine shrimp survival percentage and nematicidal LD100 per *Allium* extract. SD=standard deviation (n=3). SD for brine shrimp survival percentage and LD100 have been presented in previous related sections.

EXTRACT	TOMATO GROWTH %	SD	LETTUCE GROWTH %	SD	CRESS GROWTH %	SD	BRINE SHRIMP SURVIVAL %	LD 100 (mg/ml)
A. sativum	31.51	7.68	4.78	5.38	58.06	5.91	0.00	0.4867
A. paniculatum	43.77	4.71	37.43	8.18	83.83	4.77	88.89	0.4987
A. ampeloprasum	54.69	4.46	56.46	7.57	138.78	2.73	0.00	0.5042
A. aflatunense	1.25	6.30	14.85	9.96	85.42	1.37	3.70	0.5680
A. platyspathum	51.06	4.73	34.88	6.92	74.07	2.09	0.00	0.5690
A. moly	38.39	4.13	33.01	4.37	78.59	4.43	31.48	0.5966
A. stipitatum	6.80	9.41	20.97	7.62	107.99	2.75	1.85	0.9866
A. zebdanense	41.68	6.03	22.09	4.91	95.00	1.89	66.67	0.9882

The ideal nematicidal extract would have high toxicity against *M. incognita* nematodes at a low concentration and lowest possible or no toxicity to the crop and environment. Based on the data in Table 4.1 it is not quite possible to determine any ideal *Allium* extract, as plant extracts are rather complex matrices with many compounds which may exhibit various effects under various conditions. Ranking of *Allium* extracts based on fewer and prioritized criteria may be a more purposeful. For example, if nematode infested plant is a tomato plant, then tomato phytotoxicity criterion may be considered as a primary one. As volatile sulfur compounds are not stable for a long time, their environmental impact may be not detrimental. In addition, low water solubility and volatility of the *Allium* volatile sulfur compounds will result in greater evaporation rather than accumulation in natural water resources.

4.7. Bioactivity guided fractionation of selected Allium species

4.7.1. A. stipitatum

One of the interesting *Allium* species is probably *A. stipitatum*. It has been intensively used in Central Asia and Iran as a medicinal and edible plant [82]. Several

aromatic sulfur compounds have been reported to be found in *A. stipitatum* previously [48,82,132]. In their study, Kubec et al. reported two major peaks (74%) found in a diethyl ether extract of *A. stipitatum* when analyzed by means of HPLC-PDA at 210 nm. The peaks identified to be 2-(methyldithio)pyridine N-oxide and 2-[(methylthio)methyldithio]pyridine N-oxide. These findings are in accordance with findings reported in this study. Two *A. stipitatum* species from different collection sites were used in this study. Species of *A. stipitatum* collected in Iran was used for screening in nematicidal assay as well as in phytotoxicity and brine shrimp lethality assays. Species of *A. stipitatum* collected from Afghanistan was used in preparative HPLC isolation of fractions. Both extracts had same two major peaks of 2-(methyldithio)pyridine N-oxide and 2-[(methylthio)methyldithio]pyridine N-oxide. One main difference noted between chromatograms of the extracts is the absence of compound with molecular weight of 252 in the extract of *A. stipitatum* collected from Afghanistan. This compound is assumed to be dipyrithione (2,2'-dithiobis(pyridine-N-oxide)). The peak, assumedly representing dipyrithione, was approximately less than 5% of the whole chromatogram. Commercially available pure dipyrithione was also tested in nematicidal assay and did not show significant effect therefore it is possible to assume that, it would not contribute significantly to the nematicidal activity alone, although, synergistic effect of all aromatic sulfur compounds altogether is not excluded. Compound 2,2′-dipyridyl disulfide(Aldrithiol-2) was detected in small amounts in *A. stipitatum* from Iran, but was not detected in *A. stipitatum* from Afghanistan when compared to the HPLC-MS data of the authentic sample. When chromatograms of *A. stipitatum* from Iran and Afghanistan were compared, other compounds, which were common and different were noticed. While it is not possible to provide complete structure of them, as they were not of interest in terms of nematicidal activity and were not investigated further, it may worth to provide some information about them. Extract of *A. stipitatum* from Iran had compounds with *m/z* 204, 237, 284 and 314. While none of these compounds was reported by O'Donnel et al., only *m/z* 237 was reported by Kubec et al [132]. By using positive ion Direct Analysis in Real Time Mass Spectrometry (PI-DART-MS), proposed molecular formula was $[C_{10}H_8N_2OS_2 + H]^+$, with corresponding compound of di(2-pyridyl) disulfide N-oxide. It is proposed to be a transitional compound

at a final stage of the biosynthesis of pyridyl-containing compounds from pyrithione. Compounds with *m/z* 237, 284 and 314 were also present in *A.stipitatum* from Afghanistan, but instead of *m/z*=204, the peak with same elution time (18') had *m/z*=228. Also, a compound with *m/z*=244 was detected in the mass spectrum in *A. stipitatum* from Afghanistan, but was not detected in *A. stipitatum* from Iran. It is obvious that, working with *Allium* extracts from different collection places may yield different compounds, since sulfur soil content is one of the main factors affecting synthesis of sulfur compounds. Also, collection times as well as light amount, temperature and soil moisture content may influence biosynthesis of volatile compounds. The extraction process is also critical point, since extraction and evaporation durations and temperatures will affect amount of volatiles in the extract as well as formation of other artifacts. Isolation of fractions was performed using preparative HPLC method at 254 nm, therefore compounds which don't have chromatophore may also be present in the isolated fractions.

4.7.2. A. ampeloprasum

The nematicidal fraction of *A. ampeloprasum* was found to have allicin. Allicin is extensively researched for its biological activity *in vitro* and *in vivo*, ranging from antimicrobial to anticancer activities.

Gupta and Sharma investigated allicin against *M. incognita* in 1993 in India [84]. In this study, allicin was isolated from ethanol extract of garlic gloves and its action on nematode juveniles, egg hatching and root penetration was studied. The mortality of juveniles was assessed over 74 hours and after 48 hours allicin concentrations of 2.5 µg/ml, 5 µg/ml and 12.5 µg/ml had mortality percentages of 64%, 96% and 100%, respectively. Allicin inhibited hatching of *M. incognita* at concentrations as low as 0.5 µg/ml.

In this study, the LC50 value of the fraction with allicin was between 39-78 µg/ml after 48 hours. One of the reasons of lower activity of the allicin in current study compared to the study in by Gupta and Sharma in 1993, may be different collections of *M. incognita*.

Next reason may be instability of the allicin, so it might have started converting since extraction process and through separation by HPLC and evaporation under reduced pressure. Allicin decomposition products will produce ajoene and allyl sulfides and the composition of the products is dependent on factors such as temperature and polarity of the solvent. Another possible reason may be contamination of the fraction with a grease material used to lubricate stopcock area of a separatory funnel. Separatory funnel was used to extract allicin from HPLC isolated fraction by using liquid-liquid extraction with ethyl acetate. Some of the lubricant may have passed in the collected ethyl acetate phase and remained as part of allicin fraction which is used in the nematicidal activity. This might have increased the weight of the fraction thus resulting in dilutions with allicin amount lower than intended. As allicin and other *A. ampeloprasum* fractions were tested for purity using HPLC/MS, several *m/z* patterns, which may belong to polyethylene glycol, noticed in mass spectrum of the fractions. These patterns heavily masked the real *m/z* of the target compounds and it was not possible to confirm their structures by means of NMR. Due to insufficient plant material it was not possible to isolate more fractions and repeat the nematicidal assay.

4.7.3. A. platyspathum and A. paniculatum

Fractions collected from *A. platyspathum* and *A. paniculatum* did not have nematicidal effect within tested concentration ranges. HPLC/MS analysis of fractions revealed no significant peaks and mass fragments. Due to this fact it is possible to propose that, the compounds collected at 254 nm wavelength were not stable enough for at least 48 hours. Whether those fractions were bioactive is also not possible to say as there may be other compounds present and not detectable at wavelength of 254 nm. They may be detected at other wavelengths as well lack chromatophore, which will prevent them from being detected using UV detector. For both cases it is suggested to investigate the extracts either at different wavelengths or isolate fractions by means of column chromatography or preparative thin layer chromatography.

4.8. Previously reported phytotoxic and nematicidal sulfur compounds from Allium spp.

Dimethyl disulfide (DMDS), a compound found in garlic, was tested against potato cyst nematodes and root knot nematodes and found to be effective at all three concentrations (3 ml, 6 ml and 8 ml per 10 litre of soil) [133]. While at concentration of 8 ml/10 L DMDS was found to be toxic to plants, at a lower concentration of 3ml/10L no toxicity effect on the growth of tomato plants was observed. Low concentration (3 ml) of DMDS in 10 L soil is considered the best concentration for management of potato cyst nematodes, root knot nematodes and plant parasitic nematodes in general in the soil.

A thiosulfonate, allygrin (trans CH_3-CH = $CHSO_2$-SCH_3) was reported to be found in *A. grayi* along with five thiosulfinates isolated from an ether extract of *A. fistulosum* var. *caespitosum*. All compounds exhibited nematicidal activities at various levels with thiosulfinate compounds showing stronger nematicidal activity than disulfides and thiosulfonates with the same alkyl groups [83].

Asparagusic acid, a sulfur containing compound, although occurring naturally in roots of asparagus (*A. officinalis* var. *altilis* L.), was reported to inhibit hatching of *Heterodera rostochiensis* and *H. glycine* nematodes and killing *H. rostochiensis* (mortality 99%) and *Meloidogyne hapla* (92%), and the larvae and adults of *Pratylenchus penetrana* (82%), and *Paratylenchus curvitatus* (82%) at 50 ppm after 145 hours [134].

One of the remarkable success stories is the story of commercial garlic-based nematicidal product, NemGuard, developed by ECOspray, a UK-based business [39]. The company developed a highly refined garlic extract combining it with granulation technology, thus produced a nematicidal product capable of delivering efficacy comparable to synthetic products such as Temik (aldicarb) and Vydate (oxamyl) and Nemacur. It has been successful in field trials and already been approved by EU and UK commissions. The composition of NemGuard is presented in patent application [135] as follows:

“Analysis of the concentrate by HPLC shows the total polysulphides present are in the range of 2.4 to 3.6% w/w. Of these polysulphides, diallyl sulphides of the formula RSR, RS_2R, RS_3R and RS_4R (R= allyl group of the formula $-CH_2CHCH_2$) are present in the approximate ratio of 4%-5%:5%-8%:31%-38%:19%-22% as weight % of the total polysulphides present. These polysulphides collectively account for approximately 66% +/- 10% of all the organo-sulphur species present in the concentrate as determined by HPLC”.

Polysulfides and diallyl sulfides are more stable compounds than allicin, and in fact hydrolysis of allicin under certain conditions will yield allyl sulfides. Synergistic effect of the formed compounds must be strongly considered.

5. CONCLUSION

It is the first comprehensive study evaluating EtOAc extracts of *Allium* plants against plant parasitic nematode, *Meloidogyne incognita.* Another significant aspect of this study is evaluation of nematicidal extracts for plant and environmental toxicity.

It is worth noting that extracts are complex matrixes containing numerous compounds with possibly various effects when evaluated separately. Possible synergistic effect of compounds also should not be undermined. The concentration and ratio of the compounds will vary based on growth conditions, collection season and storage conditions. Root-knot nematodes have been a serious problem in agriculture for many years, possibly comparable to cancer disease in humans. Solutions based on synthetic chemicals have complications such as human and environmental toxicity and several have been banned due to same reasons. Therefore, it is obvious that there is a need for “green” pesticides. Ideal pesticide is expected to have an effect towards wide range of pests, while have no harm to the plants and the environment. Considering the economic damage caused by root-knot nematodes, such as *M. incognita*¸ a pesticide effective against only root-knot nematodes may also be considered a success. Combinations of various compounds may also be considered as part of solution, since nematode control seem to be complex problem involving toxicity of a nematicide to the host plant and environment.

Overall, promising results are obtained from this study and further detailed evaluation of extracts of *Allium* species may be advised. Additional bioactivity guided fractionation of *A. platyspathum* and *A. paniculatum* with an undamaged preparative HPLC column may yield better results. For example, *A. paniculatum* extract had only around 20% lethality effect on brine shrimps being closest to the blank results among other *Allium* extracts. Two compounds obtained from *A. stipitatum* may be synthesized in larger amounts in order to perform additional phytotoxic and brine shrimp lethality evaluations. They are reported to be previously patented in Japan as pesticides [48] (Nakayama, K.; Hisada, Y.; Yamashita, N. Jap. patent, JP 61056104, 1986; Sumitomo Chemical Co., Ltd. Jap. patent, JP 59163368, 1984), but unfortunately it was not possible to obtain patent texts. Extracts of *A. moly* and *A. zebdanense* also may worth

extra trials, as they had strong nematicidal activity, while being moderately toxic to plants and brine shrimps. As the stocks of *A. moly* and *A. zebdanense* depleted, it was not possible to investigate them further. *Allium* species, such as *A. aflatunense* and *A. stipitatum,* with high phytotoxicity and high nematicidal activity also may be used to treat soil before planting the seeds, thus disinfecting the soil while minimizing the harm to the germination and development of the plants.

Table 5.1. *Allium* extracts ranked based on their biological activities. Extract with Rank 1 has lowest toxicity to test plants and brine shrimps, but has highest toxicity to *M. incognita* nematodes. Extract with Rank 8 has highest toxicity against test plants and brine shrimp, but has lowest toxicity to *M. incognita* nematodes.

RANK	TOMATO	LETTUCE	CRESS	BRINE SHRIMP	NEMATICIDAL
1	*A. ampeloprasum*	*A. ampeloprasum*	*A. ampeloprasum*	*A. paniculatum*	*A. sativum*
2	*A. platyspathum*	*A. paniculatum*	*A. stipitatum*	*A. zebdanense*	*A. paniculatum*
3	*A. paniculatum*	*A. platyspathum*	*A. zebdanense*	*A. moly*	*A. ampeloprasum*
4	*A. zebdanense*	*A. moly*	*A. aflatunense*	*A. aflatunense*	*A. aflatunense*
5	*A. moly*	*A. zebdanense*	*A. paniculatum*	*A. stipitatum*	*A. platyspathum*
6	*A. sativum*	*A. stipitatum*	*A. moly*	*A. ampeloprasum*	*A. moly*
7	*A. stipitatum*	*A. aflatunense*	*A. platyspathum*	*A. platyspathum*	*A. stipitatum*
8	*A. aflatunense*	*A. sativum*	*A. sativum*	*A. sativum*	*A. zebdanense*

To rank overall effectiveness of *Allium* extracts, it is proposed to rank columns in Table 4.1. in the order of decreasing activity as shown in Table 5.1. Based on this rating, it is possible to rank extract of *A. ampeloprasum* as the most effective extract overall. The extract has lowest toxicity against tomato, lettuce and cress and very high toxicity against *M. incognita* nematodes. Although *A. ampeloprasum* ranked number 3 for its nematicidal activity, its LD100 value (0.5042 mg/mL) can be considered almost equal to the LD100 values of *Allium* extracts with nematicidal ranks 1 (*A. sativum*, 0.4867 mg/ml) and 2 (*A. paniculatum*, 0.4987 mg/mL). Brine shrimp toxicity of *A. ampeloprasum* extract is very high with 0.00 % survival, but can be ignored due to low water solubility of the extract.

Extract of *A. paniculatum* can be considered as the second most effective extract as it has low toxicity against tomato and lettuce but moderate toxicity towards cress. It has highest survival percentage of the brine shrimp with almost 90% survival. The nematicidal activity of *A. paniculatum* is also very high and comparable to the activity of *A. ampeloprasum.*

Extract of *A. platyspathum* can be ranked as the third most effective extract. It has low toxicity to tomato (51% for growth percentage), lettuce (35% growth percentage) and cress (74% growth percentage). The extract is very toxic to brine shrimps, but can be ignored due to its low water solubility. The nematicidal activity is very high with LD100 value of 0.5690 mg/ml which is comparable to the LD100 values of first two *Allium* extracts.

Extracts of *A. sativum*, *A. aflatunense*, *A. moly*, *A. stipitatum*, *A. zebdanense* although having considerable nematicidal activity, are very toxic to the tested plants. It is important to use *Allium* extracts with stable sulfur compounds. The investigation of discreet stable sulfur compounds may yield more concrete results. Therefore, it is suggested to have further investigation with the mentioned extracts with confirmed stability of sulfur compounds.

By this study it may be demonstrated that, genus *Allium* may be rich in species which can bear nematicidal compounds or combinations of compounds. In this study almost 14% of the tested *Allium* extracts showed to have high nematicidal effect. If we generalize it, then about 100 more species (out of accepted 750 species) may be waiting for discovery. Sulfur chemistry of genus *Allium* is rather rich and more structured strategy may be needed. Thus far, only a few simple sulfides, thiosulfinates and thiosulfonates have been reported in literature for their nematicidal effects. Aromatic sulfur compounds from *A. stipitatum* are lending support to the idea of possible potential. Commercial product Nemguard (ECOspray) is using a refined garlic juice and as ECOspray claims, they also have other garlic-based products targeting other pests, already approved or in the pipeline. Therefore, it may be concluded that genus *Allium* has a great potential for academic and commercial nematicidal research.

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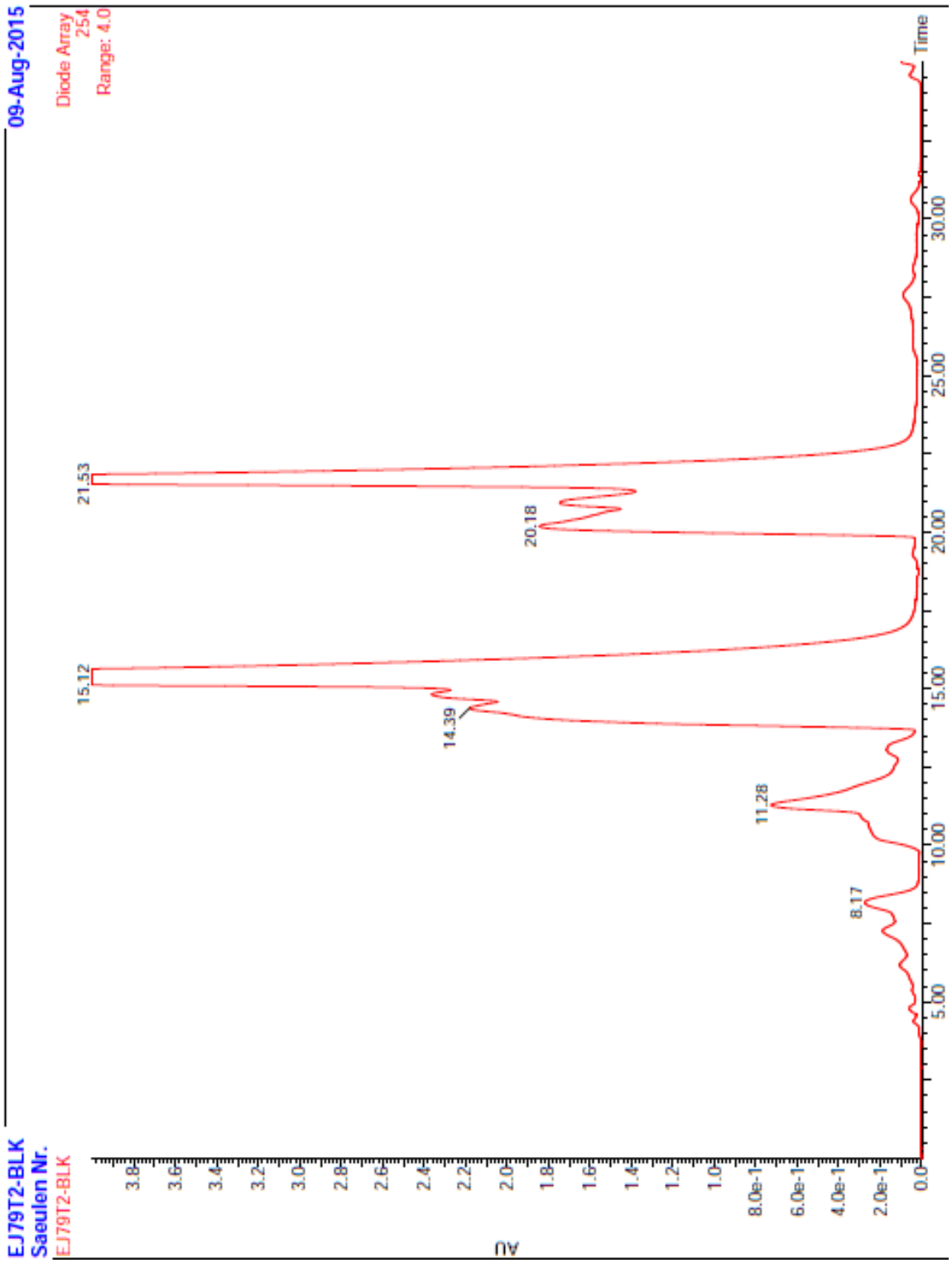
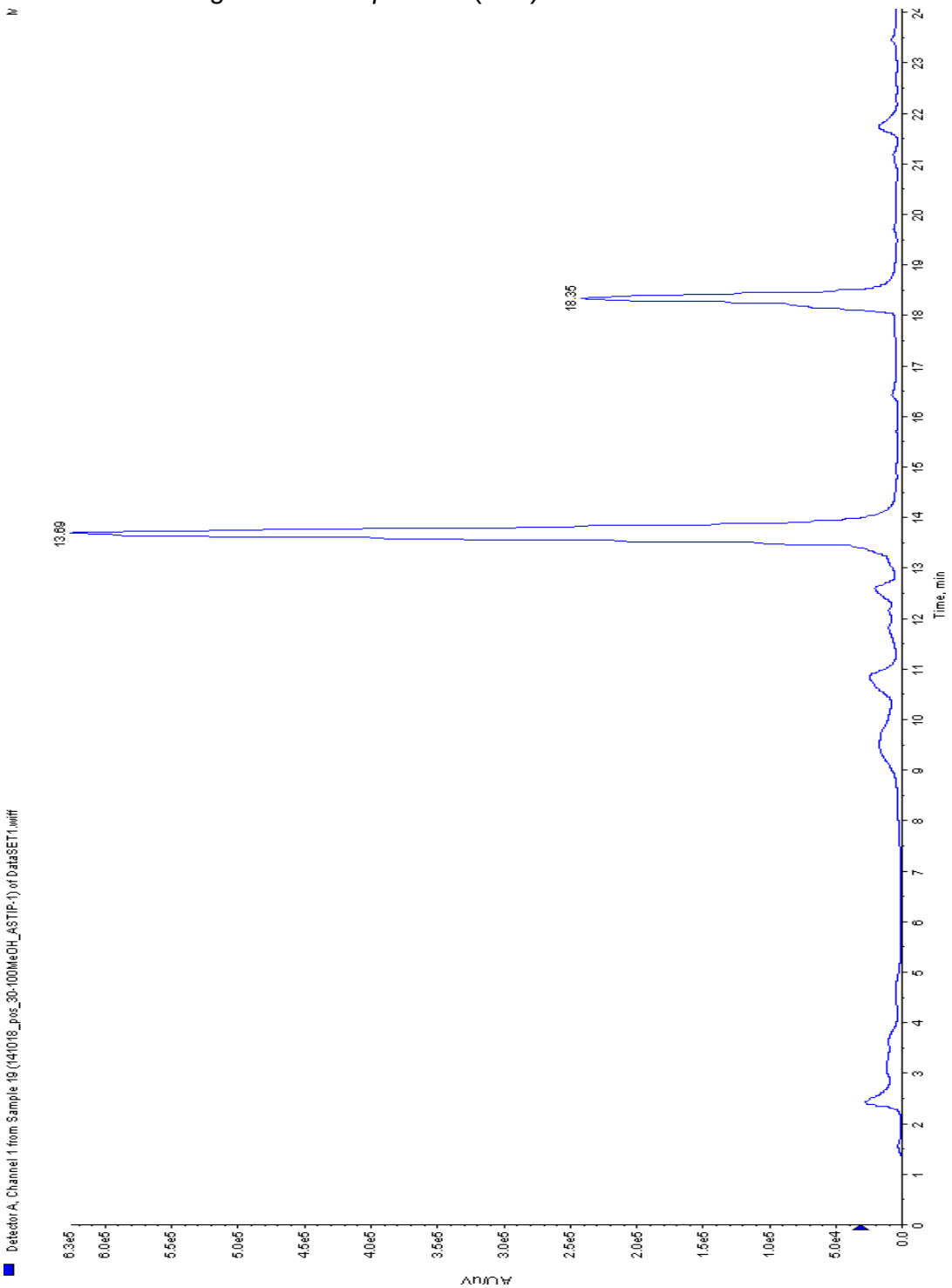
7. APPENDIX

7.1. Chromatographic and spectroscopic data

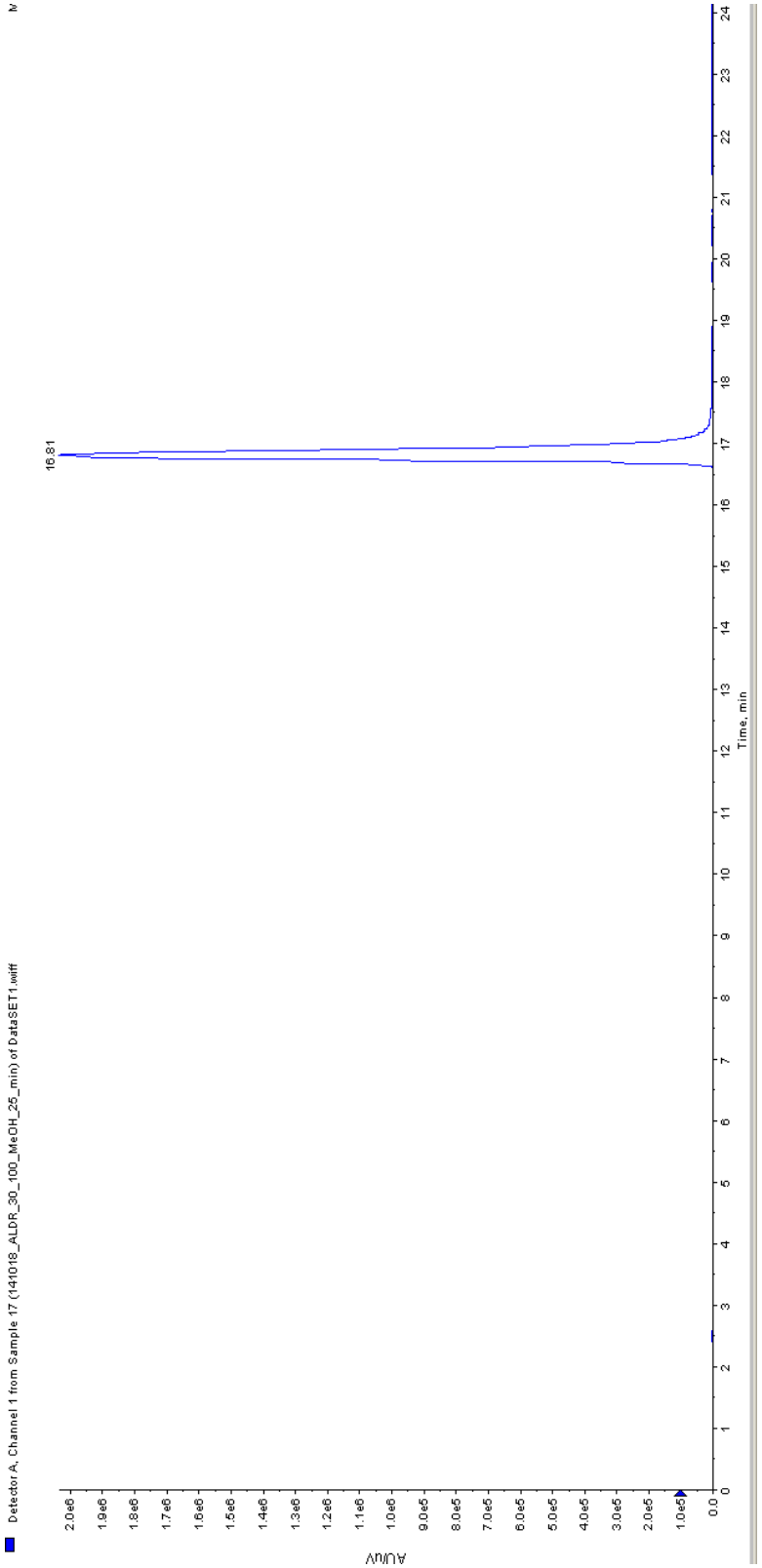
Preparative HPLC Chromatogram of 7002 *A. stipitatum*



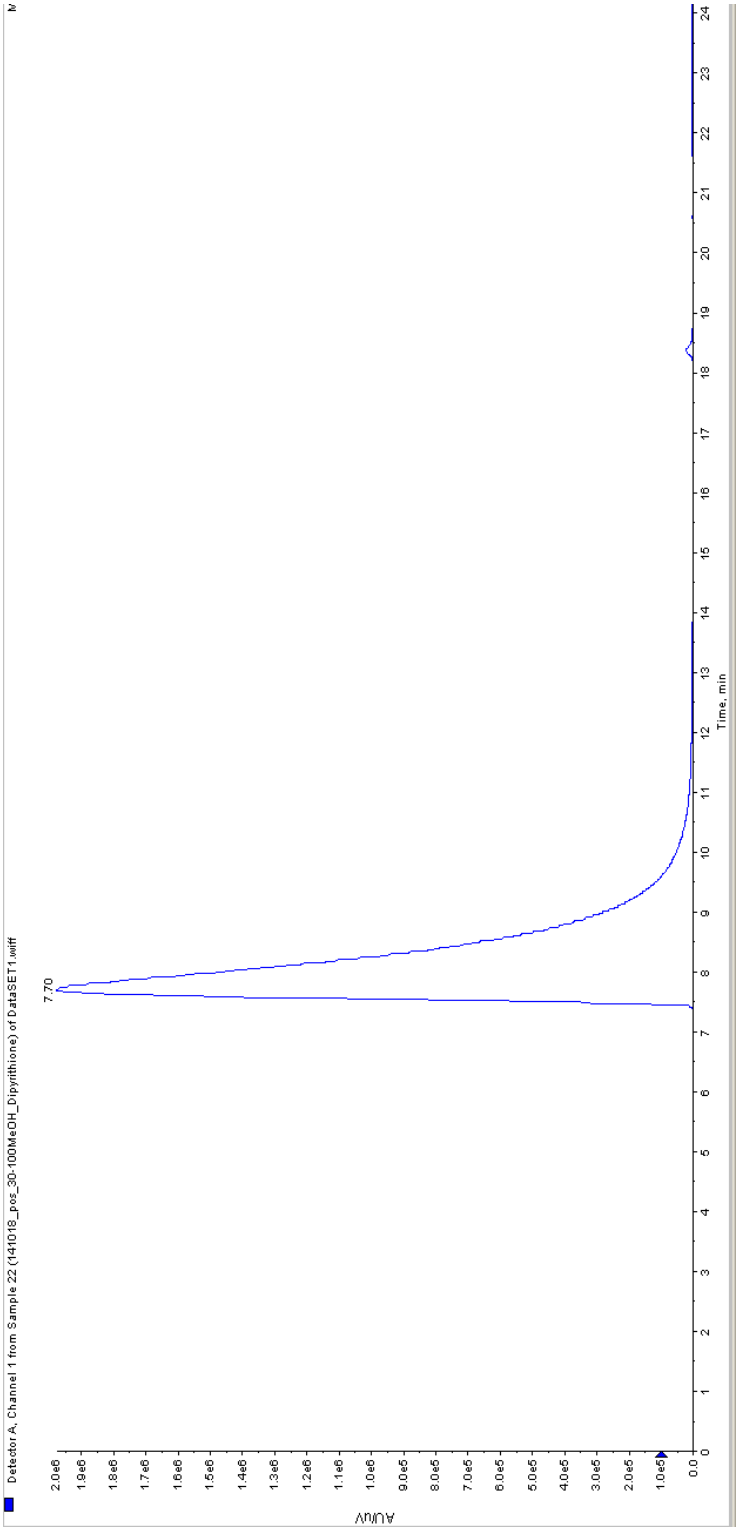
HPLC Chromatogram of *A. stipitatum* (Iran)



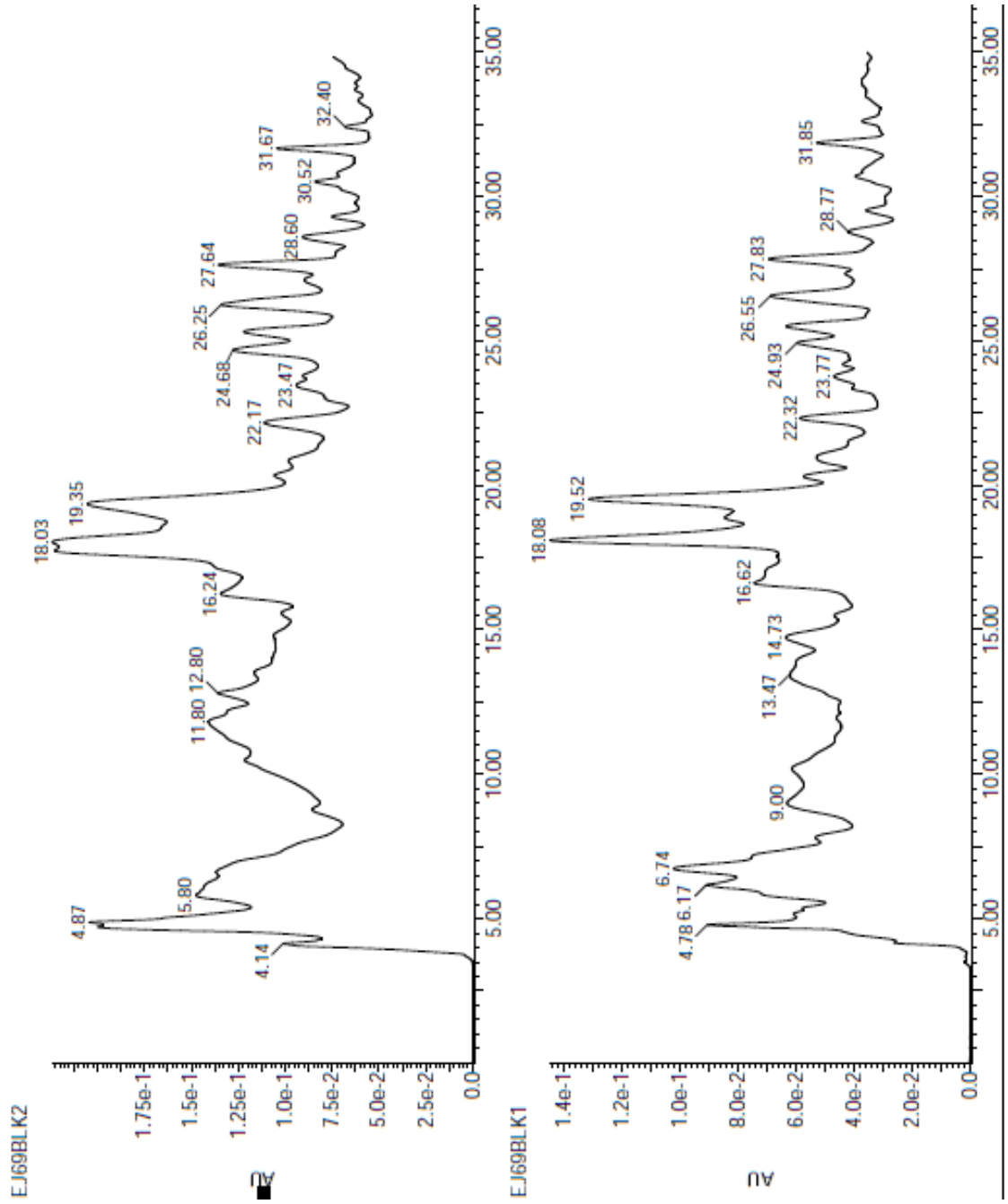
HPLC Chromatogram of Aldrithiol-2® (2,2′-dipyridyl disulfide) standard



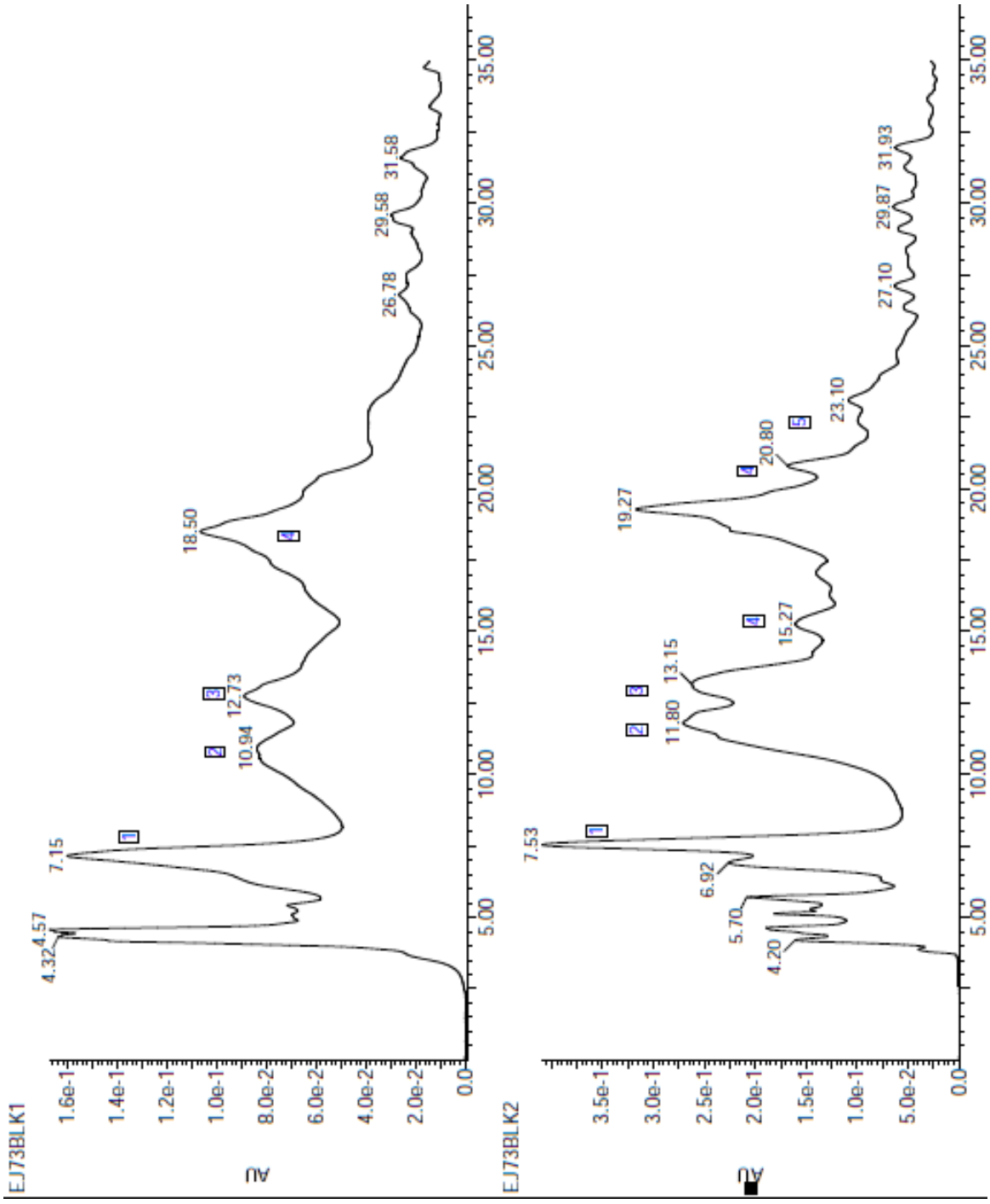
HPLC Chromatogram of dipyrithione (2,2'-dithiobis(pyridine-N-oxide)) standard



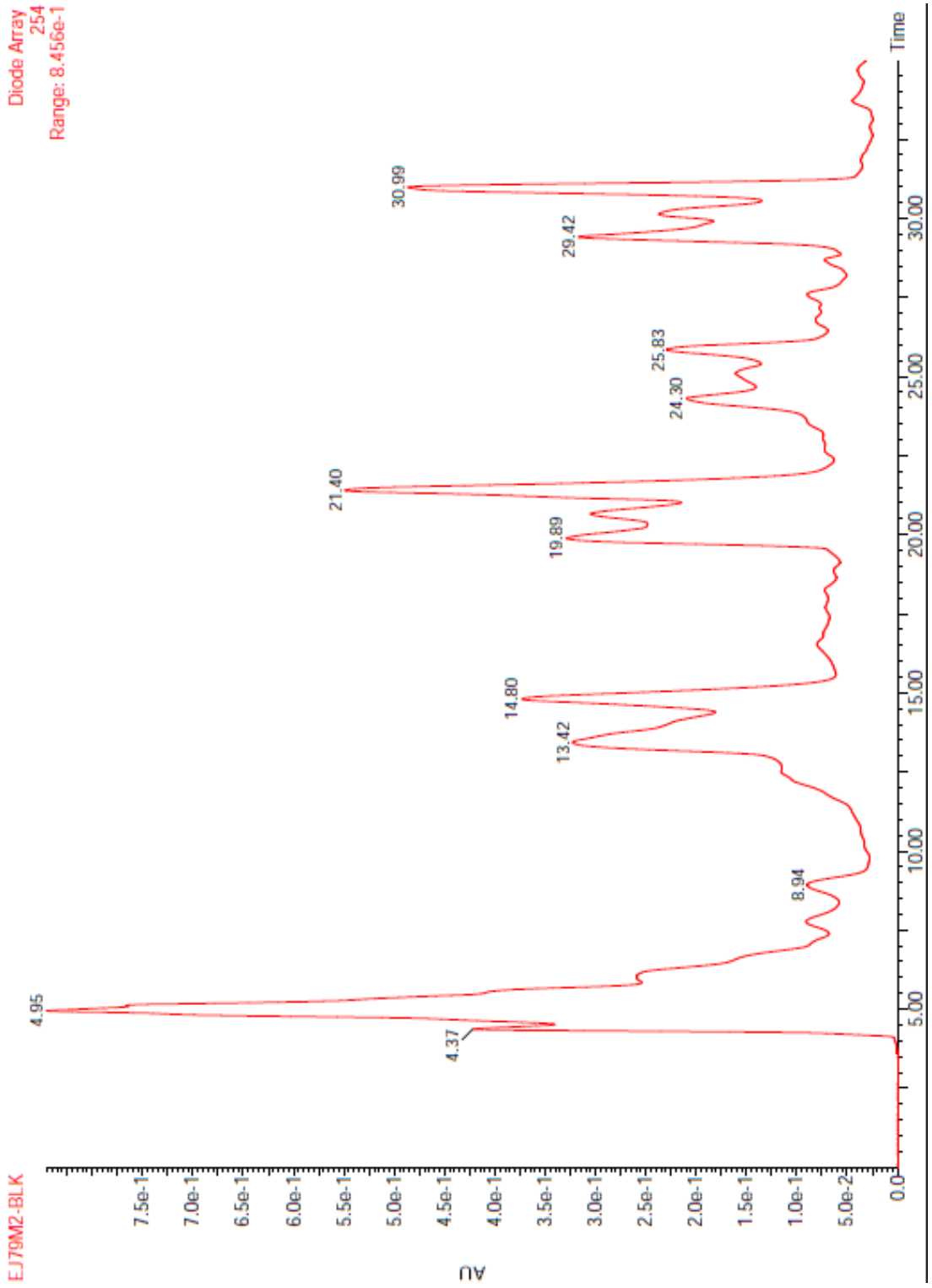
Preparative HPLC Chromatogram of 2996 *A. platyspathum*



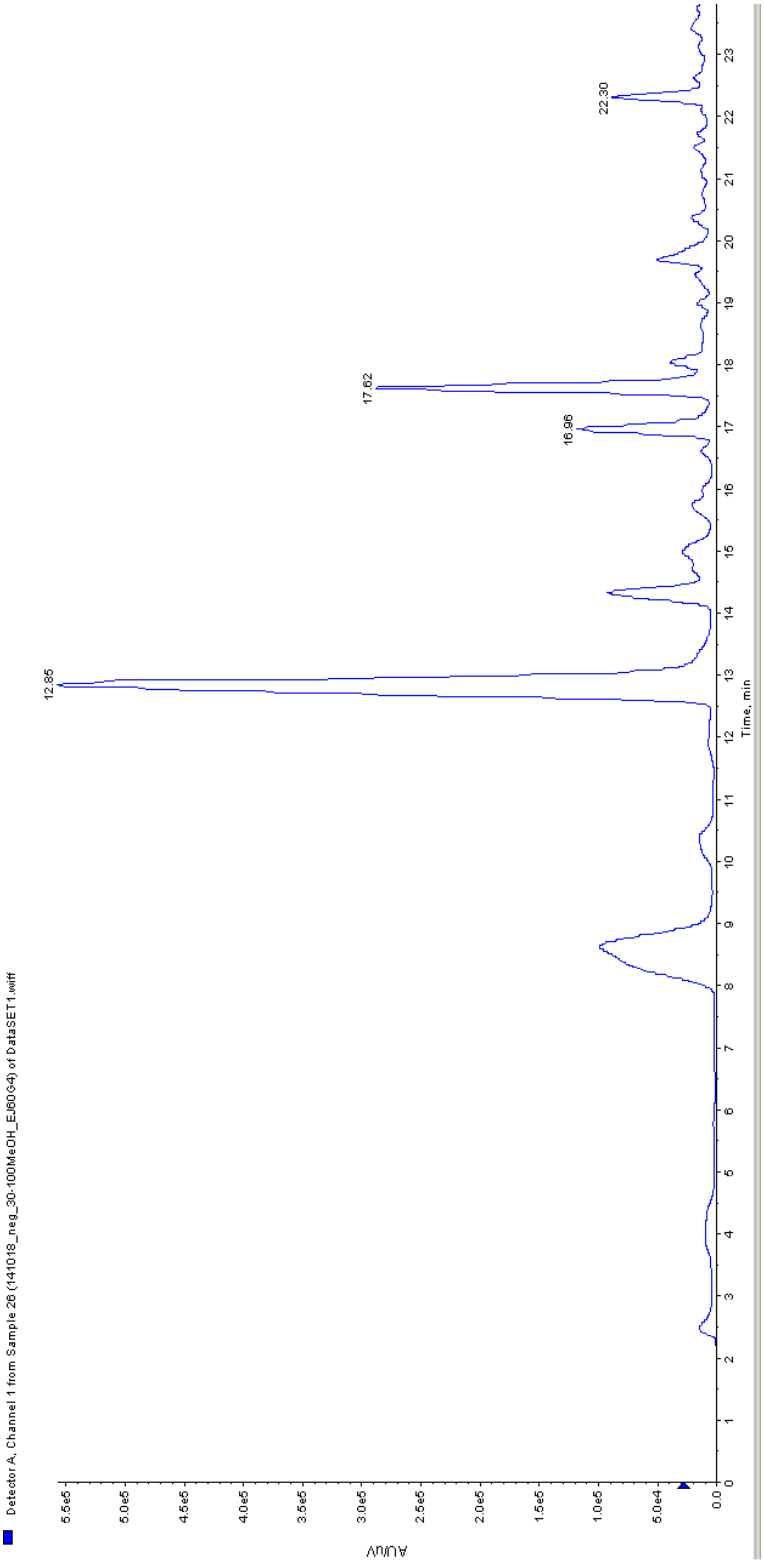
Preparative HPLC Chromatogram of 5313 *A. paniculatum*



Preparative HPLC Chromatogram of 1025 *A. ampeloprasum*

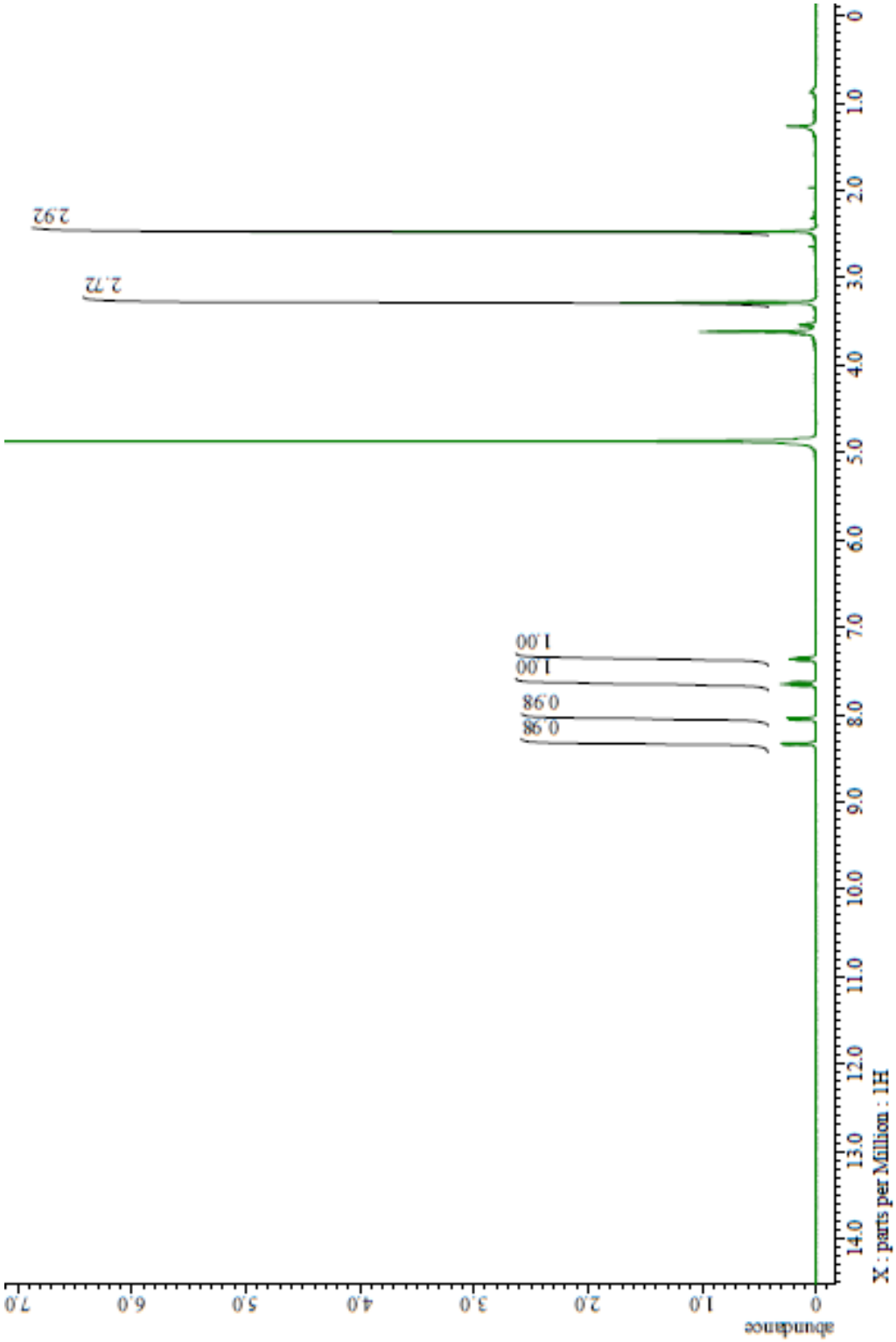


HPLC Chromatogram of *A. sativum* (commercial)



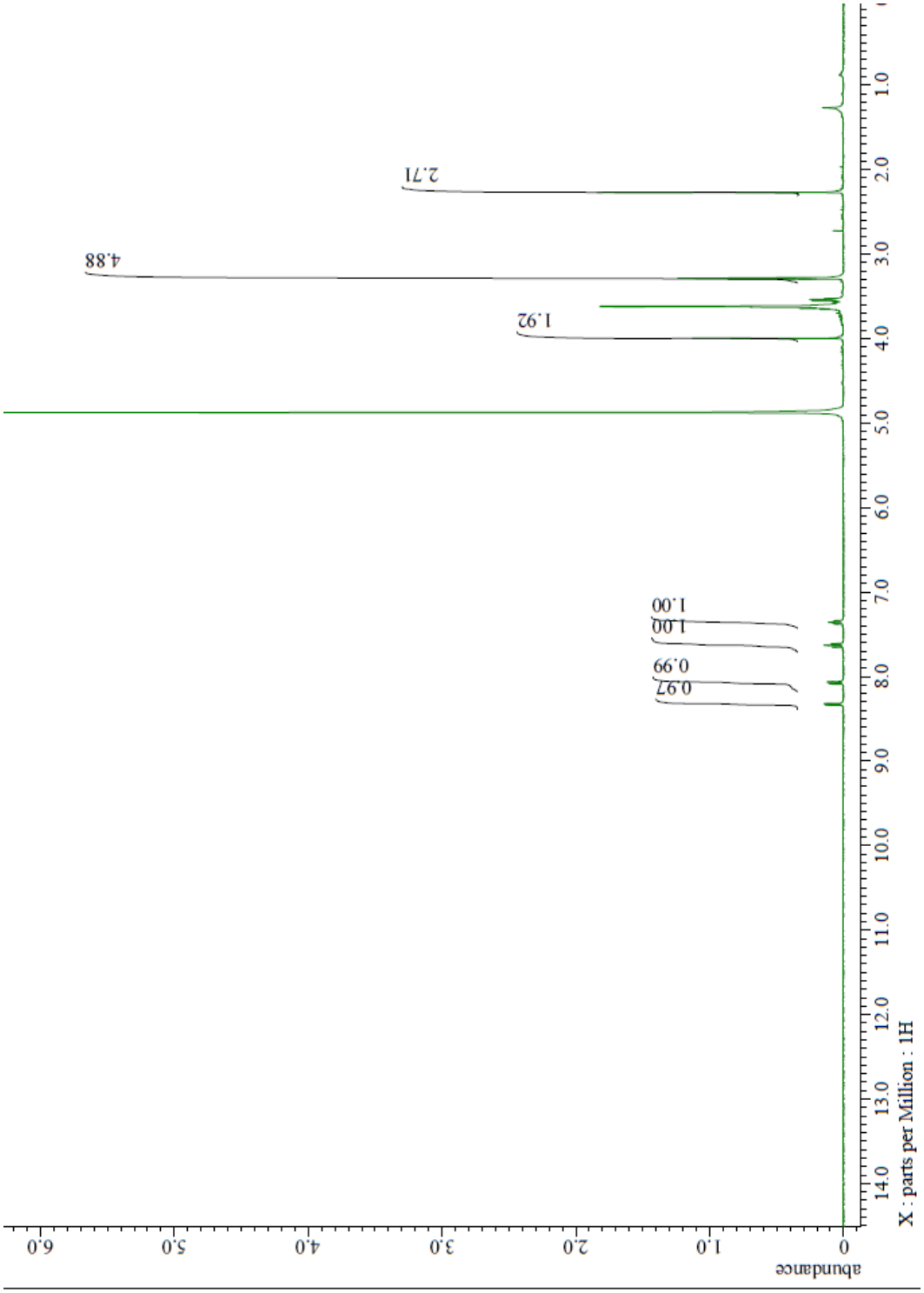
NMR Chromatogram of fraction 3 from 7002 *A. stipitatum*

(2-(methyldithio)pyridine N-oxide)



NMR Chromatogram of fraction 4 from 7002 *A. stipitatum*

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



List of Publications

Posters with Abstracts

Sevda Jivishova, Emil Jivishov, Michael Keusgen, "**Nematicidal activity of some *Allium* spp extracts against root-knot nematode *Meloidogyne incognita***", DPhG Annual Meeting 2014, Frankfurt, Germany, **Poster Presentation**

Oral Presentation

Sevda Jivishova, Emil Jivishov, Michael Keusgen, "**Nematicidal, Phytotoxic and Brine Shrimp Lethality Effects of Some *Allium* species**" Working Group Meeting "Nematology" of the German Phytomedical Society, Bayer CropScience Research Centre, Monheim, Germany, March 2015, **Invited speaker**

Publications

Sevda Jivishova, Emil Jivishov, Johannes Hallmann, Michael Keusgen, "Genus *Allium*, subgenera *Amerallium* and *Melanocrommyum*, are potent sources for nematicidal extracts against *Meloidogyne incognita***"**
In preparation

Curriculum Vitae

PERSONAL INFORMATION	
Last/First Name:	Jivishova, Sevda
Address:	Aliyar Aliyev kuc, 20/41, Narimanov r-nu AZ1033 Baku, Azerbaijan
Email:	j.sevda@gmail.com
Nationality:	Azerbaijani
Date of birth:	26.05.1979

EDUCATION	
11/2012-at present	Doctorate program at Institute of Pharmaceutical Chemistry, Philipps-Universität Marburg, Germany
02/2004-07/2006	Master's degree, Graduate School of Health Sciences, Pharmacology, Anadolu University, Eskisehir, Turkey
09/1998-02/2004	Bachelor's degree, Faculty of Pharmacy, Anadolu University, Eskisehir, Turkey
09/1994-09/1998	Baku Turkish Anadolu Lyceum, Baku, Azerbaijan
09/1986-06/1994	Public School, Baku, Azerbaijan

WORK EXPERIENCE	
01.2012-present	Lecturer - Qafqaz University, Baku, Azerbaijan
08.2010 – 10.2010	Teacher - Dove Science Academy, Oklahoma City, OK, USA)
05.2008 – 06.2008	Teacher - School of Science and Technology, San Antonio, TX, USA
09.2004 – 05.2006	Research assistant - Project funded by Anadolu University (Project No 30353) The phenolic content and antioxidant and free radical scavenging activity of some *Hypericum* species
10/2002 – 04/2003	Pharmacist assistant, "Porsuk" Pharmacy, Eskisehir, TURKEY