2012

Chemical characterization and biological activities of Himalayan aromatic medicinal plants

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CHEMICAL CHARACTERIZATION AND BIOLOGICAL ACTIVITIES OF HIMALAYAN AROMATIC MEDICINAL PLANTS

by

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A THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science
in
The Department of Chemistry
to
The School of Graduate Studies
of
The University of Alabama in Huntsville

HUNTSVILLE, ALABAMA

2012

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ABSTRACT

The School of Graduate Studies

The University of Alabama in Huntsville

Degree Master of Science College/Dept. Science/Chemistry

Name of Candidate Prabodh Satyal

Title Chemical Characterization and Biological Activties of Himalayan Aromatic Medicinal Plants

Nepal is a small country, located between two emerging giants of the Asian continent: India and China. However, like the Himalayan range, Nepal's biodiversity, a mosaic of species, is an unmatched giant in its own right. In coordination with a botanist and a chemist from Tribhuvan University in Nepal and the Natural Products Group at the University of Alabama in Huntsville, around 100 essential oil samples were collected and further analyzed using GC-MS for chemical composition and were tested for biological activity including cytotoxicity, antimicrobial, brine shrimp lethality, allelopathy, larvicidal, and insecticidal activity. In addition, separate testing of essential oil components was conducted to determine if any single component was responsible for the biological activity recorded in our samples. The results could not identify any one compound as being responsible for the observed activities in the samples. Therefore, we conclude the activities to be a result of a synergistic effect amongst the components in the samples. Aside from biological testing, the result of our systematic and detailed analyses about plant chemical composition has revealed new chemotaxonomic divisions for some

of the different species. Plant chemotypes are not only dependent on genetic differences between species but vary among geographical and climatic conditions. This was studied in some cases through comparison of oil compositions of the same species that were collected from different locations. In this thesis, out of 100 samples, I am going to present only *Acorus calamus, Amomum subulatum*, *Artemisia dubia*, *Artemisia indica*, *Artemisia vulgaris*, *Cinnamomum camphora*, *Cinnamomum glaucescens*, and *Cinnamomum tamala* essential oil compositions and their biological activities.

In conclusion, through international collaboration, this project was undertaken with the single goal of identifying potential biologically active essential oils in Nepalese plants. However, our efforts have also translated into a wider understanding of chemodiversity and species differentiation. Through our research, we believe that the Himalayan range has the potential to be one of the most important sources for alternate natural medicine.

Abstract Approval: Committee Chair _____

Department Chair _____

Graduate Dean Rhonda Kay Shrede 10/22/12

ACKNOWLEDGEMENTS

This thesis would not have been possible without the assistance of a private, anonymous donor who generously funded the purchase of the GC-MS instrument used to analyze the essential oils brought back to the lab from Nepal. I would like to thank the Biology Department for providing assistance with preparing several biological assays, especially Noura Dosoky and Dr. Debra Moriarity. I offer my sincere appreciation to Dr. Bernhard Vogler for his assistance and training on the GC-MS instrument. To Professor William N. Setzer and the members of the Natural Products Drug Discovery Research group, I offer my unreserved gratitude for allowing me to contribute to this ever-growing work. Special thanks are offered to Nawal Sherestha, Bimala Lamichhane, Akash Deo, Tilak Gautam, Anand Kafle, and Ajaya Bhattarai who helped in the identification and collection of plants as well as the extraction of essential oils in Nepal and who made this massive undertaking possible. I would also like to thank Katherine E. Woods, Brittany Kincer, Bethran Nnroom, and Dr. Meenakshi Singh for providing invaluable assistance with biological testing and analysis.

I personally would like to thank Dr. William N. Setzer for guiding me throughout this journey with patience, encouragement and tremendous enlightenment. I would also like express my gratitude to Mr. Prajwal Paudel for being with me from the beginning of this work and providing me with invaluable assistance and company throughout. Additional thanks go to Kathmandu University and Tribhuvan University for providing valuable lab equipment in Nepal. In addition, I would like to thank my parents for their unceasing support and encouragement to pursue my dreams. I would also like to thank my wife Ambika for her tremendous capacity to deal with me and her unwavering

support in the lab. Finally, I am grateful to all of the innumerable undergraduates at UAH and in Nepal for their hard and tedious work in making this research a success.

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CHAPTER I

INTRODUCTION

1.1 Medicines from Nepalese Aromatic Plants

Nepal is a small, landlocked country (147,181 km^2) lying between India and China (Figure 1.1). The country is located between latitudes of 26°22′ and 30°27′N and longitudes of 80°40′ and 88°12′E. The average length and width of the country is 885 km from east to west and 193 km from north to south, respectively. Altitudinal variation starts from near sea level (~60 m) and rises as high as 8,848 m (Mt. Everest). Hills and high mountains occupy 80% of the total land area while the remaining 14% is comprised primarily of flatlands, known as the Terai, which is less than 300 m in elevation (Bhuju *et al.*, 2007).

There are a total of 118 ecosystems observed in Nepal, and the Terai region is home to around 10, while the Siwalik Hills and Mid Hills comprise 13 each and the highlands are home to 38. In addition, Nepal has approximately 7,000 vascular plants (Table 1.1). Many of the aromatic plants of Nepal still need to be scientifically described. Therefore, vast numbers of medicinal plants available in Nepal are relatively unexplored. As mentioned above, the Indian subcontinent is the hub of medicinal plants used in Ayurvedic practices. Nepal also has its own rich Ayrvedic tradition. Ayurveda has been using roughly 1,250-1,400 plant species in the preparation of medicines. Approximately

70-80% of the rural population of Asia use medicinal plants as their primary health care agents. Therefore, exploring and testing medicinal plants will provide a basis for using these plants as medicinal and therapeutic agents in rural areas of Nepal and the rest of the world (Gewali, 2008).

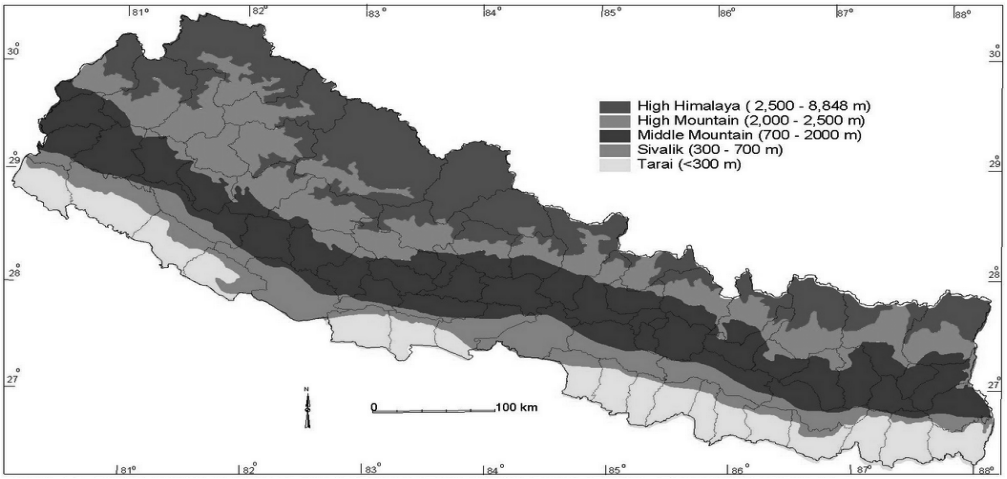

Figure 1.1 Physiographic map of Nepal (Source: Topographic Survey Branch, Department of Survey, His Majesty's Government, Nepal, 1983)

Table 1.1 Distribution of Nepalese Plants Based on Physiographic Zone (Gewali, 2008)

Physiographic Zone	Surface Area (%)	Elevation(m)	Climate	% of MAP [1]	Bryophytes	Pteridophytes	Gymno-sperms	Angio-sperms
High Himalaya	23	above 5,000	Tundra-type and Arctic	7	347	78	10	>2,000
High Mountain	20	4,000-5,000	Alpine					
		3,000-4,000	Subalpine	18				
Mid Hill	30	2,000-3,000	Cool temperate monsoon	36	493	272	16	3,364
		1,000-2,000	Warm temperate monsoon	54				
Lowland Siwalik Hill	27	500-1,000	Hot monsoon and subtropical	49	61	81	0	1,885
		below 500	Hot monsoon and tropical					

[1] Medicinal Aromatic Plants

1.2 Essential Oils and Their Uses

According to Harrewijn *et al.*, essential oils are complex mixtures of volatile compounds that are produced by living organisms and isolated by physical means, such as pressing and distillation, from a whole plant or plant part of known taxonomic origin. The essential oil components are mainly produced from three major biosynthetic pathways: the mevalonate pathway leading to sesquiterpenes, the methyl-erithrytol-pathway leading to mono- and diterpenes, and the shikimic acid pathway yielding phenylpropenes. With only these pathways, there are still large numbers of compounds with tremendous variation in the composition of essential oils (Harrewijn *et al*., 2001; Kubezka, 2010).

Essential oils (EOs) have a rich history as an important traditional medicine all around the world. The World Health Organization (WHO) defined traditional medicine as "the sum total of the knowledge, skills, and practices based on the theories, beliefs and experiences indigenous to different cultures whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness" (WHO). On the basis of this definition we can estimate the wide-ranging importance of a traditional medical system. On the Indian subcontinent, essential oils have a profound impact on the Ayurvedic "Gandhshastra"—the science that deals with flavor and fragrance. According to an Egyptian papyrus manuscript from 2800 B.C., Ancient Egyptians were the first to identify the therapeutic potential of EO. They also used EOs for flavor and fragrance during religious ceremonies in temples and pyramids. In the Chinese herbal tradition dating back 4,000 years, EO was used as a

therapeutic agent. EOs have also repeatedly been mentioned in many Judeo-Christian and Muslim religious texts (Singh, 2012).

Essential oils are stored in specialized cells/glandular cells or organelles within any plant tissue. The first recorded method of extracting EOs was written by Andalusian physician and chemist Ibn al-Baitar in the 13th century. Modern techniques rely on pressing, rubbing, or heating particular regions to rupture the cells and release the aromatic compounds. Therefore, EOs are mostly extracted by cold pressing, enfleurage, solvent extraction, steam distillation and hydrodistillation (Singh, 2012).

1.3 Nepalese Aromatic Medicinal Plants

Seven varieties of essential oil have been described in the following subsections.

1.3.1 *Acorus calamus*

Acorus calamus L., also known as "sweet flag" (Araceae), is a perennial, semi-aquatic plant indigenous to Asia, but now found growing in temperate and sub-temperate zones of Europe, East Asia, and eastern North America (Mabberley, 1997; Bruneton, 1999). The rhizome of *A. calamus* (see Figure 1.2) has been used medicinally since the time of Hippocrates (Mabberley, 1997) and previous studies have shown that the rhizomes of *A. calamus* possess several medicinally and pharmacologically important properties (Bruneton, 1999; Grieve, 1971). In Ayurvedic medicine, *A. calamus* rhizomes are used as antispasmodic, carminative, and anthelmintic therapeutics to treat disorders like epilepsy and various psychological disorders (Raina *et al.*, 2003). Additionally, in Lithuania, *A. calamus* has been used for treating diarrhea, dyspepsia, and neuralgia (Radušienė *et al.*, 2007). Although (*Z*)-Asarone ($C_{12}H_{16}O_3$) is the major constituent of

the essential oil (Bruneton, 1999), diploid forms of the plant species have been previously reported to be devoid of the compound (Bertea *et al.*, 2005). It is known for its strong antifungal and antibacterial properties, but its toxicity and carcinogenicity has limited the commercial use of (*Z*)-Asarone (Devi and Ganjewala, 2009).

1.3.2 *Amomum subulatum*

Amomum subulatum Roxb. (also known as "black cardamom" or "Nepal cardamom," Family: Zingiberaceae) is one of the major cash crops produced in the eastern Himalayas in Nepal, India, and Bhutan, also growing in a range of areas from the subtropical to cool temperate zones (Sharma *et al.*, 2000; *The Wealth of India*, 1991). The fruit of *A. subulatum* (see Figure 1.3) is trilocular and reddish brown and contains dark pink many-seeded capsules (Hussain *et al.*, 2009). This fruit has been used in Ayurvedic preparation since the 6th century B.C. as described by Susrata (Sharma *et al.*, 2000). It is mostly used in South Asia as an aromatic stimulant, stomachic, carminative, astringent, diuretic, anti-inflammatory, and an agent to combat indigestion, biliousness, abdominal pain, rectal diseases, congestive jaundice, and gonorrhea. Its rind has also been used to treat headache and stomatitis (*The Wealth of India*, 1991; Hussain *et al.*, 2009) and has also been applied as an insect-controlling agent (Singh *et al.*, 1989). In addition, the seeds of *A. subulatum* have also been used to flavor food, confections, beverages, and liquids and to preserve various foods (Kapoor *et al.*, 2008).

A. subulatum extracts have demonstrated antiulcerogenic efficacy in a rat model, consistent with the traditional use of this plant for gastrointestinal disorders (Jafri *et al.*, 2001). Additionally, the essential oil of *A. subulatum* has been found to inhibit

Aspergillus flavus growth and showed antiaflatoxigenic activity, suggesting *A. subulatum* oil's potential as an alternative to synthetic food preservatives (Singh *et al.*, 2008).

1.3.3 *Artemisia* Species

The genus *Artemisia* (Asteraceae) is made up of around 350 species of aromatic shrubs and herbs distributed over the northern hemisphere, many of which are used as traditional herbal medicines (Mabberly, 1997). Most of the *Artemisia* species possess a bitter taste and strong aromas and their leaves are covered by white hairs. In this work I have examined the essential oil compositions of three species of *Artemisia* growing wild in Nepal: *Artemisia dubia* Wall. ex Besser (see Figure 1.4), *Artemisia indica* Willd. (see Figure 1.5), and *Artemisia vulgaris* L. (see Figure 1.6). In Nepal, the leaf juice of *A. dubia* is used to treat cuts and wounds while a paste made from the plant is used against fever. In addition, *A. indica* leaf juice is used to treat ringworm (Gewali, 2008), and *A. vulgaris* has been used to treat digestive problems and intestinal worms (Tamang, 2003) as well as nose bleeds and neurological disorders (Hussain and Hore, 2007). It has also been used as a form of incense (Turin, 2003).

The composition of the essential oil of *Artemisia dubia* from China has been previously studied (Zheng *et al.*, 1996). The major components were 1,8-cineole, α-bergamotene, (*E*)-caryophyllene, and τ-cadinol. *A. dubia* extracts (Bhatt *et al.*, 2005) and essential oils (Parajuli *et al.*, 2005) from Nepal have shown antimicrobial activity. *Artemisia indica* essential oil from China has been studied and was found to be largely composed of 1,8-cineole, camphor, borneol, germacrene D, (*E*)-caryophyllene, and caryophyllene oxide (Wu *et al.*, 2009). *Artemisia vulgaris* oils have been extensively

studied and show a wide variation in chemical composition. *A. vulgaris* oils have shown allelopathic activity (Barney *et al.*, 2005), insecticidal and repellent activity (Wang *et al.*, 2006), antioxidant activity (Bhatt *et al.*, 2006; Bhatt *et al.*, 2006), and antimicrobial activity (Kovats *et al.*, 2010).

1.3.4 *Cinnamomum* Species

Cinnamomum represents a genus of evergreen aromatic trees belonging to the Lauracaeae family, which is comprised of approximately 350 species (Mabberley, 1997). In this study, three particular species—*Cinnamomum camphora* (L.) J. Pres1 (see Figure 1.7), *Cinnamomum tamala* (Buch.-Ham.) T. Nees & Nees (see Figure 1.8), and *Cinnamomum glaucescens* Hand.-Mazz. (see Figure 1.9) from Nepal—were studied for chemotypical variation in the volatile components, which were subsequently analyzed for possible biological activity.

C. camphora is primarily native to Japan and has been cultivated in the Himalayan region to commercially produce camphor, which is used in the pharmaceutical and flavor industries (Singh and Jawaid, 2012; Joshi *et al*., 2009). Traditionally, in the Ayurvedic system, *C. camphora* has been used to treat bronchitis, cold, congestion, diarrhea, dysentery, edema, flu, gas, metabolic and heart problems, and various gynecological problems (Singh and Jawaid, 2012). In addition, the Yunani, or "Greek," medicinal system also uses *C. camphora* for cephalic tonic, cardiac treatment, and also as an expectorant (Singh and Jawaid, 2012). Current research has shown that extracts from *C. camphora* can combat HF and SO_2 gas toxicity and also exhibits various biological activities including anti-inflammatory activity (Lee *et al.*, 2006), hepatotoxic and

carcinogenic activity (Tisserand and Balcas, 1995), anthelmintic activity against *Ascaridia galli* (Haque *et al.*, 2011), and anti-oxidant activity (Lee *et al.*, 2006).

C. glaucescens, commonly known as "*Sugandhkokila*" in the Nepali language, is native to the Himalayan areas of Nepal and India (Baruah *et al.*, 2006), although it is found in plains and hilly regions with altitudes below 2,500 m. Morphologically, *C. glaucescens* buds are enclosed in overlapping scales with fruits that are about 3 cm long and base enclosed in a cup that is 10-12 mm across, while the leaves alternate between 7-10 cm long with an elliptical shape, well-marked midrib, and 4-5 pairs of lateral veins. *C. glaucescens* is extensively used by locals as a spice, in medicine and in smoking tobacco (Ravindran *et al.*, 2011). Therapeutically, *C. glaucescens* has been traditionally used as a demulcent and stimulant and has shown analgesic, antiseptic, astringent, and carminative properties. Furthermore, *C. glaucescens* seeds are used for the treatment of the common cold, coughs, toothaches and taeniasis (tapeworm infection by *Taenia* spp.). In addition, seed paste is applied to treat muscular swellings, and seed oil has also been demonstrated to treat muscular spasms, joint pains, and body aches (Ravindran *et al.*, 2011).

C. tamala, commonly known as *Tezpat* in the Nepali language, is widely distributed throughout tropical and subtropical regions of Australia, the Pacific region, South America, and the Himalayan region of Asia (Gupta *et al.*, 2008), and typically grows in altitudes between 900-2,500 m (Ahmed *et al.*, 2000). Morphologically, *C. tamala* is a perennial or small evergreen tree, attaining 8-12 m and a girth of 150 cm, with the leaves ranging from 12-20 cm large and 5-8 cm broad with white flowers (Sharma *et al.*, 2009). Pharmacologically, the plant has been reported to exhibit

antihyperglycemic, antidiarrheal, antihyperlipidermic, antioxygenic, antiinflammatory, acaricidal, hepatoprotective, gastroprotective, antioxidant, antibacterial, α-amylase inhibitory, and immunomodulatory activities (Kumar *et al.*, 2012).

1.4 Photographs of the Nepalese Aromatic Medicinal Plants

The following section contains photographs of the aforementioned Nepalese aromatic medicinal plants.

Figure 1.2 *Acorus calamus*

Figure 1.3 *Amomum subulatum*

Figure 1.4 *Artemisia dubia*

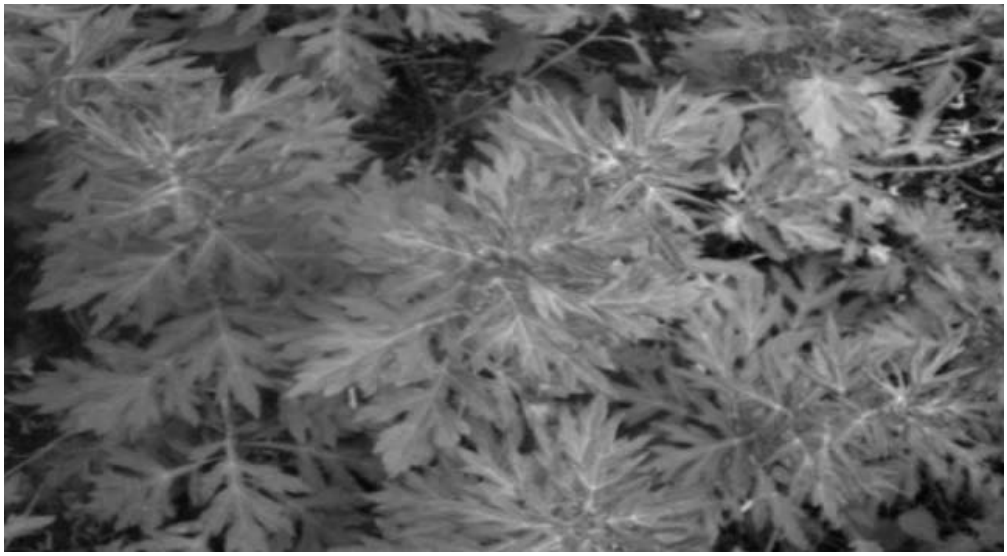

Figure 1.5 *Artemisia indica*

Figure 1.6 *Artemisia vulgaris*

Figure 1.7 *Cinnamomum camphora*

Figure 1.8 *Cinnamomum glaucescens*

Figure 1.9 *Cinnamomum tamala*

CHAPTER II

MATERIALS AND METHODS

2.1 Plant Material

The following plant materials have been reported in this thesis work.

2.1.1 *Acorus calamus*

The leaves and rhizomes of P23 and P24 samples of *A. calamus* were collected from the city of Biratnagar (26°28′N, 87°16′E, 72 m above sea level) in the Morang district of the Koshi Zone in Nepal on May 16, 2011. The plant was identified by Tilak Gautam, and a voucher specimen has been deposited in the herbarium of the Botany Department at Tribhuwan University's postgraduate campus in Biratnagar. The fresh leaf sample, P23 (110 g), was crushed and hydrodistilled using a Clevenger-type apparatus for 4 h to give clear but pale yellow essential oil (0.8 g), which was stored at 4ºC until analysis. Hydrodistillation of the rhizome, P24 (100 g), gave 0.4 g of oil. The P19 rhizome sample was collected from Sindhuli, Nepal (27°12′15″N, 85°54′44″E, 1,500 m above sea level) on May 21, 2011, and the other rhizome sample, P22, was collected from Hetauda Makwanpur, Nepal (27°25′N, 85°02′E, 1,500 m from sea level) on the same day May 21, 2011. Voucher specimens of P19 and P22 were also deposited in the Botany Department's herbarium at Tribhuvan University. Rhizome samples (100 g each)

of P19 and P22 were hydrodistilled to give 2.0 g and 1.5 g of essential oil, respectively.

2.1.2 *Amomum subulatum*

The dried fruits of *A. subulatum* were collected from the Terahthum district (27°8'39"N, 87°32'35"E, and 2,288 m above sea level) in the Mechi Zone of Nepal on June 1, 2011. The dried fruit was identified by Mr. Tilak Goutam. The seeds (P58, 110 g) and rinds (P59, 100 g) were separated, pulverized, and subjected to hydrodistillation using a Likens-Nickerson apparatus with continuous extraction with CH_2Cl_2 for 4 h to yield a clear reddish-brown essential oil (5.0 and 1.0 g, respectively), which were stored at -25°C until analysis.

Figure 2.1 Site of Plant Material Collection A (Kirtipur) (P3), B (Dhulikhel) (P67, P75), C (Hetauda) (P11, P12, P15, P19, P21), D (Sindhuli) (P22), E (Terhathum) (P58, P59), and F (Biratnagar) (P23, P24). Source: Google Map

2.1.3 *Artemisia* Species

A. dubia was collected on May 16, 2011, from the Kirtipur Municipality (27°40'12"N, 85°16'50"E, 1,360 m above sea level) in Kathmandu, Nepal. The plant was identified by Dr. Krishna Shrestha, and a voucher specimen (number TUCH 49) has been deposited in Tribhuvan University's Central Herbarium. Fresh leaves (60.0 g) were shredded and hydrodistilled for 4 h using a Clevenger apparatus to give 0.025 g of essential oil, which was stored at -25ºC until analysis.

A. indica was collected on July 1, 2011, from Dhulikhel (27°36'36"N, 85°33'E, 1,550 m above sea level) in Kavre, Nepal. The plant was identified by Sameer Thapa, and a voucher specimen (number NH5401) has been deposited in the National Herbarium and Plant Laboratories in Kathmandu, Nepal. Fresh leaves (80.0 g) were shredded and hydrodistilled for 4 h using a Clevenger apparatus to give 0.042 g of essential oil, which was stored at -25°C until analysis.

A. vulgaris was collected on May 21, 2011, from Hetauda Makwanpur (27°25'12"N, 85°1'50"E, 1,550 m above sea level) in Nepal. The plant was identified by Tilak Gautam and a voucher specimen (number 1204) has been deposited in the Botany Department of Tribhuvan University's postgraduate campus in Biratnagar. Fresh leaves (100.0 g) were shredded and hydrodistilled for 4 h using a Clevenger apparatus to give 0.8 g of essential oil, which was stored at -25°C until analysis.

2.1.4 *Cinnamomum* Species

All of the three *Cinnamomum* species—*C. camphora* (P15), *C. tamala* (P12), and *C. glaucescens* (P21)—were collected on May 18, 2011, from Hetouda, Makwanpur

(27.42°N, 85.03°E, and 1,550 m above sea level) in Nepal. Additional samples of *C. camphora* (P75) were collected from Dhulikhel, Kavre (27.61°N 85.59°E, 1,512 m above sea level) on July 12, 2011. The plants were identified by Kiran Pokharel, and voucher specimens (M001, M002, M003, and K004) have been deposited in the National Herbarium and Plant Laboratories. Dried leaves (300.0 g) of *C. camphora* (P15), dried leaves of *C. camphora* (P75) (100 g), roots (200.0 g) of *C. tamala*, and berries (300 g) of *C. glaucescens* were shredded and hydrodistilled for 4 h using a Clevenger-type apparatus to give 3.1 mL, 0.5 mL, 3.0 mL, and 2.5 mL, respectively, which were stored at -25ºC until analysis.

2.2 Gas Chromatographic Mass Spectral (GC-MS) Analysis

The *Artemisia* essential oils were analyzed by GC-MS using an Agilent 6890 GC with an Agilent 5973 mass selective detector [MSD, operated in the EI mode (electron energy = 70 eV, scan range = 45-400 amu, and scan rate = 3.99 scans/sec)], and an Agilent ChemStation data system. The GC column was an HP-5ms fused silica capillary with a 5% phenyl-polymethylsiloxane stationary phase, a film thickness of 0.25 μm, a length of 30 m, and an internal diameter of 0.25 mm. The carrier gas was helium with a column head pressure of 48.7 kPa and a flow rate of 1.0 mL/min. The injector temperature was 200°C, and the detector temperature was 280°C. The GC oven temperature program was used as follows: 40°C initial temperature, held for 10 min; increased at 3°C/min to 200°C; increased 2°/min to 220°C. A 1 % w/v solution of the sample in CH_2Cl_2 was prepared, and 1 μL was injected using a splitless injection technique.

Identification of the oil components was based on their retention indices, which were determined by reference to a homologous series of *n*-alkanes and by comparison of their mass spectral fragmentation patterns with those reported in the literature (Adams, 2007) and stored on the MS library [NIST database (G1036A, revision D.01.00)/ChemStation data system (G1701CA, version C.00.01.080)]. The percentages of each component are reported as raw percentages based on the total ion current without standardization.

2.3 Biological Testing

Various biological testings were performed they are as follows:

2.3.1 Antimicrobial Screening

The essential oils were screened for antimicrobial activity against the Gram-positive bacteria *Bacillus cereus* (ATCC No. 14579) and *Staphylococcus aureus* (ATCC No. 29213) and the Gram-negative bacteria *Pseudomonas aeruginosa* (ATCC No. 27853) and *Escherichia coli* (ATCC No. 10798). Minimum inhibitory concentrations (MIC) were determined using the microbroth dilution technique (Sham and Washington, 2012). Dilutions of the essential oil were prepared in cation-adjusted Mueller Hinton broth (CAMHB) beginning with 50 μL of 1% w/w solutions of essential oil in DMSO plus 50 μL CAMHB. The extract solutions were serially diluted (1:1) in CAMHB in 96-well cell culture plates. Organisms at a concentration of approximately 1.5×10^8 colony-forming units (CFU)/mL were added to each well. Plates were incubated at 37°C for 24 hr; the final minimum inhibitory concentration (MIC) was determined as the lowest

concentration without turbidity. Gentamicin was used as a positive antibiotic control while DMSO was used as a negative control. Antifungal activity was determined as described above using *Aspergillus niger* (ATCC No. 16888) in yeast-nitrogen base growth medium with approximately 7.5×10^7 CFU/mL. Amphotericin B was the positive control with *A. niger* hyphal culture diluted to a McFarland turbidity of 1.0. Amphotericin B was the positive control.

2.3.2 Brine Shrimp Lethality

The brine shrimp (*Artemia salina*) lethality test was carried out using a modification of the procedure by McLaughlin (1991). *Artemia salina* eggs were hatched in a sea salt solution (Instant Ocean®, 38 g/L) with an incandescent light bulb as the heat source. After 48 hours, the newly hatched nauplii were counted using a micropipette and transferred to 20-mL vials. Nine vials, each containing 10 *A. salina* nauplii in 10 mL of sea salt solution (the same as the hatching solution), were prepared. Three vials were labeled as controls with the first one containing no DMSO, another with 10 µL of DMSO, and the last one with 100 µL of DMSO. Three replicate vials contained 10 µL of 1% essential oil solution in DMSO, and the other three were prepared by adding 100 µL of 1% essential oil solution in DMSO. Surviving *A. salina* were counted after 24 hours (Satyal *et al.*, 2012).

2.3.3 Cytotoxic Activity

Human MCF-7 breast adenocarcinoma cells (ATCC No. HTB-22) (Soule *et al.*, 1973) were grown in a 3% CO_2 environment at 37°C in RPMI-1640 medium, supplemented with 10% fetal bovine serum, 100,000 units of penicillin, 10.0 mg of

streptomycin per liter of medium, and 15 mM of Hepes and buffered with 26.7 mM of $NaHCO_3$, pH 7.35. Cells were plated into 96-well cell culture plates at 2.5×10^4 cells per well. The volume in each well was 100 µL. After 48 h, supernatant fluid was removed by suction and replaced with 100 µL growth medium containing 1.0 µL of DMSO solution of the essential oil (1% w/w in DMSO), giving a final concentration of 100 µg/mL for each well. Solutions were added to wells in four replicates. Medium controls and DMSO controls (10 µL DMSO/mL) were used. Tingenone (Setzer *et al.*, 1998) was used as a positive control. After the addition of compounds, plates were incubated for 48 h at 37°C in 5% CO_2. Medium was then removed by suction, and 100 µL of fresh medium was added to each well. In order to establish percent kill rates, the MTT assay for cell viability was carried out (Ferrari *et al.*, 1990). After colorimetric readings were recorded (using a Molecular Devices SpectraMAX Plus microplate reader, 570 nm), average absorbances, standard deviations, and percent kill ratios ($\%kill_{cmpd}/\%kill_{DMSO}$) were calculated. The cytotoxicity assays were carried out by Noura S. Dosoky.

2.3.4 Allelopathic Activity

An allelopathic bioassay based on lettuce (*Lactuca sativa*) and perennial rye grass (*Lolium perenne*) germination and subsequent radicle and hypocotyl growth was measured to study the effects of the essential oils and their components (Kennedy *et al.*, 2011). Stock solutions of each essential oil (2.0 g/L of essential oil and 1.0 g/L of Tween-80 in water) were prepared and used for the assays. Two-fold serial dilutions of the stock test solutions were prepared to give test concentrations of 4000, 2000, 1000,

500, and 250 μg/mL with the control being 1.0 g/L of aqueous Tween-80. Seeds were placed in 6-well test plates (10 seeds per well) each well lined with two layers of Whatman No. 1 filter paper moistened with test solution. The test plates were then sealed with Parafilm® and incubated at room temperature in the dark for 5 days, after which the number of germinated seeds was determined and the root (radicle) and shoot (hypocotyl) lengths were measured. A student's *t*-test (Zar, 1996) was used to compare radicle and hypocotyl test means with controls. Seed germination IC_{50} values were determined using the Reed-Muench method (Reed and Muench, 1938).

2.3.5 *Chaoborus plumicornis* and *Culex pipiens* Larvicidal Activity

Mosquito larvae (*Culex pipiens*) were obtained from Carolina Biological Supply (Item number 144478) while glassworm (*Chaoborus plumicornis*) larvae were bought from a local aquarium shop. For the bioassay, 10 mL of sterile water was placed in five 20-mL vials. Ten mosquito larvae were transferred into each vial using a soft brush. Three vials were labeled as control with the first containing 10 μL DMSO, the second containing 100 μL DMSO, and the third containing only sterile water. Then, 10 μL of 1% solution of essential oil in DMSO and 100 μL of 1% essential oil/DMSO solution (*i.e.*, final concentrations of 10 and 100 μg/mL) were added to the remaining two vials. Surviving mosquito larvae were counted after 24 hours. The experiments were carried out at 23 ± 2ºC.

2.3.6 Fire Ant Bioassay

Worker red imported fire ants, probably *Solenopsis invicta* × *richteri* hybrid (Chen *et al.*, 2012) were collected from the University of Alabama in Huntsville. Sample

solutions of 1000 μg/mL, 500 μg/mL and 250 μg/mL were prepared in 1% aqueous Tween-80® solution. The control was 1% Tween solution. Each assay was carried out using a 400-mL beaker, fitted with a filter paper disk on the bottom. The filter paper was sprayed with 600 μL of sample solution, and 20 fire ant workers were transferred to the beaker. The beaker was then sealed with Parafilm®. The mortality of fire ants was recorded after 24 h. The bioassay was carried out in room temperature. LC_{50} values were calculated using the Reed-Muench method (Reed and Muench, 1938).

2.3.7 Fruit Fly Bioassay

Wild type *Drosophila melanogaster* were obtained from Carolina Biological Supply, and a breeding colony was maintained using a *Drosophila* culture kit. *Drosophila* medium (2 mL) was placed into each of five 20-mL glass vials. Three vials were labeled as control, the first being filled with only *Drosophila* medium, the second with 20 μL DMSO, and the third with 150 μL of DMSO. Another two vials contained 20 μL of 1% essential oil solution in DMSO and 150 μL of 1% essential oil solution in DMSO, respectively. Ten individual fruit flies were transferred into each vial and the assay was carried out in triplicate. Surviving fruit flies were counted after 24 h.

2.3.8 Nematicidal Activity

A nematocidal assay using *Caenorhabditis elegans* was carried out using a modification of the procedure of Park and others (2007). A 1% solution of *A. sublatum* seed oil in DMSO was prepared. Dilutions of the sample solution were prepared in a sterile water solution beginning with 50 μL of the 1% essential oil solution plus 50 μL sterile water. The sample solution was serially diluted (1:1) with sterile water in a

96-well plate. Then, 10 to 30 *C. elegans* (mixtures of juvenile and adult nematodes, male:female:juvenile ~1:1:2) were added into each well per 50 μL of sample solution. Sterile water and serially diluted DMSO were used as controls. The dead and living nematodes were counted after 24 h under a microscope. Dead nematodes were identified by their immobility and straight bodies, even after transfer to clean water. Nematocidal activities of the major essential oil components were carried out similarly. LC_{50} values were determined using the Reed-Muench method (Reed and Muench, 1938).

CHAPTER III

RESULTS AND DISCUSSION

3.1 Analysis of *Acorus calamus* Leaf and Rhizome Essential Oils

Leaf and rhizome essential oils of *A. calamus* were obtained in 0.7% (P23), 0.4% (P24), 2.0% (P19), and 1.5% (P22) product yields. The total number of compounds identified for each sample is as follows: 46 compounds (94.3%) for sample P19, 37 compounds (96.2%) for sample P22, 26 compounds (97.6%) for sample P24, and 38 compounds (94.1%) for sample P23. All essential oils (see Table 3.1) were dominated by the presence of (*Z*)-asarone (78.1-86%). The essential oils also contained (*E*)-asarone (1.9%-9.9%) and small amounts of γ-asarone (2.0-2.3%), (*Z*)-methylisoeugenol (1.5-2.0%), and linalool (0.2-4.3%).

Table 3.1 Chemical compositions of *Acorus calamus* rhizome and leaf essential oils

RI[i]	Compound	Percent Composition			
		P19[a]	P22[b]	P24[c]	P23[d]
781	Methyl 2-methylbutanoate	---	---	---	0.1
799	*n*-Octane	---	---	---	0.1
941	α-Pinene	tr[e]	---	tr[e]	---
953	Camphene	tr[e]	---	tr[e]	---
975	Sabinene	tr[e]	tr[e]	tr[e]	---
978	β-Pinene	tr[e]	tr[e]	tr[e]	---
992	Myrcene	tr[e]	---	---	---
994	Mesitylene	---	---	---	0.1
1024	*p*-Cymene	---	---	tr[e]	---
1028	Limonene	0.1	---	0.1	tr[e]
1030	1,8-Cineole	0.1	0.3	0.1	tr[e]
1038	(*Z*)-β-Ocimene	tr[e]	---	---	0.2
1048	(*E*)-β-Ocimene	tr[e]	---	---	tr[e]
1100	Linalool	0.4	0.2	4.3	0.3
1105	Nonanal	---	---	---	tr[e]
1105	α-Thujone	tr[e]	---	tr[e]	---
1124	Chrysanthenone	---	tr[e]	---	---
1143	Camphor	0.1	tr[e]	0.3	tr[e]
1153	Menthone	tr[e]	---	---	---
1155	(3*Z*)-Nonen-1-ol	---	---	---	tr[e]
1163	*iso*-Menthone	tr[e]	---	---	---

Table 3.1 (continued)

RI[i]	Compound	Percent Composition			
		P19[a]	P22[b]	P24[c]	P23[d]
1165	Borneol	tr[e]	---	---	---
1171	Menthol	0.1	---	---	---
1176	Terpinen-4-ol	tr[e]	tr[e]	---	---
1189	α-Terpineol	tr[e]	0.1	---	---
1197	Myrtenol	---	---	---	tr[e]
1205	Decanal	---	---	---	tr[e]
1235	Neral	tr[e]	---	---	---
1304	(*Z*)-Methyl cinnamate	tr[e]	tr[e]	---	---
1356	Eugenol	tr[e]	---	---	---
1375	α-Copaene	---	tr[e]	---	---
1384	(*E*)-Methyl cinnamate	---	0.1	---	---
1392	β-Elemene	tr[e]	tr[e]	---	---
1405	Methyl eugenol	tr[e]	tr[e]	---	tr[e]
1418	(*E*)-Caryophyllene	0.1	---	0.1	0.1
1431	β-Gurjunene (= Calarene)	0.2	0.1	---	0.1
1453	α-Humulene	---	---	tr[e]	---
1459	(*Z*)-Methyl isoeugenol	2.0	1.6	1.5	2.0
1475	*n*-Dodecanol	---	---	0.2	tr[e]
1494	Unidentified[f]	0.6	0.6	---	0.6
1499	(*E*)-Methyl isoeugenol	---	tr[e]	tr[e]	tr[e]
1516	Unidentified[g]	2.5	2.2	1.2	2.0
1524	δ-Cadinene	0.1	0.1	tr[e]	tr[e]

Table 3.1 (continued)

RI[i]	Compound	Percent Composition			
		P19[a]	P22[b]	P24[c]	P23[d]
1528	Kessane	0.1	0.1	---	tr[e]
1544	α-Calacorene	tr[e]	0.1	---	tr[e]
1550	Elemol	tr[e]	tr[e]	---	tr[e]
1559	Elimicin	0.2	0.2	tr[e]	0.2
1565	(*E*)-Nerolidol	---	---	tr[e]	---
1577	γ-Asarone	2.3	2.0	2.2	2.1
1603	Unidentified[g]	1.1	1.0	0.4	0.7
1624	(*Z*)-Asarone	84.9	86.9	84.0	78.1
1660	α-Cadinol	tr[e]	tr[e]	---	---
1663	*cis*-Calamenen-10-ol	0.3	0.3	---	tr[e]
1669	*trans*-Calamenen-10-ol	tr[e]	tr[e]	---	tr[e]
1682	Khusinol	---	0.3	---	---
1685	(*E*)-Asarone	2.3	1.9	4.0	9.9
1689	Shyobunol	0.1	tr[e]	1.0	0.2
1711	Unidentified[h]	0.7	0.6	tr[e]	0.9
1722	Asaraldehyde	0.5	0.5	---	0.7
1750	Isocalamendiol	0.3	0.2	---	tr[e]
1755	Aspidinol	0.2	0.2	---	0.1
	Total Identified	94.3	96.2	97.6	94.1

[a] Rhizome essential oil from Sindhuli, Nepal
[b] Rhizome essential oil from Hetauda Makwanpur, Nepal
[c] Rhizome essential oil from Biratnagar, Nepal
[d] Leaf essential oil from Biratnagar, Nepal
[e] tr = "trace" (< 0.05%)
[f-h] Unknown compounds
[i]Retention Indices on HP-5ms fused silica capillary column

A. calamus essential oils from Nepal, therefore, are qualitatively similar to previously reported samples from India (Raina *et al.*, 2003, Osman *et al.*, 2008), but notably different from samples from Mongolia (Todorova *et al.*, 1995) or Lithuania (Radušienė *et al.*, 2007). Large chemical variations have been noted in *A. calamus* oils with (*Z*)-asarone dominating Indian oils, lesser amounts of (*Z*)-asarone being found in European oils, and no (*Z*)-asarone existing in American varieties (Bruneton, 1999).

Table 3.2 Allelopathic activities of essential oils and their components on lettuce (*Lactuca sativa*) and perennial ryegrass (*Lolium perenne*)

Material (Concentration, μg/mL)	Germination Inhibition (%)		IC50 (μg/mL)		Seedling Growth (% of Controls)			
	Lactuca sativa	*Lolium perenne*	*L. sativa*	*L. perenne*	*Lactuca sativa*		*Lolium perenne*	
					Radical	hypocotyl	radicle	hypocotyl
A. calamus(1000)	43.33	60	450	737	71.94[a]	28.31[a]	8.96[a]	0.00[a]
A. calamus(500)	46.67	35			84.16[b]	33.87[a]	19.87[a]	8.62[a]
A. calamus(250)	25	21.67			88.28[b]	45.65[a]	35.12[a]	16.10[a]
A. subulatum(4000)	90	55	1583	1674	72.8[c]	50.8	43.7[a]	13.0[a]
A. subulatum(2000)	61.7	51.7			74.8[c]	74.2[c]	65.7[a]	38.1[a]
A. subulatum(1000)	23.3	28.3			96.2[m]	99.7[m]	87.8[a]	85.2[c]
A. dubia (1000)	100	100	160	657	---	---	---	---
A. dubia (500)	76.67	13.33			18.31[a]	55.17[a]	30.41[a]	71.31[e]
A. dubia (250)	65	15			41.45[a]	79.22[a]	56.19[a]	73.99[f]
A. dubia (125)	46.67	15			70.00[a]	> 100[m]	96.77[m]	> 100[m]
A. indica(1000)	96.67	48.33	250	1015	41.20[a]	0.00[a]	16.78[a]	3.01[a]
A. indica(500)	53.33	20			73.42[e]	74.18[f]	48.32[a]	38.72[a]
A. indica(250)	50	13.33			81.25[f]	77.32[f]	53.17[a]	29.10[a]
A. vulgaris (2000)	100	60	125	1000	---	---	47.06[a]	15.93[a]
A. vulgaris(1000)	96.67	50			74.45[e]	36.83[a]	51.13[a]	18.71[a]
A. vulgaris (500)	58.33	43.33			96.28[m]	64.04[e]	70.18[f]	56.81[f]
A. vulgaris (250)	58.33	36.67			> 100[m]	> 100[m]	>100[m]	67.88[g]
A. vulgaris (125)	63.33	nt[f]			> 100[m]	> 100[m]	---	---
C. camphora oil (1000)	98.3	31.7	218	1000	69.4[a]	37.9[a]	>100[m]	>100[m]
C. camphora oil (500)	71.7	25			81.6[k]	82.9[l]	>100[m]	>100[m]

Material (Concentration, μg/mL)	Germination Inhibition (%)		IC50 (μg/mL)		Seedling Growth (% of Controls)			
	Lactuca sativa	*Lolium perenne*	*L. sativa*	*L. perenne*	*Lactuca sativa*		*Lolium perenne*	
					Radical	hypocotyl	radicle	hypocotyl
C. camphora oil (250)	66.7	18.3			80.2[h]	>100[m]	>100[m]	>100[m]
C. glaucescens oil (2000)	50	45			46.4[a]	45.5[a]	52.2[a]	4.8[a]
C. glaucescens oil (1000)	35	30	1620	>1773	54.7[a]	51.6[a]	60.4[a]	28.0[a]
C. glaucescens oil (500)	30	18.3			82.0[h]	66.2[a]	83.0[j]	72.6[j]
C. glaucescens oil (250)	15	15			87.0[j]	76.8[a]	90.9	73.3[k]
C. tamala oil (2000)	98.3	63.3			0.0[a]	0.0[a]	60.6[a]	21.0[a]
C. tamala oil (1000)	41.7	16.7	1084	1630	86.6[j]	64.7[a]	70.1[a]	88.1[l]
C. tamala oil (500)	23.3	10			96.0[r]	77.8[a]	93.0[l]	99.1[m]
C. tamala oil (250)	18.3	6.7			97.7[m]	79.4[a]	97.8[m]	99.7[m]
Camphor (1000)	91.7	50			19.9[a]	4.8[a]	15.8[a]	3.5[a]
Camphor (500)	91.7	43.3	91.7	50	21.1[a]	9.7[a]	17.3[a]	5.3[a]
Camphor (250)	58.3	35			51.6[a]	19.3[a]	41.5[a]	7.8[a]
Coumarin(500)	100	100			---	---	---	---
Coumarin(250)	93.33	43.33	51.4	265	21.80[a]	42.05[a]	41.66[a]	24.71[a]
Coumarin(125)	85	30			29.07[a]	48.06[a]	59.92[a]	33.75[a]
Coumarin(62.5)	73.33	21.67			52.53[a]	88.61[g]	78.46[f]	58.09[a]
Methyl (*E*)-cinnamate (2000)	48.3	100			46.9[a]	46.9[a]	---	---
Methyl (*E*)-cinnamate (1000)	20	93.3	1828	610	63.3[a]	52.0[a]	16.9[a]	2.4[a]
Methyl (*E*)-cinnamate (500)	1.7	30			89.8[j]	75.8[a]	42.2[a]	10.6[a]
Methyl (*E*)-cinnamate (250)	---	20			---	---	45.4[a]	28.4[a]
α-Pinene (4000)	6.67	13.3	nt	nt	>100[m]	99.6[m]	90.0[m]	>100[m]
β-Pinene (4000)	13.3	18.3	nt	nt	84.1[k]	58.8[a]	77.0[k]	11.5[a]

Material (Concentration, μg/mL)	Germination Inhibition (%)		IC50 (μg/mL)		Seedling Growth (% of Controls)			
	Lactuca sativa	*Lolium perenne*	*L. sativa*	*L. perenne*	*Lactuca sativa*		*Lolium perenne*	
					Radical	hypocotyl	radicle	hypocotyl
β-Pinene (2000)	1.7	25			98.6[m]	85.1[m]	82.5[m]	39.4[a]
α/β-Thujones(3000)	98.33	78.33	448	1060	15.28[a]	0.00[a]	44.04[a]	0.00[a]
α/β-Thujones(1500	93.33	60			62.40[a]	22.17[a]	67.41[a]	12.31[a]
α/β-Thujones(750)	90	40			96.26[m]	71.13[e]	76.29[f]	20.51[a]
α/β-Thujones(375)	35	35			> 100[m]	97.11[m]	> 100[m]	65.13[f]

[a] $P < 0.001$, [b] $P \approx 0.1$, [c] $0.01 < P < 0.1$, [d] $0.1 < P < 0.5$, [e] $0.001 < P < 0.01$, [f] $0.01 < P < 0.05$,
[g] $0.5 < P < 0.1$, [h] $P \approx 0.005$, [i] $P \approx 0.01$, [j] $P \approx 0.05$, [k] $P \approx 0.2$, [l] $P \approx 0.5$, [m] not significant, [n] not tested

Table 3.3 Biological activities of essential oils and their components on various organisms

Bioassay/Materials	*A. salina* lethality (LC_{50}, μg/mL)	MCF-7 cytotoxicity (% kill at 100 μg/mL)	Antimicrobial (MIC, μg/mL)					*C. elegans* nematocidal (LC_{50}, μg/mL)	Insecticidal (LC_{50}, μg/mL)		
			B. cereus	*S. aureus*	*E. coli*	*P. aeruginosa*	*A. niger*		*Chaoborus plumicornis* (larvae)	*Drosophila melanogaster*	*Solenopsis invicta* × *richteri*
A. calamus oil	9.48	92.2±5.1	2500	1250	1250	nt[a]	19.5	64	nt[a]	229	287
A. subulatum seed oil	28.1±3.0	19.4±0.9	625	313	625	625	313	341	nt[a]	188	1500
A. subulatum rind oil	15.0±9.0	31.2±10.6	313	625	1250	1250	19.5	nt[a]	nt[a]	493	1149
A. dubia oil	12	100	1250	625	1250	2500	313	352	200	196	500
A. indica oil	100	15	625	1250	625	625	625	56.2	140	501	886
A. vulgaris oil	34	32.2±12.8	2500	2500	2500	2500	2500	1100	304	100	339
C. camphora oil	2.5±2.5	71.2±26.8	313	313	1250	625	19.5	461	54.4	153±50	176±53
C. glaucescens oil	20.1±2.6	55.1±4.8	625	313	625	313	313	97.4	178	348±83	325±90
C. tamala oil	28.1±3.0	41.2±13.3	625	625	625	625	156	252	104	245±25	191±59
Camphor	21.7±9.9	100	313	1250	1250	1250	625	244	187	117±58	286±23
1,8-Cineole	26.9±13.1	100	313	625	1250	1250	625	227	287	160±81	375±74
p-Cymene	4.7±1.0	100	1250	1250	625	625	313	nt[a]	79.6	18.4±4.0	518±56
Limonene	6.3±3.4	90.78±9.6	625	313	625	1250	1250	85.1	92.8	249±62	1285±80
Linalool	36.0±3.9	18.0±9.8	625	156	625	1250	625	1575	278	28.0±7.3	613±65
methyl (*E*)-cinnamate	20.7±3.1	0	1250	1250	625	625	nt[a]	138±28	nt[a]	167±67	333±39
α-Pinene	21.4±5.7	68.3±4.9	1250	1250	1250	1250	156	> 2500	97.4	220±54	> 1000
β-Pinene	15.7±6.1	78.0±4.2	1250	1250	1250	1250	156	> 2500	97.4	260±123	1506

Table 3.3 (continued)

Bioassay/Materials	*A. salina* lethality (LC_{50}, µg/mL)	MCF-7 cytotoxicity (% kill at 100 µg/mL)	Antimicrobial (MIC, µg/mL)					*C. elegans* nematocidal (LC_{50}, µg/mL)	Insecticidal (LC_{50}, µg/mL)		
			B. cereus	*S. aureus*	*E. coli*	*P. aeruginosa*	*A. niger*		*Chaoborus plumicornis* (larvae)	*Drosophila melanogaster*	*Solenopsis invicta* × *richteri*
α-Terpineol	> 100	100	625	1250	1250	1250	313	2176	140	383±184	287
αβ-Thujone	42	14	2500	2500	2500	2500	625	1539.55	632.46	229	1000
Coumarin	19	46	625	625	625	625	625	1088.19	126.19	128	854

[a]"nt" Not tested

A.calamus rhizome oil (P19) showed notable germination inhibition on both lettuce (*Lactuca sativa*) and perennial rye grass (*Lolium perenne*) seeds (see Table 3.2). The IC_{50} values for germination inhibition were determined to be 450 μg/mL and 737 μg/mL, respectively, for *L. sativa* and *L. perenne*. Thus, lettuce germination was more susceptible to inhibition by *A. calamus* oil. Interestingly, however, *L. perenne* seedlings showed greater growth inhibition (both radicle and hypocotyl elongation) than *L. sativa*.

Both the growing plants (Zhang *et al.*, 2009) as well as aqueous extracts (Hu *et al.*, 2009) of *A. calamus* have previously shown allelopathic activity against the phytoplankton *Microcystis aeruginosa* and *Chlorella pyreniodosa*. A "sandwich method" of allelopathic screening using *A. calamus* dried leaf litter showed little allelopathic activity against lettuce (*L. sativa*) (Fujii *et al.*, 2003). These previous works, however, did not provide phytochemical information about the extracts or leachates used.

A. calamus rhizome oil (P19) showed notable activity in the brine shrimp (*Artemia salina*) lethality test (LC_{50} = 9.48 μg/mL), *in-vitro* cytotoxic activity on MCF-7 cells (92.2 ± 5.1% kill at 100 μg/mL), and antifungal activity against *Aspergillus niger* (MIC = 19.5 μg/mL).

The high concentration of (*Z*)-asarone in *A. calamus* oil is likely responsible for the biological activities observed. Crude extracts of Indian *A. calamus* leaves and rhizomes as well as (*Z*)-asarone itself have shown antifungal activities (Devi and Ganjewala, 2009; McLaughlin, 1991), while Lithuanian *A. calamus* leaf oil, 16-25% (*Z*)-asarone, has shown low antifungal activity (Radušienė *et al.*, 2007). Crude ethanol extracts of *A. calamus* rhizome had previously demonstrated *in-vitro* cytotoxic activity against several human and murine cell lines (Mehrotra *et al.*, 2003). (*Z*)-Asarone has also

shown anthelmintic (McGraw *et al.*, 2002) as well as insect antifeedant and growth inhibitory activities (Koul *et al.*, 1990). Although present in only 2.3% of sample P19, γ-asarone had previously shown antifungal activity (Varma *et al.*, 2002) and may, therefore, contribute to the bioactivity of *A. calamus* oil.

3.2 *Amomum subulatum* Seed and Rind Essential Oils

The seed and rind essential oils of *A. subulatum* were obtained in 4.5% (seed oil) and 1.0% (rind oil) product yields. A total of 85 compounds were identified, accounting for 99.1% and 99.0% of the seed oil and rind oil, respectively. The seed oil contained 70 of the identified compounds while the rind oil contained 64 of these compounds. Both of the essential oils (see Table 3.4) were dominated by the presence of 1,8-cineole (60.8% and 39.0%, respectively). The essential oils also contained α-pinene (6.4% and 4.8%), β-pinene (8.3% and 17.7%), α-terpineol (9.8% and 12.3%), and terpinen-4-ol (3.4% and 3.2%). Spathulenol (3.4%) was relatively abundant in the rind oil only.

Table 3.4 Chemical composition of *Amomum subulatum* essential oils

RI[a]	Compound	% Composition		RI[a]	Compound	% Composition	
		seed	rind			seed	rind
834	2-Furaldehyde	0.4	0.8	1195	Myrtenol	0.2	1.5
854	2-Furanmethanol	tr[b]	---	1208	*trans*-Piperitol	0.1	---
909	2-Acetylfuran	---	0.1	1217	*trans*-Carveol	0.1	0.2
935	α-Thujene	0.6	0.4	1222	*exo*-2-Hydroxycineole	0.1	---
941	α-Pinene	6.4	4.8	1229	*cis*-Carveol	tr[b]	---
951	α-Fenchene	---	tr[b]	1238	*endo*-2-Hydroxycineole	0.1	---
953	Camphene	0.2	0.2	1241	Carvone	0.1	---
958	Thuja-2,4(10)-diene	---	tr[b]	1273	*p*-Menth-1-en-7-al	0.1	---
966	5-Methylfurfural	0.2	0.3	1278	*p*-Ethylguaiacol	0.1	---
975	Sabinene	tr[b]	tr[b]	1282	α-Terpinen-7-al	tr[b]	---
979	β-Pinene	8.3	17.7	1285	Bornyl acetate	tr[b]	---
992	Myrcene	0.9	0.2	1290	*p*-Cymen-7-ol	tr[b]	---

Table 3.4 (continued)

RI[a]	Compound	% Composition		RI[a]	Compound	% Composition	
		seed	rind			seed	rind
1004	α-Phellandrene	tr[b]	tr[b]	1294	2-Undecanone	tr[b]	---
1016	α-Terpinene	0.5	0.5	1297	Perilla alcohol	tr[b]	---
1024	*p*-Cymene	---	0.4	1325	*p*-Mentha-1,4-dien-7-ol	---	0.1
1028	Limonene	0.5	1.1	1356	Eugenol	tr[b]	0.3
1032	1,8-Cineole	60.8	39.0	1366	Dihydroeugenol	---	0.1
1043	Phenylacetaldehyde	0.1	0.2	1375	α-Copaene	tr[b]	0.1
1054	*o*-Cresol	0.1	0.1	1392	β-Elemene	tr[b]	0.1
1055	(*E*)-β-Ocimene	0.1	---	1419	(*E*)-Caryophyllene	tr[b]	0.2
1058	γ-Terpinene	1.4	0.8	1454	α-Humulene	tr[b]	0.1
1070	*cis*-Sabinene hydrate	0.1	---	1460	Alloaromadendrene	tr[b]	0.1
1076	*p*-Cresol + *m*-Cresol	0.1	0.2	1478	γ-Muurolene	tr[b]	0.5
1088	Terpinolene	0.5	0.7	1481	Germacrene D	0.1	0.2
1096	Methyl benzoate	tr[b]	---	1486	β-Selinene	0.2	0.7
1099	*trans*-Sabinene hydrate	0.1	---	1495	δ-Selinene	tr[b]	tr[b]

Table 3.4 (continued)

RI[a]	Compound	% Composition		RI[a]	Compound	% Composition	
		seed	rind			seed	rind
1100	Linalool	0.1	tr[b]	1497	Bicyclogermacrene	tr[b]	---
1103	Perillene	tr[b]	---	1501	α-Muurolene	---	tr[b]
1114	*endo*-Fenchol	0.1	0.2	1514	γ-Cadinene	tr[b]	0.2
1117	*trans*-Thujone	tr[b]	---	1524	δ-Cadinene	tr[b]	0.3
1121	*cis*-*p*-Menth-2-en-1-ol	0.2	0.1	1543	α-Calacorene	---	tr[b]
1126	α-Campholenal	0.1	0.1	1565	(*E*)-Nerolidol	1.4	1.3
1137	Nopinone	---	tr[b]	1578	Spathulenol	0.1	3.4
1139	*trans*-Pinocarveol	0.3	1.3	1584	Caryophyllene oxide	tr[b]	0.5
1144	*trans*-Verbenol	---	tr[b]	1594	Salvial-4(14)-en-1-one	---	0.1
1147	Camphene hydrate	---	tr[b]	1609	Humulene epoxide II	---	0.1
1158	Sabina ketone	---	tr[b]	1628	1-*epi*-Cubenol	---	0.3
1162	Pinocarvone	0.1	0.3	1635	Isospathulenol	tr[b]	0.4
1164	Borneol	---	0.2	1639	τ-Muurolol	---	0.4
1166	δ-Terpineol	1.6	1.1	1647	α-Muurolol	---	0.3

RI[a]	Compound	% Composition		RI[a]	Compound	% Composition	
		seed	rind			seed	rind
1177	Terpinen-4-ol	3.4	3.2	1654	α-Cadinol	---	0.4
1185	*p*-Cymen-8-ol	---	0.2	1670	14-Hydroxy-9-*epi*-(*E*)-caryophyllene	---	0.3
1191	α-Terpineol	9.8	12.3	1686	Germacra-4(15),5,10(14)-trien-1α-ol	---	0.3
					Total Identified	99.1	99.0

[a] "RI" = Retention Indices on HP-5ms fused silica capillary column

[b] "tr" = Trace components (< 0.5%)

A. subulatum essential oils from Nepal, therefore, are qualitatively similar to previously reported samples from India (Kapoor *et al.*, 2008; Gurudutt *et al.*, 1996; Naik *et al.*, 2004) and Pakistan (Atta-Ur-Rahman *et al.*, 2000).

A. subulatum seed oil showed notable germination inhibition on both lettuce (*Lactuca sativa*) and perennial ryegrass (*Lolium perenne*) seeds (see Table 3.2). The IC_{50} values for germination inhibition were determined to be 1583 μg/mL and 1674 μg/mL, respectively, for *L. sativa* and *L. perenne*. Thus, lettuce germination was slightly more susceptible to germination inhibition by *A. subulatum* oil. Interestingly, however, *L. perenne* seedlings showed greater growth inhibition (both radicle and hypocotyl elongation) than *L. sativa* (see Table 3.2). The phytotoxic activity of *A. subulatum* seed oil can be attributed to the high concentrations of 1,8-cineole and α-terpineol (Kennedy *et al.*, 2011).

A. subulatum seed oil showed moderate activity in the brine shrimp (*Artemia salina*) lethality test (see Table 3.3) with an LC_{50} of 28.1 ± 3.0 μg/mL, comparable to many other essential oils (Werka *et al.*, 2007). It also showed marginal activity on all of the microorganisms tested (see Table 3.3): *Bacillus cereus* (MIC = 625 μg/mL), *Pseudomonas aeruginosa* (MIC = 625 μg/mL), *Staphylococcus aureus* (MIC = 313 μg/mL), *Escherichia coli* (MIC = 313 μg/mL), and *Aspergillus niger* (MIC = 313 μg/mL). Several previous studies have reported antibacterial (Gilani *et al.*, 2006; Arora and Kaur, 2007; Agnihotri and Wakode, 2010; Aneja and Joshi, 2009) and antifungal (Atta-Ur-Rahman *et al.*, 2000; Jain and Agrawal, 1978; Mishra and Dubey, 1990; Dubey *et al.*, 2008) activities for *A. subulatum* essential oils that are consistent with the results of the present study. Specifically, 1,8-cineole, especially in synergy with other

essential oil components, is likely responsible for the antimicrobial activities (Viljoen *et al.*, 2003).

Neither *A. subulatum* seed oil nor rind oil demonstrated notable *in-vitro* cytotoxic activity against MCF-7 breast tumor cells (19.4% kill and 31.2% kill at 100 μg/mL, respectively). Note that 1,8-cineole, α-pinene, β-pinene, and α-terpineol are not appreciably cytotoxic, either alone (see Table 3.3) or in combination on this cell line (Wright *et al.*, 2007).

A. subulatum seed oil was moderately nematocidal to *Caenorhabditis elegans* (LC_{50} = 341 μg/mL). This activity is likely due to the oil's major component, 1,8-cineole, which was also nematocidal (LC_{50} = 227 μg/mL). The other major components in the seed oil—α-pinene, β-pinene, and α-terpineol—were not nematocidal ($LC_{50} \geq 2000$ μg/mL).

The seed oil of *A. subulatum* was more toxic toward *D. melanogaster* (LC_{50} = 188 μg/mL) than it was against *Solenopsis* (LC_{50} = 1500 μg/mL). The major components of *A. subulatum* oil had similar activities against *D. melanogaster* (see Table 3.3), consistent with fruit fly insecticidal activity.

3.3 Analysis of the *Artemisia* Species Essential Oil

Artemisia essential oils were obtained in 0.04%, 0.05%, and 0.8% product yields for *A. dubia*, *A. indica*, and *A. vulgaris*, respectively. The chemical compositions of the oils are summarized in Table 3.5. *A. dubia* oil from Nepal was dominated by chrysanthenone (29.0%), coumarin (18.3%), and camphor (16.4%), with lesser amounts of verbenone (5.2%) and borneol (3.1%), in marked contrast to *A. dubia* essential oil

from China (Zeng *et al.*, 1996). *Artemisia herba-alba* essential oils from Algeria (Dob and BenabDelkader, 2006) and Morocco (Ouachikh *et al.*, 2009) have been characterized as having high concentrations of both chrysanthenone and camphor. Chrysanthenone is known to be a photoisomerization product of verbenone (Kostyk *et al.*, 1993), and filifilone has been shown to be a thermal decomposition product of chrysanthenone (Asfaw *et al.*, 2001).

Likewise, Nepalese *A. indica* essential oil was notably different from Chinese *A. indica* oil (Wu *et al.*, 2009). The *A. indica* oil in this present study showed high concentrations of ascaridole (15.4%) and isoascaridole (9.9%), along with *trans*-*p*-mentha-2,8-dien-1-ol (9.7%), *trans*-verbenol (8.4%), *cis*-*p*-mentha-1(7),8-dien-2-ol (6.0%), α-terpineol (5.6%), and menthol (5.4%). The essential oil of *Artemisia molinieri* from France has been reported to contain high levels of ascaridole (Carnat and Lamaison, 1992; Masotti *et al.*, 2003) as did the essential oil of *Artemisia persica* from Iran (Mirjalili *et al.*, 2006). Ascaridole is the active component of *Chenopodium ambrosioides* oil and is responsible for the antiparasitic effects of the oil (Monzote *et al.*, 2011). The compound is known to be thermally labile, however, and isomerizes to isoascaridole (Cavalli, 2004).

A. vulgaris oils are generally dominated by the monoterpenoids camphor, 1,8-cineole, borneol, α-thujone, and β-thujone, as well as the sesquiterpene hydrocarbons α-humulene and (*E*)-caryophyllene, but the relative abundance of these constituents and the presence and abundance of other components is variable and likely depends on geographical, ecological, and phenological factors. The literature review indicates that the sample from Nepal in this study is more closely related, chemically, to samples from

Turkey (Baykan *et al.*, 2011) and India (Mishra and Singh, 1986) and represents an α-thujone-rich chemotype. In addition to α-thujone (30.5%), the Nepalese *A. vulgaris* oil was also rich in 1,8-cineole (12.4%) and camphor (10.3%).

The allelopathic potential of *Artemisia* essential oils from Nepal have been assessed in terms of their inhibition of seed germination as well as their inhibition of seedling growth against a representative dicot (lettuce, *Lactuca sativa*) and a representative monocot (perennial ryegrass, *Lolium perenne*). The allelopathic activities of *A. dubia*, *A. indica*, and *A. vulgaris* essential oils are summarized in Table 3.2. *Lactuca sativa* seed germination is notably inhibited by all three essential oils: *A. dubia* IC_{50} = 160 μg/mL, *A. indica* IC_{50} = 250 μg/mL, and *A. vulgaris* IC_{50} < 125 μg/mL. *Lolium perenne* was less susceptible to germination inhibition (IC_{50} = 657, 1015, and 1000 μg/mL for *A. dubia*, *A. indica*, and *A. vulgaris*, respectively).

Both root (radicle) and shoot (hypocotyl) growth of *L. sativa* and *L. perenne* were notably inhibited by the three *Artemisia* essential oils. *A. dubia* and *A. indica* oils significantly inhibited the growth of both seedlings at concentrations of 250 μg/mL and higher. *A. vulgaris* oil, however, was somewhat less active in terms of growth inhibition, with significant activity starting at 500 μg/mL and higher.

The allelopathic activity of *A. dubia* essential oil can be attributed to the high concentrations of coumarin and camphor. Coumarin has previously shown allelopathic potential against *Zoysia japonica* (seedling growth inhibition) (Yamamoto, 1995), *Daucus carota* (*in-vitro* cell culture) (Abenavoli *et al.*, 2003), and *Zea mays* (root growth and morphology) (Abenavoli *et al.*, 2004). In the present work, I have found that coumarin inhibits both seed germination of *L. sativa* and *L. perenne* (IC_{50} = 51.4 and

265 μg/mL, respectively) and seedling growth (significant inhibition of radicle and hypocotyl elongation of both species at 62.5 μg/mL). Additionally, camphor has demonstrated allelopathic activity on *Brassica campestris* seedlings (Nishida *et al.*, 2005) and *Oryza sativa* seedlings (Yumi *et al.*, 2011), as well as germination and seedling growth inhibition of *L. sativa* and *L. perenne* (Kennedy *et al.*, 2011).

The allelopathic activity of *A. indica* essential oil from Nepal is likely due to the high concentration of ascaridole (15.4%), a compound that has demonstrated broad bioactivity (Yen *et al.*, 2007; Torres *et al.*, 2008; Monzote *et al.*, 2009; Chu *et al.*, 2011; Bai *et al.*, 2011) as well as allelopathic activity (Jiménez-Osornio *et al.*, 1996). Further, α-Terpineol (5.6%) is also allelopathic (Kennedy *et al.*, 2011; De Martino *et al.*, 2010) and may contribute to the activity of *A. indica* oil.

The allelopathy of *A. vulgaris* has been reported previously (Barney *et al.*, 2005), and this current work complements those previous reports. The phytotoxic activity of *A. vulgaris* oil against *L. sativa* and *L. perenne* in this study can be attributed to relatively high concentrations of the known allelopathic agents 1,8-cineole (Nishida *et al.*, 2005; Kennedy *et al.*, 2011; Koitabashi *et al.*, 1997; Romagni *et al.*, 2000; Singh *et al.*, 2002), α- and β-thujone (Halligan, 1975; Jassbi *et al.*, 2010), and camphor (Nishidha *et al.*, 2005; Yumi *et al.*, 2011; Kennedy *et al.*, 2011). In the present work, a mixture of α- and β-thujones has been found to weakly inhibit *L. sativa* and *L. perenne* germination with IC_{50} values of 448 and 1060 μg/mL, respectively.

Of the three *Artemisia* oils examined in this study, only *A. dubia* showed notable *in-vitro* cytotoxic activity, killing 100% of MCF-7 human breast adenocarcinoma cells at a concentration of 100 μg/mL. Neither *A. dubia* nor *A. vulgaris* essential oils showed

notable antimicrobial activity in this study. *A. dubia* was marginally active against *Staphylococcus aureus* (MIC = 625 μg/mL) and *Aspergillus niger* (MIC = 313 μg/mL), while *A. indica* oil was marginally active against *B. cereus*, *E. coli*, *P. aeruginosa*, and *A. niger* (MIC = 625 μg/mL). The crude ethanol extract of *A. dubia* showed weak antibacterial activity against *S. aureus* and *S. epidermidis* (MIC = 800 μg/mL) (Bhatt *et al.*, 2005), while *A. dubia* essential oil showed weak antifungal activity against *Alternaria brassicicola* (64% growth inhibition at 10 μL/mL) (Parajuli *et al.*, 2005), consistent with the present results. A thujones-rich *A. vulgaris* oil from Turkey demonstrated antibacterial and antifungal activity in a zone-of-inhibition assay, but minimum inhibitory concentrations were not determined (Baykan *et al.*, 2011).

3.4 Analysis of the *Cinnamomum* Species Essential Oils

Cinnamomum essential oils were obtained in 1.3%, 0.9%, 1.0%, and 1.5% product yields for *C. camphora* (P15), *C.camphora* (P75), *C.tamala*, and *C. glaucescens*, respectively. The chemical compositions of the oils are summarized in Tables 3.6 through 3.9. *C. camphora* (P15) oil was dominated by camphor (36.5%), camphene (11.7%), and limonene (9.0%), with lesser amounts of sabinene (6.3%) and β-pinene (6.3%), while the *C. camphora (*P75) oils contained almost exclusively camphor (98.0%). Currently there are five different chemotypes observed worldwide for *C. camphora*: camphor, linalool, 1,8-cineole, neroldiol, and borneol (Ho *et al*., 2009). Specifically camphor content varied from a Japanese oil sample with 51.5% composition (Senanayake, 1977) to a Chinese oil sample with 83.9% and an Ivory Coast leaf oil sample containing 74% camphor.

Table 3.5 Chemical composition of *Cinnamomum camphora* (P15) leaf oil from Nepal

RI[a]	Compound	%	RI[a]	Compound	%
916	Tricyclene	0.7	1219	*trans*-Carveol	0.2
936	α-Thujene	7.7	1228	Citronellol	tr[b]
942	α-Pinene	4.7	1226	*cis*-Carveol	0.1
954	Camphene	11.7	1237	Cuminaldehyde	0.1
977	Sabinene	6.3	1240	Neral	tr
981	β-Pinene	6.3	1242	Carvone	0.2
993	Myrcene	1.2	1251	Piperitone	0.1
1001	δ-2-Carene	tr[b]	1283	Safrole	tr[b]
1010	δ-3-Carene	tr[b]	1286	*p*-Cymen-7-ol	0.1
1026	*p*-Cymene	1.8	1348	α-Cubebene	tr[b]
1029	*o*-Cymene	1.8	1376	α-Copaene	0.2
1034	Limonene	9.0	1381	*trans*-Soberol	0.3
1035	1,8-Cineole	2.7	1390	β-Cubebene	0.2

Table 3.5 (continued)

RI[a]	Compound	%	RI[a]	Compound	%
1059	γ-Terpinene	tr[b]	1393	β-Elemene	0.1
1069	*cis*-Sabinene hydrate	tr[b]	1419	(*E*)-Caryophyllene	0.1
1073	*cis*-Linalool oxide (furanoid)	tr[b]	1426	Carvone hydrate	0.1
1076	*trans*-Dihydrorose oxide	tr[b]	1439	Aromadendrene	0.2
1087	Fenchone	0.1	1454	a-Humulene	0.1
1093	6,7-Epoxymyrcene	tr[b]	1461	Alloaromadendrene	0.1
1097	α-Pinene oxide	tr[b]	1477	γ-Muurolene	tr[b]
1102	Perillene	0.2	1487	β-Selinene	tr[b]
1108	Linalool	0.6	1496	*epi*-Cubebol	tr[b]
1146	Camphor	36.5	1501	α-Muurolene	tr[b]
1167	Pinocarvone	0.2	1515	γ-Cadinene	tr[b]
1169	Borneol	0.2	1517	Cubebol	0.1
1180	Terpinen-4-ol	1.4	1524	*trans*-Calamenene	tr[b]
1186	*p*-Methylacetophenone	0.1	1585	Caryophyllene oxide	0.4

Table 3.5 (continued)

RI[a]	Compound	%	RI[a]	Compound	%
1188	*p*-Cymen-8-ol	0.2	1593	Viridiflorol	tr[b]
1193	α-Terpineol	1.5	1595	Cubeban-11-ol	tr[b]
1197	Myrtenal	0.3	1604	Ledol	tr[b]
1199	Myrtenol	0.3	1611	Humulene epoxide II	0.3
1208	trans-Piperitol	tr[b]	1630	1-*epi*-Cubenol	tr[b]
1210	Verbenone	0.1	1643	τ-Muurolol	tr[b]
				Total Identified	98.5

[a] "RI" = Retention Indices on HP-5ms fused silica capillary column
[b] "tr" = Trace components (< 0.5%)

Table 3.6 Chemical composition of *Cinnamomum camphora* (P75) leaf oil from Nepal

RI[a]	Compound	%	RI[a]	Compound	%
941	α-Pinene	tr[b]	1227	Nerol	tr[b]
953	Camphene	0.2	1419	(*E*)-Caryophyllene	tr[b]
978	β-Pinene	0.1	1453	α-Humulene	tr[b]
1031	1,8-Cineole	0.2	1497	Bicyclogermacrene	tr[b]
1146	Camphor	98.0	1550	Elemol	tr[b]
1165	Borneol	1.4	1578	Spathulenol	tr[b]
1177	Terpinen-4-ol	tr[b]	1584	Caryophyllene oxide	tr[b]
1190	α-Terpineol	0.2	1654	Selin-11-en-4α-ol	tr[b]
				Total Identified	100

[a] "RI" = Retention indices on HP-5ms fused silica capillary column
[b] "tr" = Trace components (< 0.5%)

Table 3.7 Chemical composition of *Cinnamomum glaucescens* (P21) leaf oil from Nepal

RI[a]	Compound	%	RI[a]	Compound	%
933	Methyl hexanoate	tr[b]	1235	Ascaridole	tr[b]
935	α-Thujene	0.4	1236	Cuminal	0.1
941	α-Pinene	2.6	1236	Neral	tr[b]
953	Camphene	0.1	1243	Carvone	tr[b]
958	Thuja-2,4(10)-diene	tr[b]	1253	Piperitone	tr[b]
964	Benzaldehyde	0.2	1255	Geraniol	0.1
978	Sabinene	5.7	1270	Geranial	tr[b]
980	β-Pinene	2.8	1281	Neryl formate	tr[b]
993	Myrcene	0.1	1285	*neo*-*iso*-3-Thujanol acetate	tr[b]
1005	α-Phellandrene	tr[b]	1290	*p*-Cymene-7-ol	0.1
1016	α-Terpinene	0.3	1302	Methyl (*Z*)-cinnamate	0.5
1024	*p*-Cymene	0.3	1321	Methyl geranate	tr[b]
1028	*o*-Cymene	0.2	1332	8-Hydroxy-*neo*-menthol	tr[b]
1034	1,8-Cineole	24.8	1349	α-Cubebene	tr[b]
1060	γ-Terpinene	1.0	1375	α-Copaene	0.3
1067	*cis*-Sabinene hydrate	tr[b]	1391	Methyl (*E*)-cinnamate	40.5
1072	*cis*-Linalool oxide (furanoid)	0.2	1422	(*E*)-Caryophyllene	tr[b]
1079	*p*-Cresol	tr[b]	1428	Carvone hydrate	0.3
1087	Terpinolene	0.3	1437	Coumarin	tr[b]
1102	Linalool	3.7	1455	α-Humulene	tr[b]
1113	*endo*-Fenchol	tr[b]	1497	*epi*-Cubebol	0.1
1121	*cis*-*p*-Menth-2-en-1-ol	0.1	1518	Cubebol	0.1

Table 3.7 (continued)

RI[a]	Compound	%	RI[a]	Compound	%
1126	Methyl octanoate	tr[b]	1525	δ-Cadinene	0.1
1138	*trans*-Pinocarveol	0.1	1579	Spathulenol	tr[b]
1139	*trans*-Sabinol	0.1	1584	Caryophyllene oxide	0.3
1144	*trans*-Verbenol	tr[b]	1592	Viridiflorol	tr[b]
1157	Sabina ketone	tr[b]	1604	Ledol	tr[b]
1162	Pinocarvone	0.1	1609	Humulene epoxide II	0.1
1165	Isoborneol	0.1	1628	1-*epi*-Cubenol	tr[b]
1168	δ-Terpineol	0.9	1643	τ-Muurolol	tr[b]
1171	*cis*-Linalool oxide (pyranoid)	tr[b]	1650	β-Eudesmol	tr[b]
1180	Terpinen-4-ol	4.8	1655	α-Cadinol	tr[b]
1184	*p*-Methylacetophenone + Thuj-3-en-10-al	0.1	1659	*cis*-Calamenen-10-ol	tr[b]
1186	Cryptone	0.1	1666	*trans*-Calamenen-10-ol	tr[b]
1192	α-Terpineol	7.4	1669	14-Hydroxy-9-*epi*-(*E*)-caryophyllene	tr[b]
1198	Myrtenol	0.3	1673	Cadalene	tr[b]
1207	*cis*-Piperitol	tr[b]	1678	Mustakone	tr[b]
1208	Verbenone	0.1	1722	(2*Z*,6*E*)-Farnesol	tr[b]
1217	*trans*-Carveol	tr[b]	1741	(2*E*,6*E*)-Farnesal	tr[b]
1227	Nerol	0.2		Total Identified	99.4

[a] "RI" = Retention indices on HP-5ms fused silica capillary column
[b] "tr" = Trace components (< 0.5%)

The *C. tamala* root essential oil (P12) was also found to be a camphor chemotype, with 35.0% camphor composition followed by linalool (10.6%), *p*-cymene (8.5%), *o*-cymene (6.8%), and 1,8-cineole (6.1%). From a review of the relevant literature, it appears that this is the first study of the root essential oil of *C. tamala* from Nepal. However, previous leaf essential oil studies have shown that eugenol and cinnamaldehyde are major components, with Indian samples reporting 81.7% to 78% eugenol composition (Chopra *et al.*, 1956; Rana and Blazquez, 2005). However, other studies have shown that cinnamaldehyde (12.7-8.7%, 52.8%, and 41.2-55.2%) and linalool (50.3-33.7%, 19.7%, and 15.7-15.3%), rather than eugenol, are the main components of *C. tamala* leaf oil (Sood *et al.*, 1979; Gulati *et al.*, 1977).

Table 3.8 Chemical composition of *Cinnamomum tamala* (P12) root oil from Nepal

RI[a]	Compound	%	RI[a]	Compound	%
928	Tricyclene	0.1	1337	δ-Elemene	tr[b]
933	α-Thujene	0.7	1349	α-Cubebene	0.1
941	α-Pinene	4.7	1366	Neryl acetate	tr[b]
952	Camphene	2.0	1371	Hydrocinnamyl acetate	0.1
955	Thuja-2,4(10)-diene	tr[b]	1379	α-Copaene	1.3
960	Benzaldehyde	0.1	1382	*trans*-*p*-Menth-6-ene-2,8-diol	tr[b]
973	Sabinene	0.5	1385	Methyl (*E*)-cinnamate	tr[b]
979	β-Pinene	4.1	1387	Geranyl acetate	0.1
991	Myrcene	1.1	1393	β-Cubebene	0.8
998	δ-2-Carene	tr[b]	1394	β-Elemene	0.2

Table 3.8 (continued)

RI[a]	Compound	%	RI[a]	Compound	%
1007	δ-3-Carene	0.3	1422	(*E*)-Caryophyllene	1.0
1025	*p*-Cymene	8.5	1429	Carvone hydrate	tr[b]
1035	*o*-Cymene	6.8	1430	β-Copaene	tr[b]
1039	1,8-Cineole	6.1	1439	Coumarin	0.6
1043	Salicylaldehyde	0.3	1440	Aromadendrene	tr[b]
1053	*cis*-Sabinene hydrate	tr[b]	1451	(*E*)-Cinnamyl acetate	1.2
1059	*cis*-Linalool oxide (furanoid)	0.3	1457	α-Humulene	1.2
	trans-Linalool oxide (furanoid)	0.3	1462	9-*epi*-(*E*)-Caryophyllene	tr[b]
1089	Linalool	10.6	1479	γ-Muurolene	0.1
1147	Camphor	35.0	1483	Amorpha-4,7(11)-diene	0.1
1162	Isoborneol	tr[b]	1485	Widdra-2,4(14)-diene	tr[b]
1164	Borneol	0.8	1488	β-Selinene	tr[b]
1165	Pinocarvone	0.2	1493	*trans*-Muurola-4(14),5-diene	tr[b]
1169	*trans*-Linalool oxide (pyranoid)	tr[b]	1497	*epi*-Cubebol	0.2
1179	Terpinen-4-ol	1.8	1502	α-Muurolene	0.1
1182	*p*-Methylacetophenone	tr[b]	1507	Germacrene A	tr[b]
1185	Cryptone	0.3	1516	γ-Cadinene	tr[b]
1193	α-Terpineol	2.2	1518	Cubebol	0.1
1196	Myrtenal	0.2	1526	δ-Cadinene	0.3
1196	Myrtenol	0.1	1529	(*E*)-*o*-Methoxycinnamaldehyde	tr[b]
1197	Estragole (= Methyl chavicol)	0.1	1538	α-Cadinene	tr[b]
1199	*cis*-Piperitol	tr[b]	1543	α-Calacorene	tr[b]
1206	*trans*-Piperitol	tr[b]	1554	(*Z*)-Caryophyllene oxide	0.1
1207	Verbenone	0.1	1558	Germacrene B	tr[b]
1217	*trans*-Carveol	0.1	1565	β-Calacorene	tr[b]

Table 3.8 (continued)

RI[a]	Compound	%	RI[a]	Compound	%
1227	Citronellol	0.1	1582	Spathulenol	0.2
1237	Cuminal	0.1	1587	Caryophyllene oxide	1.3
1239	*o*-Anisaldehyde	0.1	1609	Humulene epoxide II	0.4
1242	Carvone	0.1	1623	α-Corocalene	tr[b]
1251	Piperitone	0.1	1629	1-*epi*-Cubenol	0.1
1254	Geraniol	tr[b]	1634	Caryophylla-4(12),8(13)-dien-5-ol	0.1
1260	(2*E*)-Decenal	tr[b]	1643	τ-Muurolol	0.1
1269	(*E*)-Cinnamaldehyde	0.4	1646	α-Muurolol (= Torreyol)	tr[b]
1270	Geranial	tr[b]	1656	Selin-11-en-4α-ol	0.1
1286	Bornyl acetate	0.8	1659	*cis*-Calamenen-10-ol	0.1
1290	*p*-Cymen-7-ol	0.1	1668	*trans*-Calamenen-10-ol	tr[b]
1299	Terpinen-4-ol acetate	0.1	1672	14-Hydroxy-9-*epi*-(*E*)-caryophyllene	0.1
1301	Carvacrol	tr[b]	1674	Cadalene	tr[b]
1305	(*E*)-Cinnamyl alcohol	0.2	2037	Kaur-16-ene	0.2
				Total Identified	99.4

[a] "RI" = Retention indices on HP-5ms fused silica capillary column
[b] "tr" = Trace components (< 0.5%)

C. glaucescens fruit (drupes) were distilled to collect their essential oil, which was found to primarily contain methyl (*E*)-cinnamate (40.5%) and 1,8-cineole (24.8%). Smaller quantities of α-terpineol (7.4%), sabinene (5.7%), terpinen-4-ol (4.8%), and linalool (3.7%) were also present. In comparison, the leaf oil of *C. glaucescens* from India contained elemicin (92.9%) and methyl eugenol (4.9%) as the major components

(Baruah and Nath, 2006). Another study of the fruit oil of *C. glaucescens* from Nepal showed a similar essential oil composition, with the major components being 1,8-cineole (13%), methyl (*E*)-cinnamate (14%), and α-terpineol (7%) (Adhikary *et al.*, 1992).

The allelopathic activities of *C. cinnamomum*, *C. glaucescens*, and *C. tamala* essential oils are summarized in Table 3.2. *Lactuca sativa* seed germination is notably inhibited by all three essential oils: *C. cinnamomum* IC_{50} = 218μg/mL, *C. glaucescens* IC_{50} = 1620 μg/mL, and *C. tamala* IC_{50} = 1630 μg/mL. *Lolium perenne* was less susceptible to germination inhibition (IC_{50} = 1000, >1773, and 1630 μg/mL, respectively, for *C. cinnamomum*, *C. glaucescens*, and *C. tamala*). Their major components camphor, Methyl (*E*)-cinnamate have shown allelopathic activity as compared to other components α-Pinene and α-Pinene(see Table 3.2)

Essential oils from the three *Cinnamomum* species were analyzed for antibacterial and antifungal activities while the major components were also tested to determine the compounds that were responsible for the sample oil results. Of the three essential oils tested for antimicrobial activities—*C. camphora*, *C. tamala*, and *C. glaucescens*—only *C. camphora* showed noteworthy antifungal activity against *A. niger* (MIC = 20 μg/mL). This is hypothesized to be a result of synergism among the major components [camphor (36.5%), limonene (9.0%), and β-pinene (6.3%)], all of which have exhibited MIC = 156 μg/mL against *A. niger* as shown in Table 3.2. However, mixtures of essential oil components were not tested for antifungal activity in this study. In addition, *C. camphora* showed marginal activity against *B. cereus* and *S. aureus*, all with MIC = 313 μg/mL. The Nepalese *C. camphora* exhibited antifungal and antibacterial

activity in accordance with literature reports from various regions (Rangari, 2011; Singh *et al.*, 2008; Ho *et al.*, 2009).

Likewise, *C. tamala* also showed notable antifungal activity against *A. niger* (MIC = 156 μg/mL) and marginal activity against *C. albicans*, which is likely due to the presence of camphor and linalool. Of the major components of Nepalese *C. tamala*, 1,8-cineole exhibited notable antibacterial activity against *S. aureus* (MIC = 313 μg/mL), while *p*-cymene was only active against *A. niger* (MIC = 313 μg/mL). Other reports are also consistent with the activity shown from this sample in its display of potent antibacterial activity against *B. subtilis*, *E. coli*, *S. cerevisae*, and *S. aureus* (Girgune *et al.*, 1978) as well as its antifungal activity against *Microsporum adudouinii* and *Tricophyton mentagrophytes* (Yadav *et al.*, 1999).

Similar antifungal activity was also observed for *C. glaucescens*, with marginal activity against *A. niger* (MIC = 313 μg/mL). This observation is mainly due to the fact that methyl (*E*)-cinnamate (MIC = 156 μg/mL) comprises 40.5% of the *C. glaucescens* fruit oil. Marginal activity against *B. cereus* (MIC = 313 μg/mL) and *P. aeruginosa* (MIC = 313 μg/mL) was also observed.

Of the three samples tested, *C. camphora* (P15) and *C. glaucescens* had substantial cytotoxic activities, with kill rates of 71.3% and 72.0%, respectively. The major components for all three samples demonstrate cytotoxicity, particularly limonene (90.8% kill) in *C. camphora* and *p*-cymene (> 100% kill) in *C. tamala*. Some correlation between cytotoxic activity and brine shrimp lethality was observed where *C. camphora* (P15) exhibited strong activity with LC_{50} of 2.52±2.51 μg/mL. Both *C. tamala* and *C. glaucescens* samples displayed brine shrimp lethality (28.1±3.0 and

20.1±2.6 μg/mL, respectively) due to the presence of 1,8-cineole (LC_{50} = 26.9±13.1 μg/mL) in both samples. In addition, *p*-Cymene, a major component of *C. tamala* was observed to have inconsistent properties, with strong activity against brine shrimp (LC_{50} = 4.65±0.99 μg/mL) while displaying virtually no cytotoxic activity.

A test of nematocidal activity showed *C. glaucescens* essential oil as having the strongest activity, which is shown in Table 3.3. The activity of *C. glaucescens* could possibly be caused by the presence of methyl (*E*)-cinnamate, which has shown significant activity (LC_{50} = 138±28 μg/mL) in this study. Of all the individual major components tested, limonene showed the strongest nematocidal activity, while such activity in α-pinene, β-pinene, and α-terpineol was virtually non-existent.

Minimal fruit fly activity was observed for the three *Cinnamomum* species, with the strongest activity exhibited by *C. camphora* (P15) (LC_{50} = 100 μg/mL). Although *C. tamala* did not show any appreciable activity, analysis of the major components exhibited significant activity as detailed in Table 3.3. Therefore, antagonistic effects among the remaining components are hypothesized to account for the disparity between the oil and components' activity.

There was appreciable insecticidal activity against fire ants among the three oil samples. *C. camphora* (P15) was the most active (LC_{50} = 176 ± 53 μg/mL), followed by *C. tamala* (LC_{50} = 191 ± 59 μg/mL). Again, additive effect m was observed among the major oil components as presented in Table 3.3.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Using essential oils is of paramount importance in the field of natural medicine. Nepal is one of the most biodiversically rich countries, with many additional plant species and medicinally valuable compounds that have yet to be characterized and discovered. This research has been carried out to characterize the chemical and biological properties of the following eight essential oils and their major components as well as their biological activities regarding allelopathy, antimicrobial function, cytotoxicity, nematocidal activity, and insecticidal value: *Acorus calamus*, *Amomum subulatum*, *Artemisia dubia*, *Artemisia indica*, *Artemisia vulgaris*, *Cinnamomum camphora*, *Cinnamomum glaucescens* and *Cinnamomum tamala*.

Four essential oils from the leaf (P24) and rhizomes (P19, P22, P23) of *Acorus calamus* L., collected from various parts of Nepal, were obtained by hydrodistillation and analyzed by GC-MS. From a total of 61 peaks, 57 compounds were identified among the four essential oils, accounting for 94.3%, 96.2%, 97.6%, and 94.1% of the oils, respectively. All of the essential oils were dominated by (*Z*)-asarone (78.1%-86.9%). The essential oils also contained (*E*)-asarone (1.9%-9.9%) and small amounts of γ-asarone (2.0-2.3%), (*Z*)-methyl isoeugenol (1.5-2.0%), and linalool (0.2-4.3%). Allelopathic testing of sample P19 showed an inhibition of seed germination of *Lactuca*

sativa and *Lolium perenne* with IC_{50} values of 450 and 737 μg/mL, respectively. However, the P19 oil demonstrated stronger seedling growth inhibition of *L. perenne* than of *L. sativa*. Sample P19 also showed notable brine shrimp lethality (LC_{50} = 9.48 μg/mL), cytotoxic activity (92.2% kill on MCF-7 cells at 100 μg/mL), and antifungal activity against *Aspergillus niger* (MIC = 19.5 μg/mL). In addition, this sample has shown noteworthy insecticidal activities.

Two essential oils from the seed and rind of *Amomum subulatum* Roxb. (collected from Nepal) were obtained by hydrodistillation and were analyzed by GC-MS. Out of 85 peaks, components were identified among the two essential oils, accounting for 99.1%, and 99.0% of the oils' compositions, respectively. The two essential oils were dominated by the monoterpenoids 1,8-cineole (60.8% and 39.0%), α-pinene (6.4% and 4.8%), β-pinene (8.3% and 17.7%), and α-terpineol (9.8% and 12.3%). Allelopathic testing of seed essential oil showed an inhibition of seed germination of *Lactuca sativa* and *Lolium perenne*, with IC_{50} values of 1583 and 1674 μg/mL, respectively. The seed essential oil demonstrated a stronger seedling growth inhibition of *L. perenne* than of *L. sativa*. *A. subulatum* seed oil also showed moderate brine shrimp lethality (LC_{50} = 28.1 ± 3.0 μg/mL). The seed and rind oils were only marginally cytotoxic (20% and 30% kill on MCF-7 cells at 100 μg/mL, respectively) and antibacterial. However, *A. subulatum* rind oil was appreciably antifungal against *Aspergillus niger* (MIC = 19.5 μg/mL). The seed essential oil of *A. subulatum* was also screened for nematocidal activity against *Caenorhabditis elegans* and insecticidal activity against the fruit fly (*Drosophila melanogaster*) and the red imported fire ant (*Solenopsis invicta* × *richteri*). The oil was only marginally toxic to the fire ant (LC_{50} = 1500 μg/mL), but

moderately toxic to the nematode and the fruit fly (LC_{50} = 341 and 188 μg/mL, respectively).

The essential oils from the aerial parts of *Artemisia dubia*, *Artemisia indica*, and *Artemisia vulgaris* growing wild in Nepal were obtained by hydrodistillation and analyzed by GC-MS. The major components of *A. dubia* oil were chrysanthenone (29.0%), coumarin (18.3%), and camphor (16.4%). *A. indica* oil was dominated by ascaridole (15.4%), isoascaridole (9.9%), *trans*-*p*-mentha-2,8-dien-1-ol (9.7%), and trans-verbenol (8.4%). The essential oil of Nepalese *A. vulgaris* was rich in α-thujone (30.5%), 1,8-cineole (12.4%), and camphor (10.3%). In addition, the essential oils were screened for phytotoxic activity against *Lactuca sativa* (lettuce) and *Lolium perenne* (perennial ryegrass), using both seed germination and seedling growth, and all three *Artemisia* oils exhibited notable allelopathic activity. *A. dubia* oil showed *in-vitro* cytotoxic activity on MCF-7 cells (100% kill at 100 μg/mL) and was also marginally antifungal against *Aspergillus niger* (MIC = 313 μg/mL).

The essential oils from the leaves of two geographically distinct *C. camphora* (P15 and P75), roots of *C. tamala*, and fruits of *C. glaucescens* growing wild in Nepal were obtained by hydrodistillation and analyzed by GC-MS. The major components in *C. camphora* (P15) oil were camphor (36.5%) and camphene (11.7%). *C. tamala* oil was also dominated by camphor (35.0%) followed by a smaller amount of linalool (10.9%) and *p*-cymene (8.4%). The berry essential oil of *C.glaucescens* was rich in (*E*)-methyl cinnamate (40.5%) and 1,8-cineole (24.8%). The three *Cinnamomum* essential oils were screened for multiple biological functions, including phytotoxicity, cytotoxicity, brine shrimp lethality, and pesticidal activity such as mosquito larvicidal and nematocidal

activity as well as fruit fly and fire ant toxicity. In addition to testing essential oils, subsequent tests of the individual major components of the three oils were also performed. The results show a wide variety of activity, with *C. tamala* being active against mosquito larvea, *C. glaucescens* exhibiting significant nematocidal activity, and *C. camphora* (P15) being most active in cytotoxicity and brine shrimp lethality. An individual components analysis showed evidence of both synergism and antagonism among the components.

A final comparison of all of the essential oils has shown that *A. dubia* was most active in *in-vitro* cytotoxic activity on MCF-7 cells (100% cell killing at 100 μg/mL) and germination inhibition of *L. sativa* (IC_{50} = 125 μg/mL). Similarly *A. vulgaris* has shown good activity in a fruit fly lethality bioassay (LC_{50} = 100 μg/mL) and on germination inhibition of *L. perenne* (IC_{50} = 657 μg/mL). Further, *A. calamus* has shown the strongest activity for *C. elegans* (LC_{50} = 64 μg/mL) and *A. niger* (MIC = 19.5 μg/mL). Meanwhile, *C. camphora* is most active for brine shrimp lethality (LC_{50} = 2.5±2.5 μg/mL) and glass worm larvicidal activity (LC_{50} =54.4 μg/mL), and *C. tamala* has shown the strongest activity for red imported fire ants (LC_{50} = 191±59 μg/mL). Antibacterial activity is not shown to be noteworthy with the tested organism; however, *S. aureus* activity (MIC = 313 μg/mL) has been shown by *C. camphora*, *C. glaucescens*, and *A. subulatum* seed oil. *C. glaucescens* is the most active essential oil for *P. aeuriginosa* (MIC = 313 μg/mL), while *A. subulatum* seed oil and *C. camphora* oil are most active (MIC = 313 μg/mL) for *B. cereus*. Most of the oils have not shown any remarkable activity against *E. coli*.

In conclusion, the studied plants have a comparatively wide availability and high essential oil yield. The *Artemisia* species is found to be the best plant genus for biological control of plant species. Similarly, the *Cinnamomum* species is found to be the best agent for insect control. Furthermore, essential oils have been proven to be environmentally friendly, and all of the essential oils have shown marginal activity in antimicrobial screening. Thus, this research proves that essential oils are truly essential as medicinally valuable substances with a siginificant impact on the traditional medical system.

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Appendix A

Gas Chromatography Traces

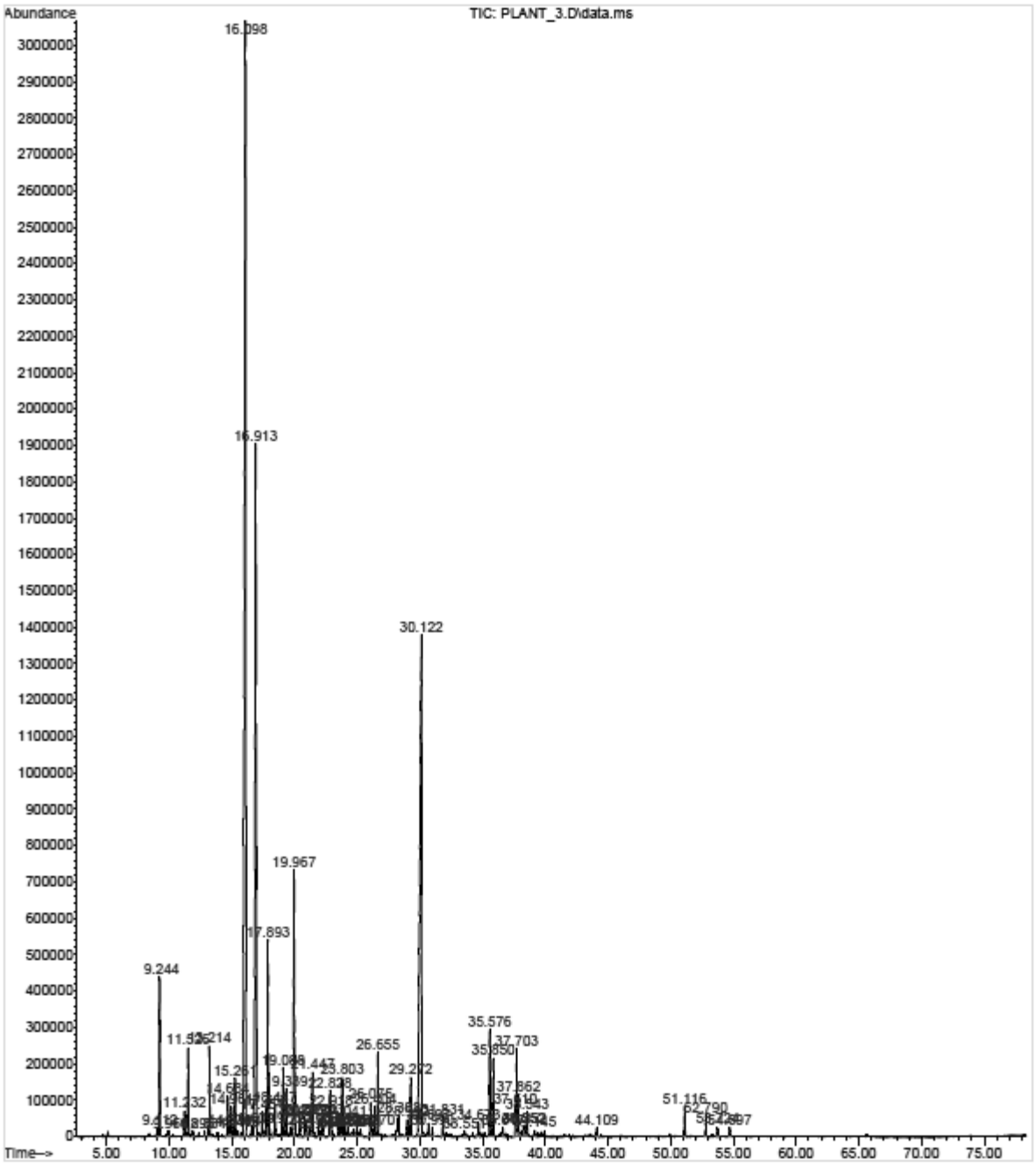

Figure A.1 GC trace of *Artemisia dubia* (P3) leaf essential oil

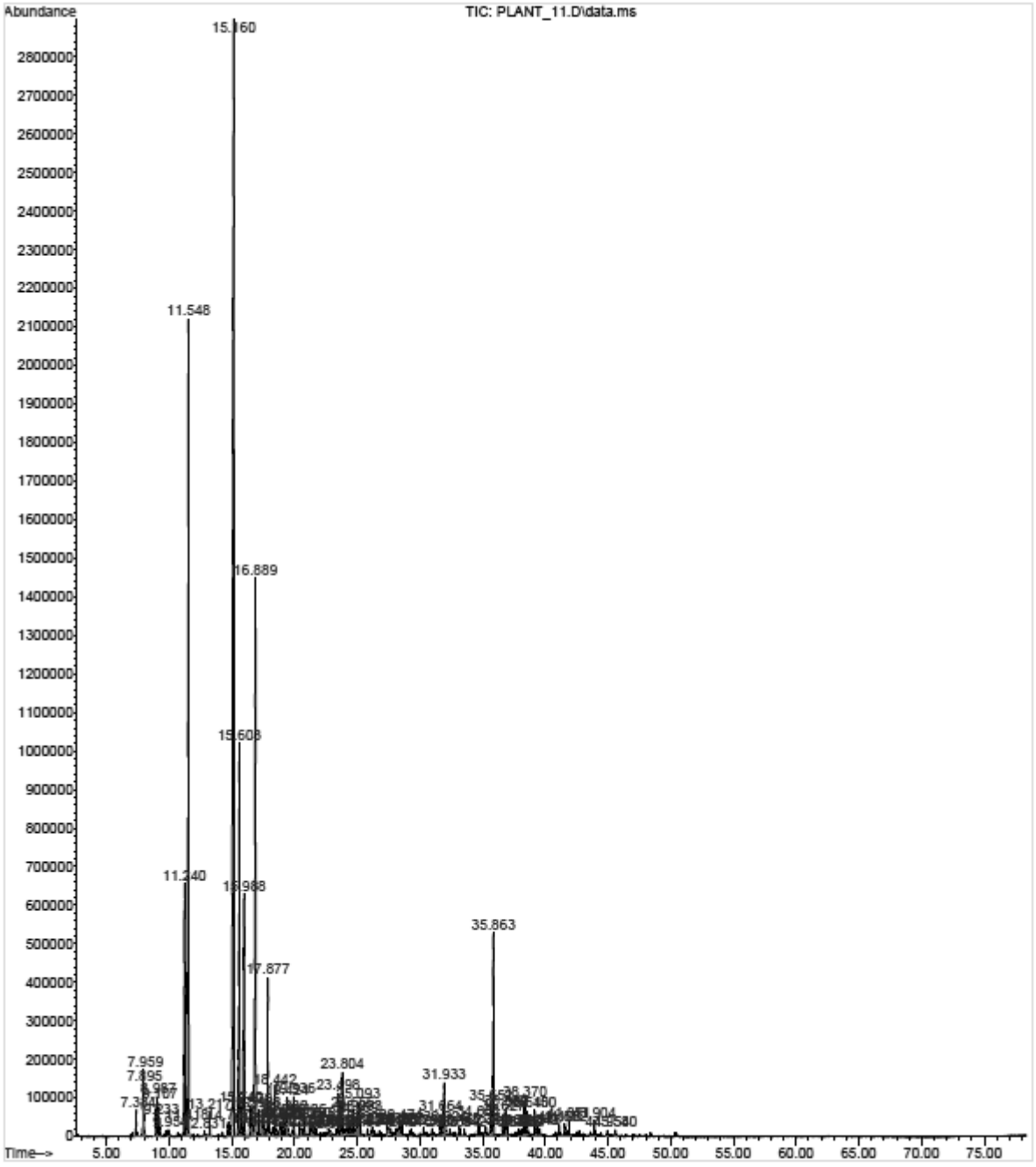

Figure A.2 GC trace of *Artemisia vulgaris* (P11) leaf essential oil

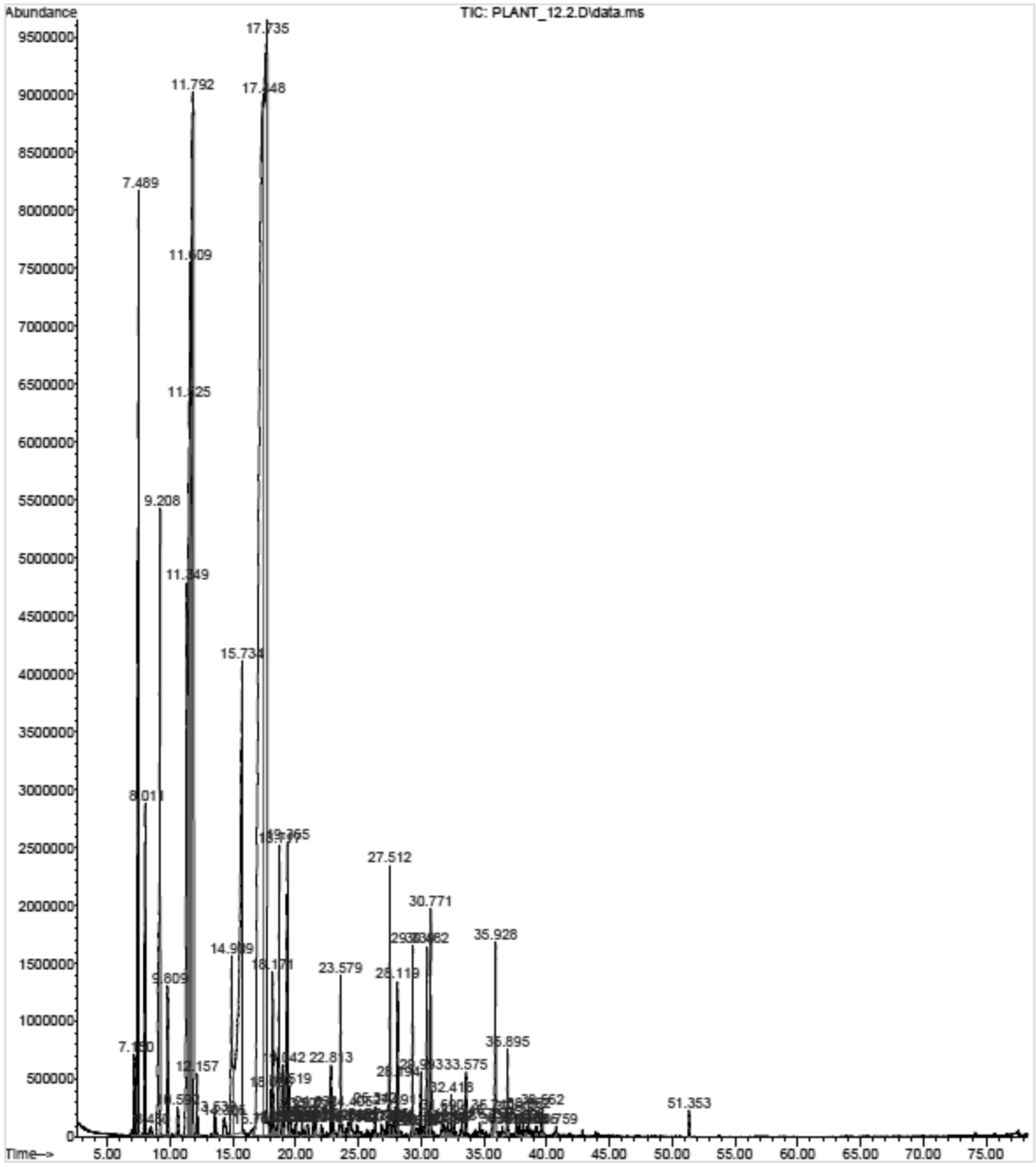

Figure A.3 GC trace of *Cinnamomum tamala* root (P12) essential oil

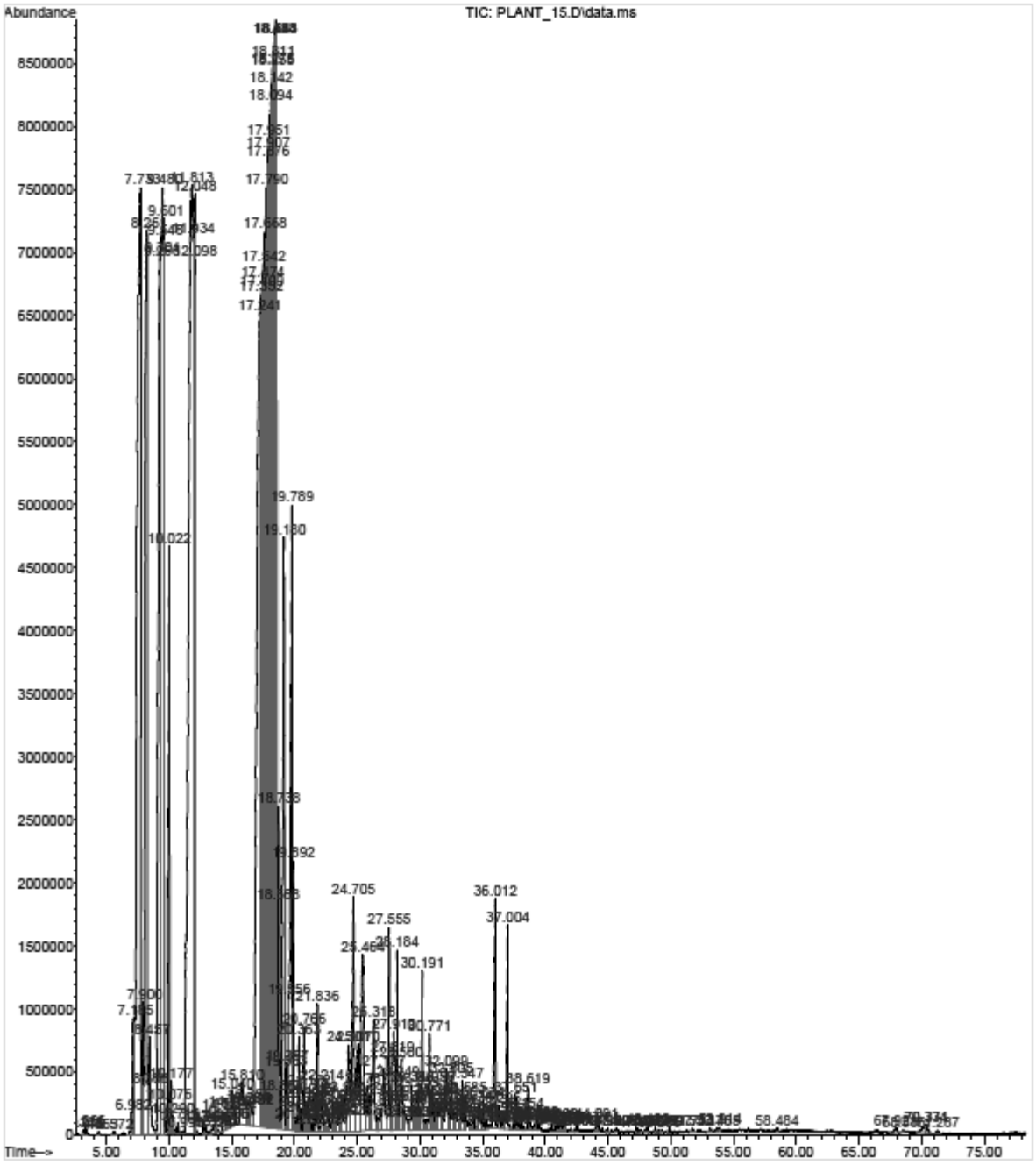

Figure A.4 GC trace of *Cinnamomum camphora* (P15) leaf essential oil

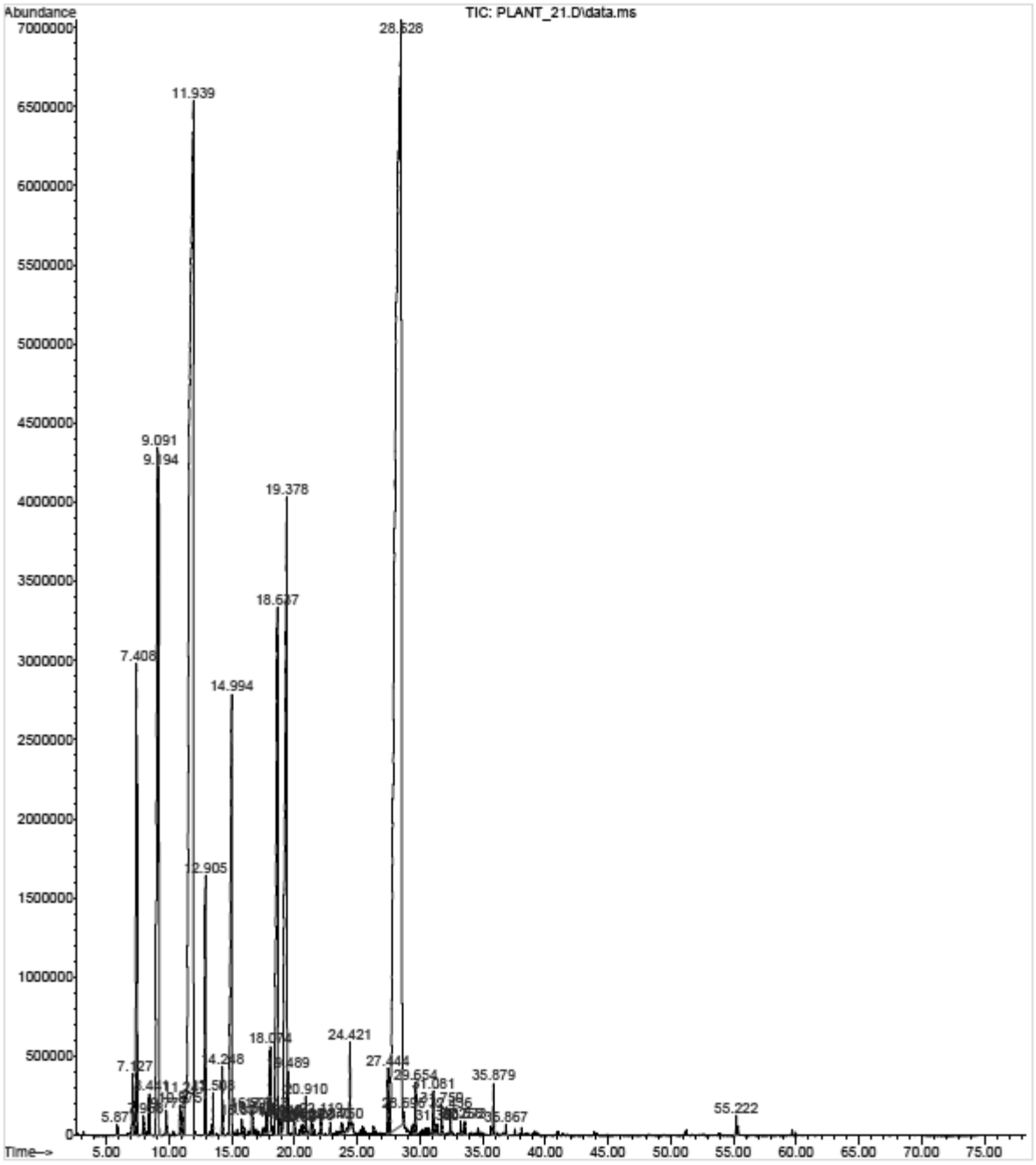

Figure A.5 GC trace of *Cinnamomum glaucescens* (P21) drupe essential oil

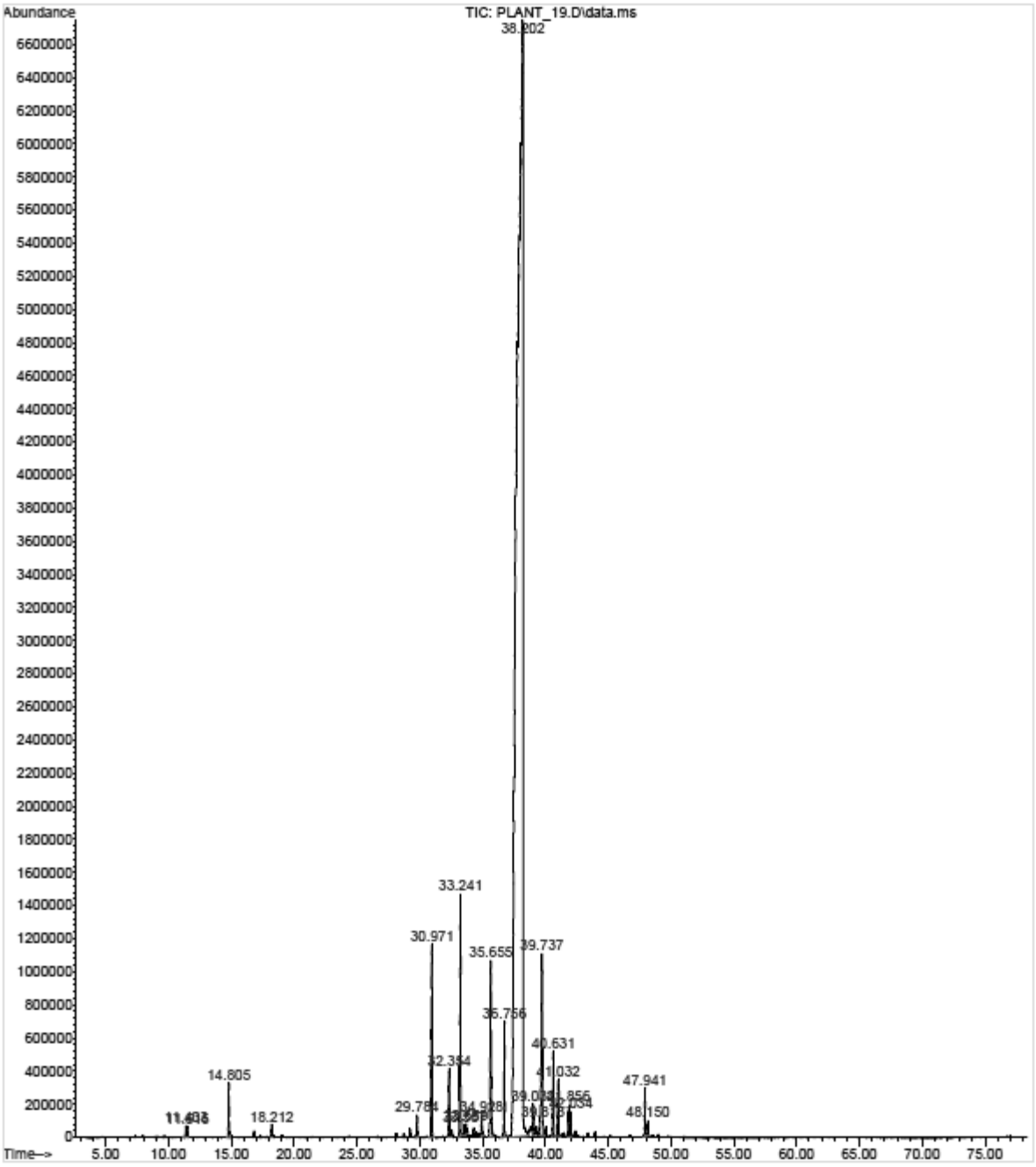

Figure A.6 GC trace of *Acorus calamus* (P19) rhizome essential oil

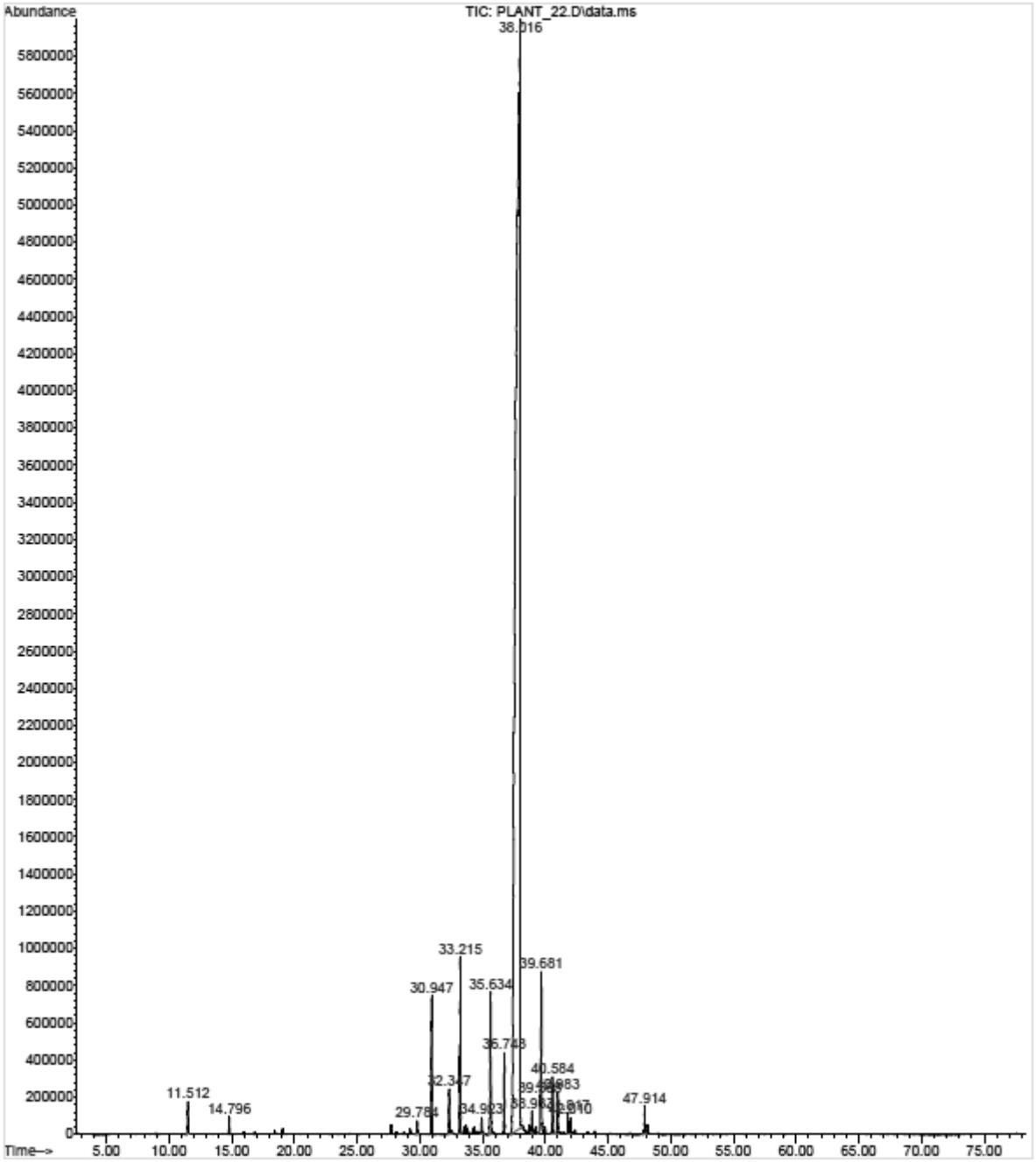

Figure A.7 GC trace of *Acorus calamus* (P22) rhizome essential oil

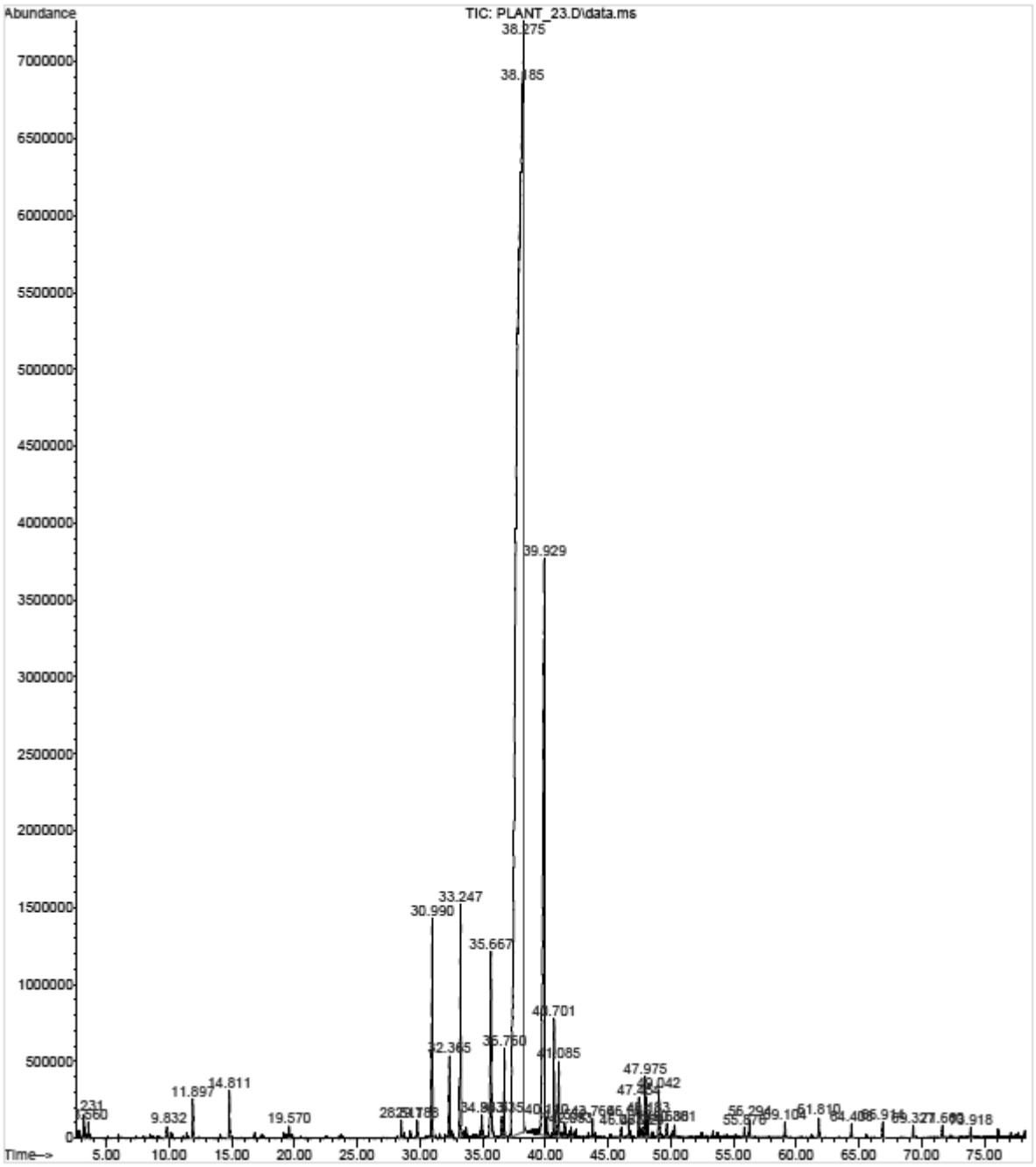

Figure A.8 GC trace of *Acorus calamus* (P23) rhizome essential oil

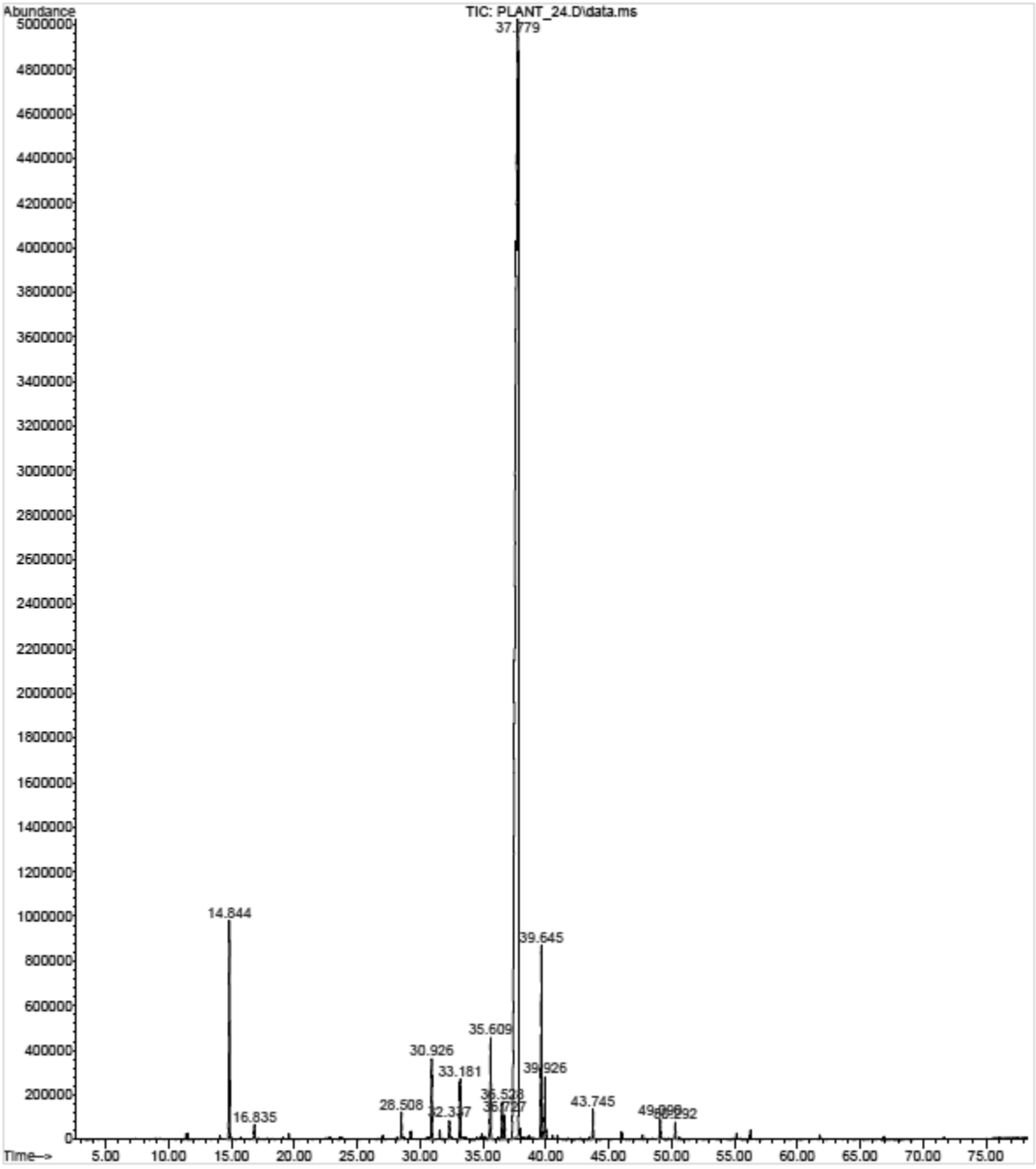

Figure A.9 GC trace of *Acorus calamus* (P24) leaf essential oil

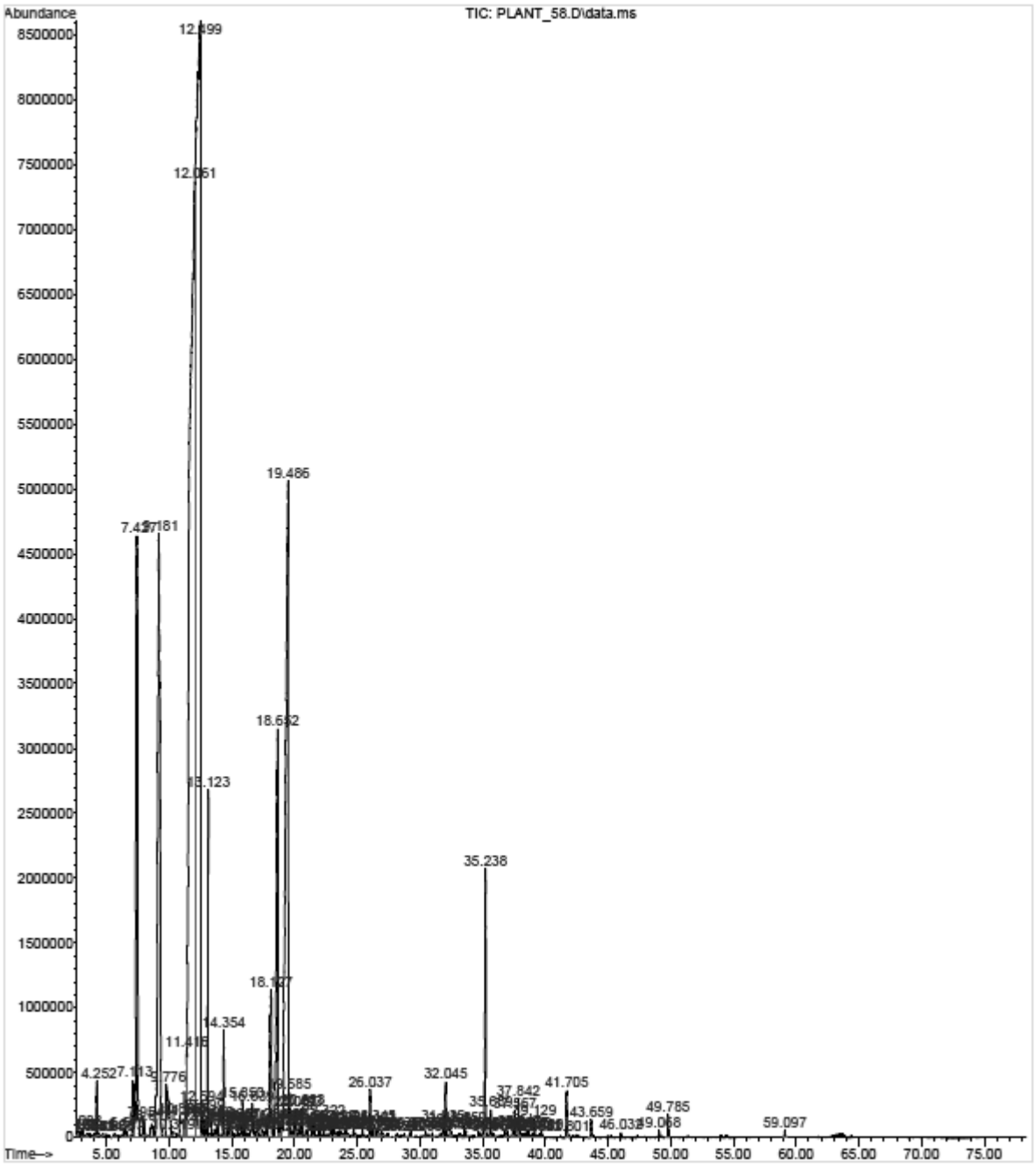

Figure A.10 GC trace of *Amomum subulatum* (P58) seed essential oil

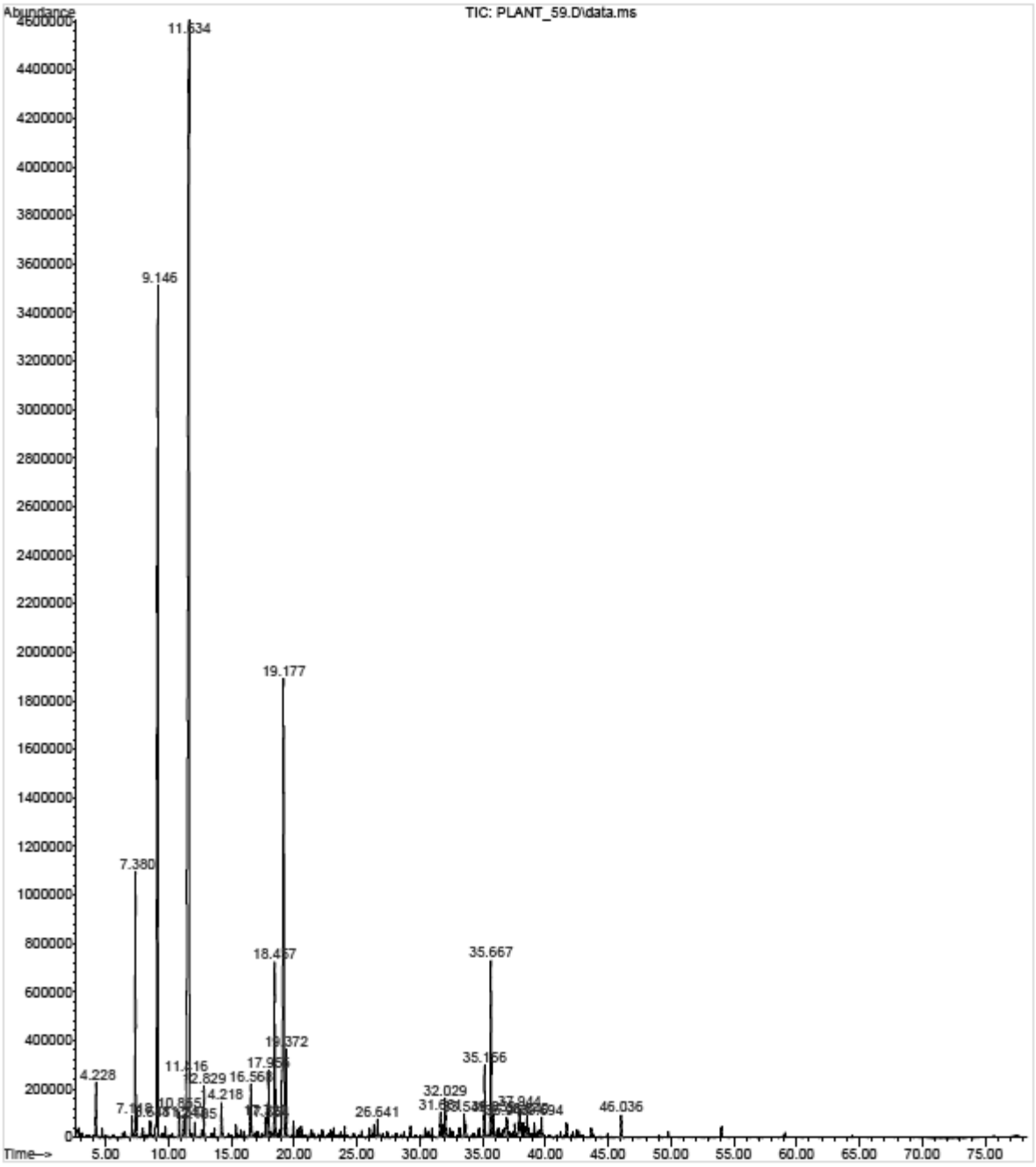

Figure A.11 GC trace of *Amomum subulatum* (P59) rind essential oil

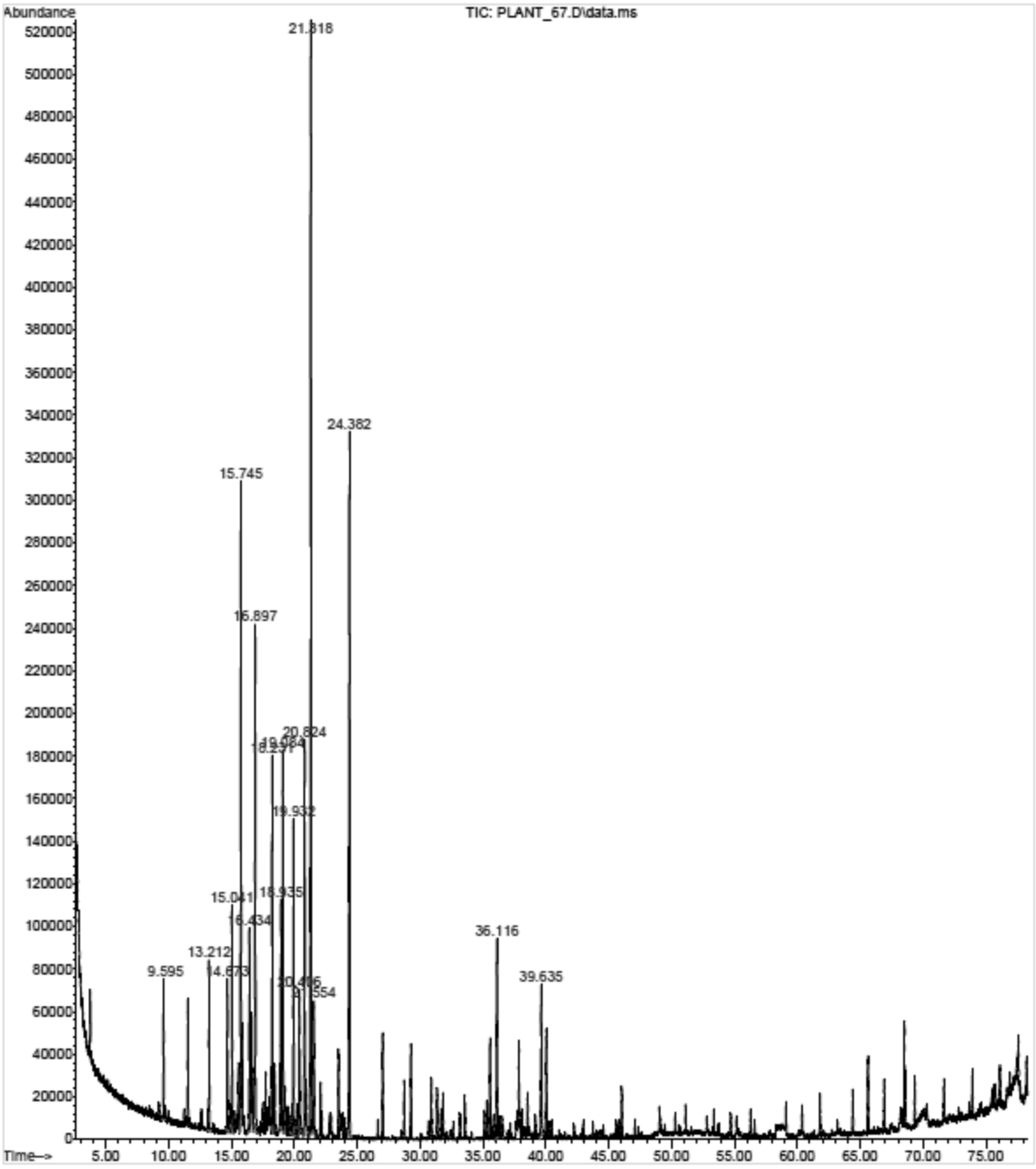

Figure A.12 GC trace of *Artemisia indica* (P67) leaf essential oil

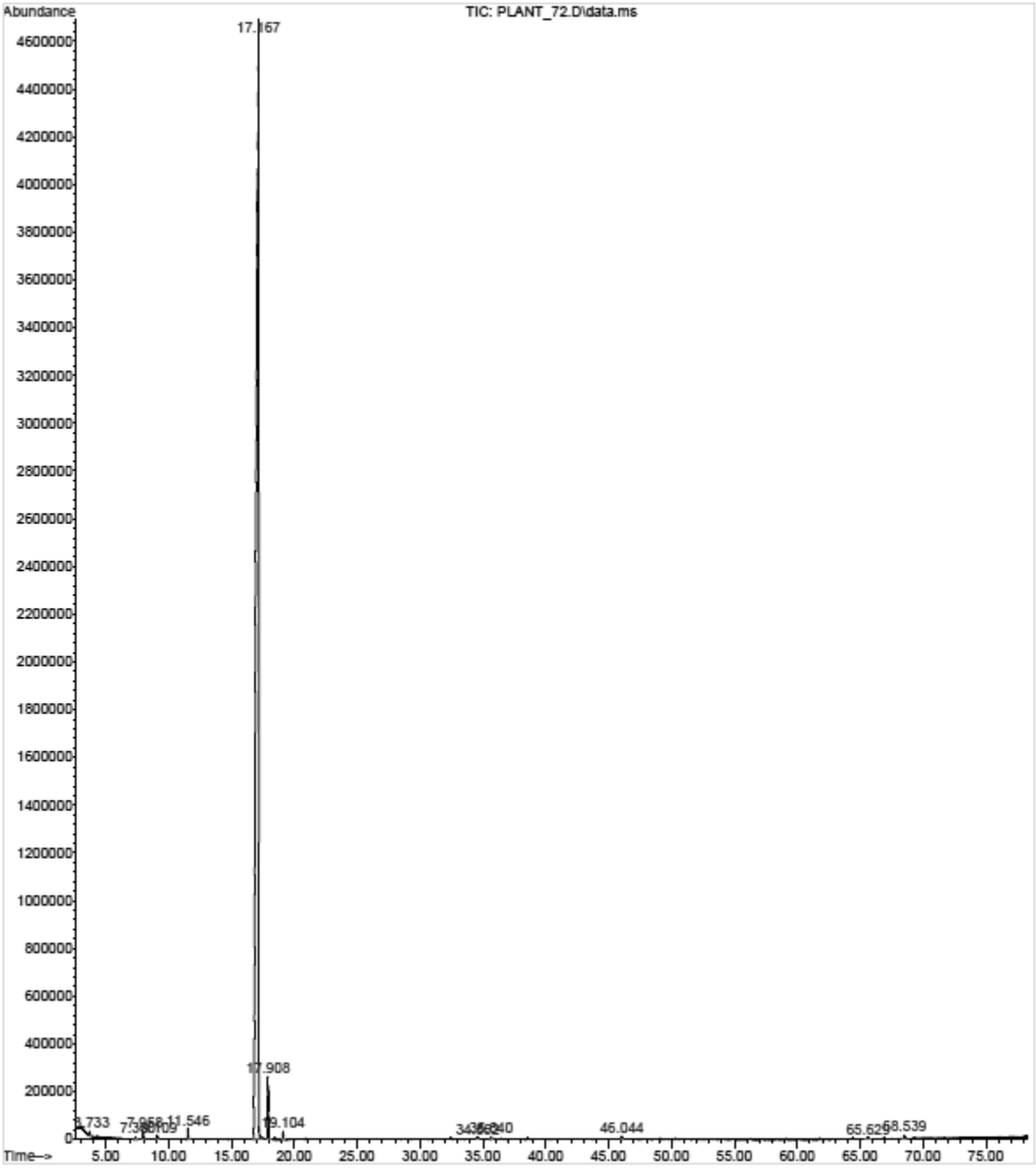

Figure A.13 GC trace of *Cinnamomum camphora* (P75) leaf essential oil.

Appendix B

MASS SPECTRA OF UNKNOWN COMPONENTS

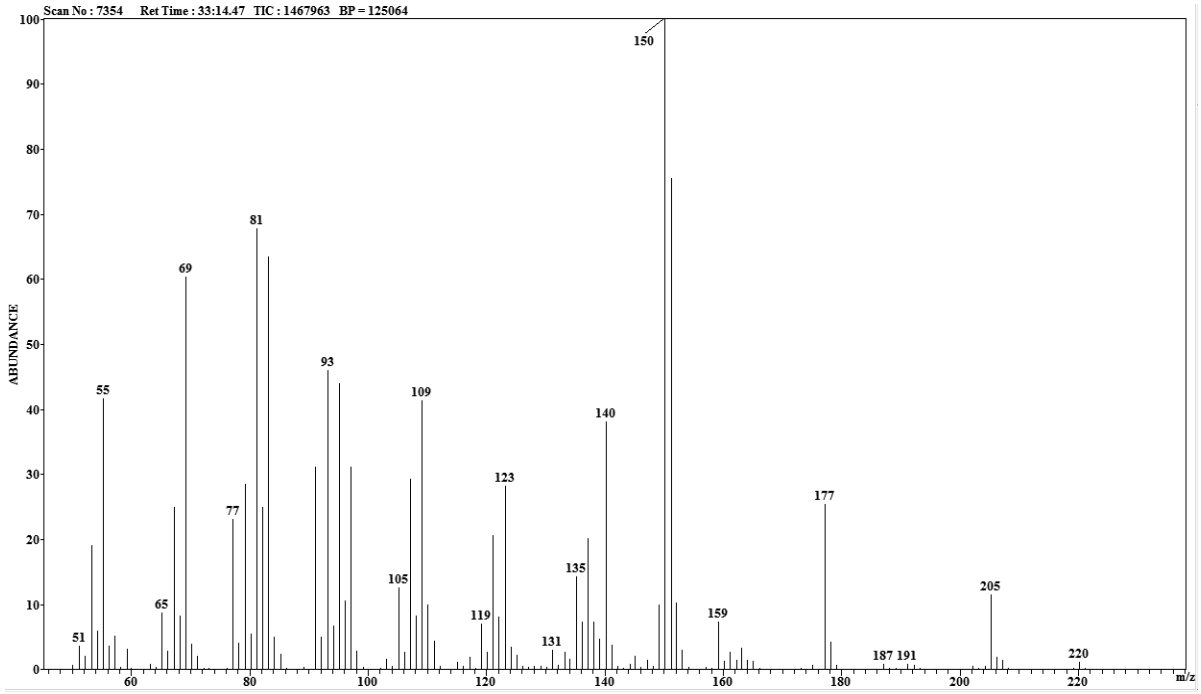

Figure B.1 Unknown 1 from the the *Acorus calamus* essential oil (P19).

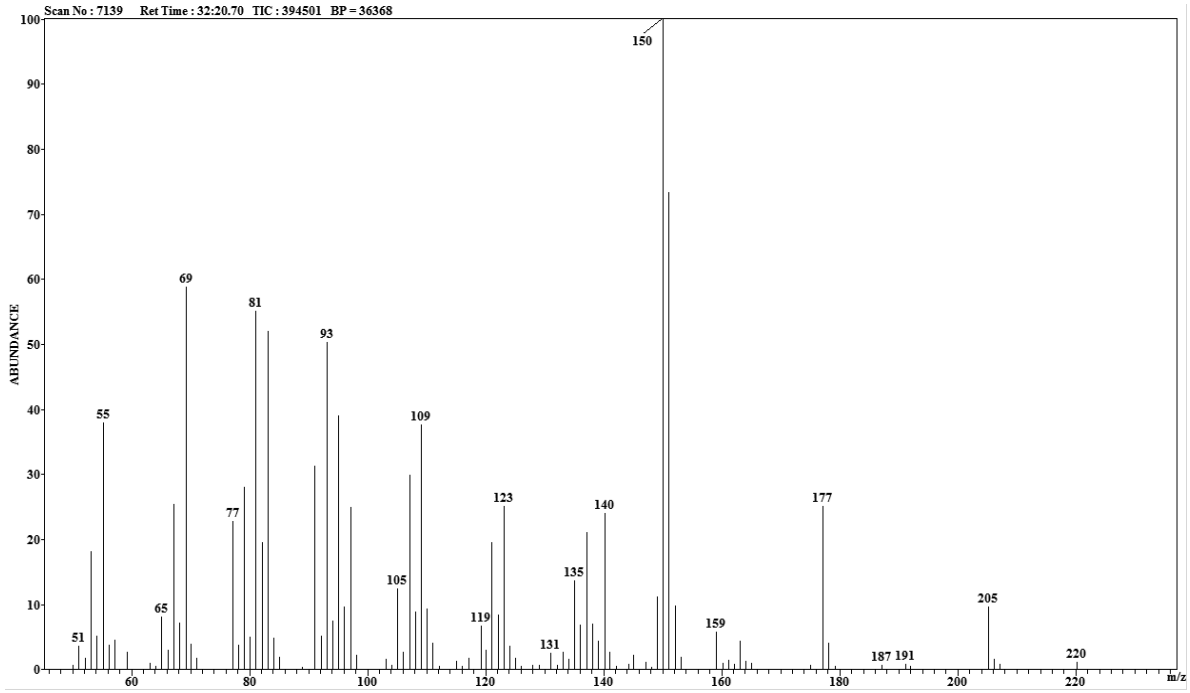

Figure B.2 Unknown 2 from the the *Acorus calamus* essential oil (P19).

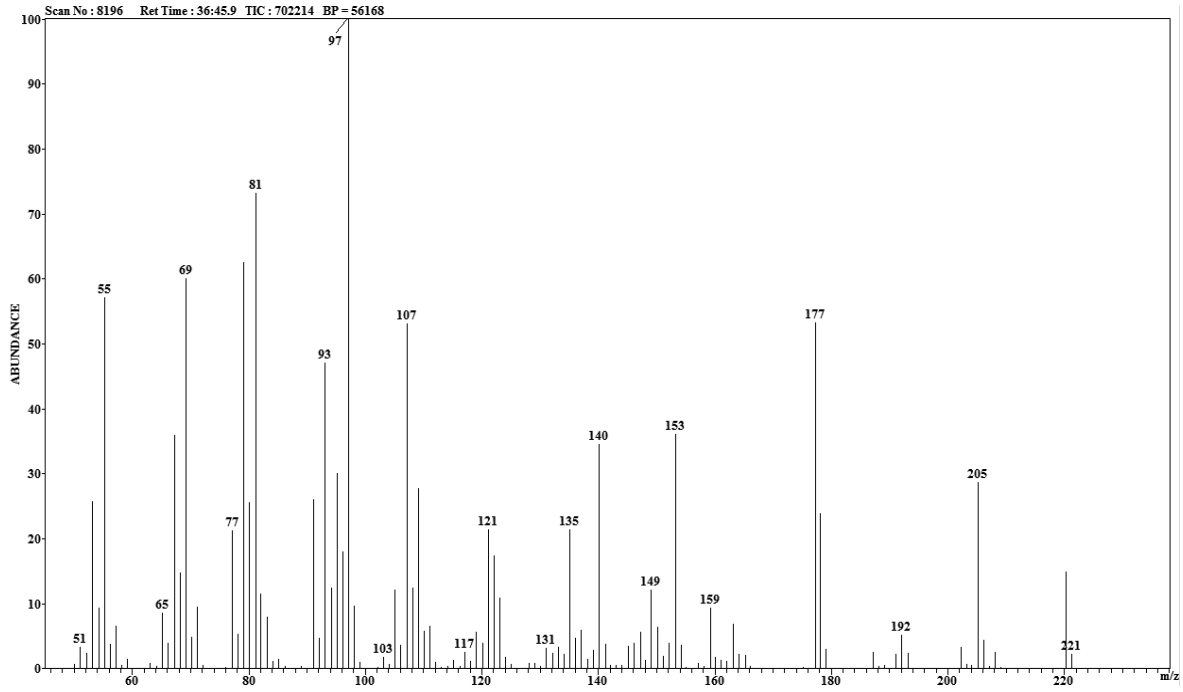

Figure B.3 Unknown 3 from the the *Acorus calamus* essential oil (P19).

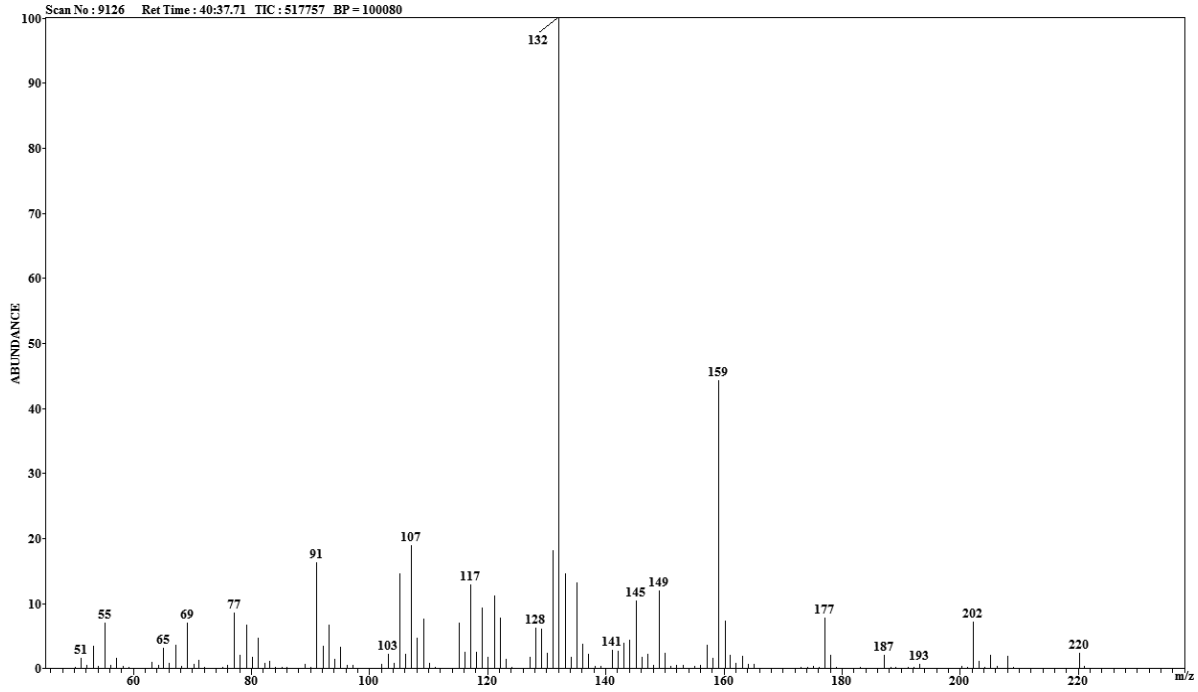

Figure B.4 Unknown 4 from the the *Acorus calamus* essential oil (P19).

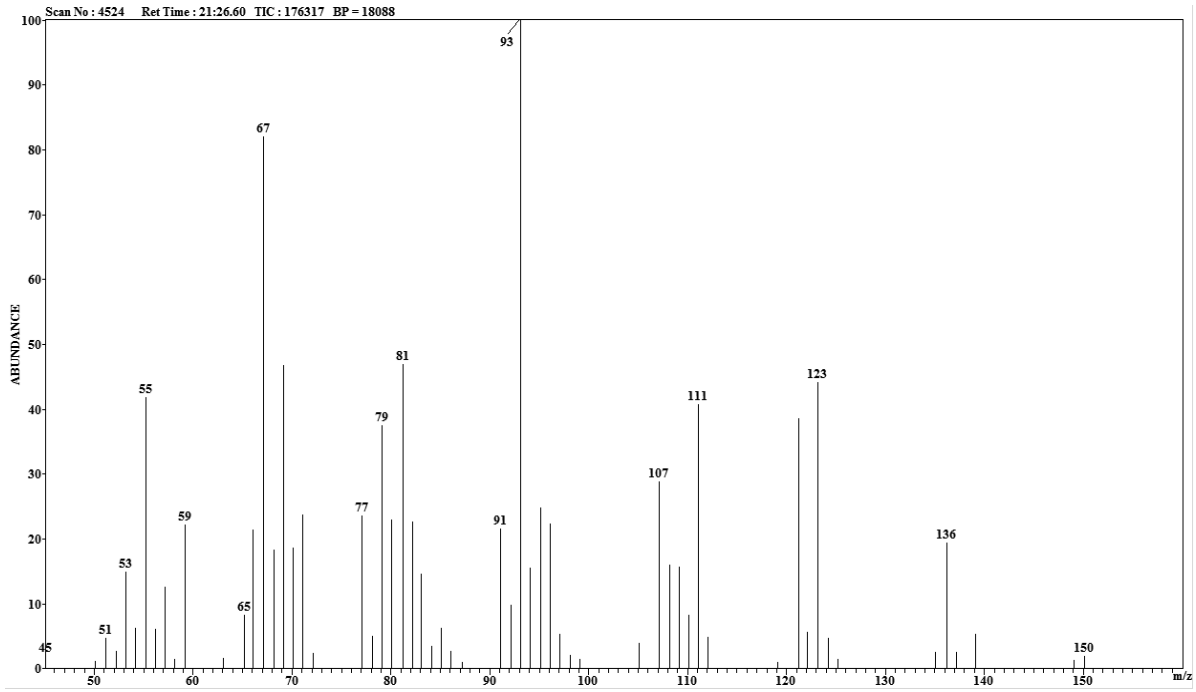

Figure B.5 Unknown 1 from the the *Artemisia dubia* essential oil (P3).

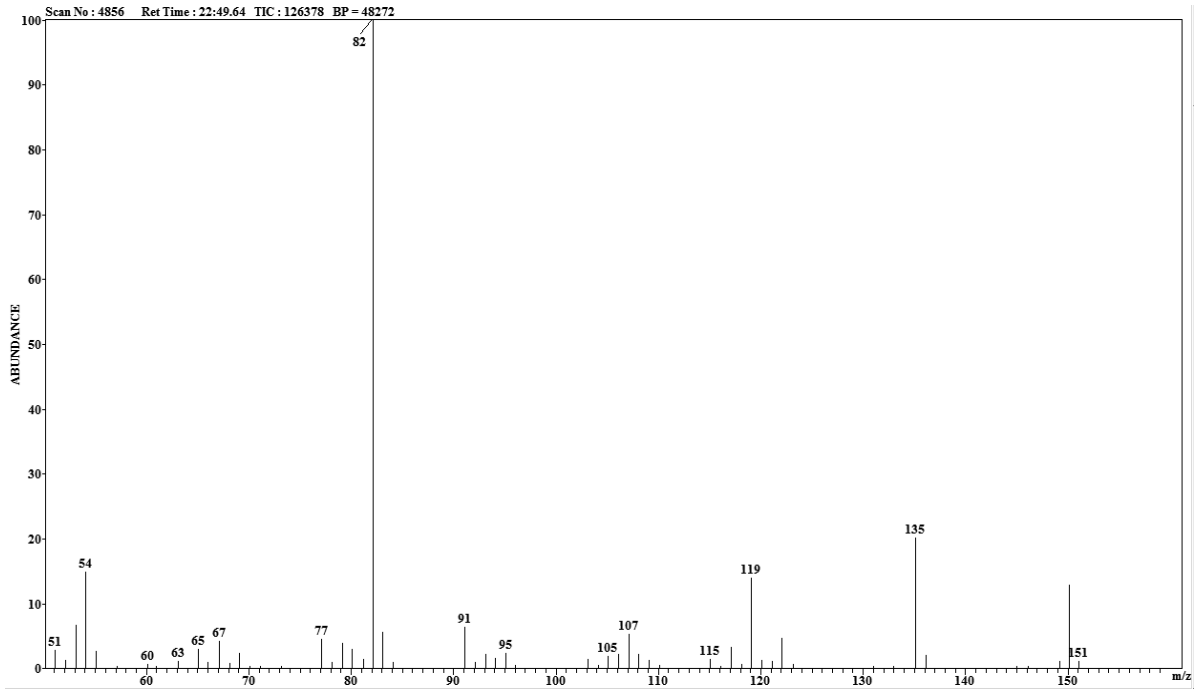

Figure B.6 Unknown 2 from the the *Artemisia dubia* essential oil (P3).

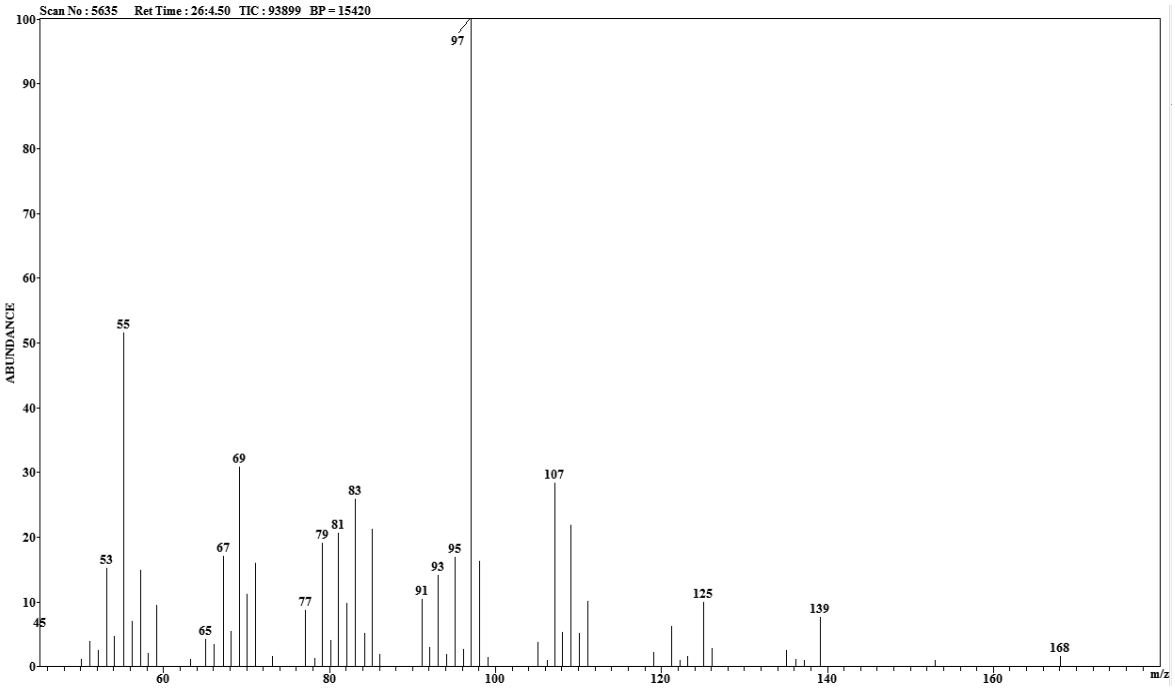

Figure B.7 Unknown 3 from the the *Artemisia dubia* essential oil (P3).

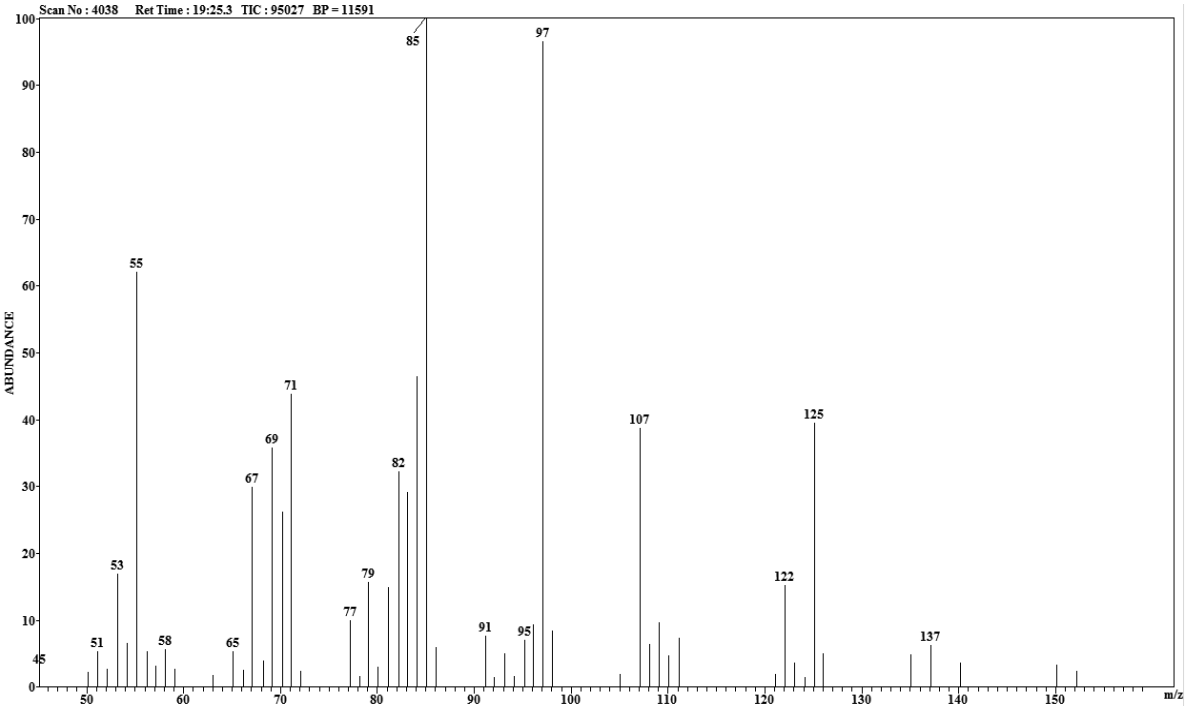

Figure B.8 Unknown 1 from the the *Artemisia vulgaris* essential oil (P11).

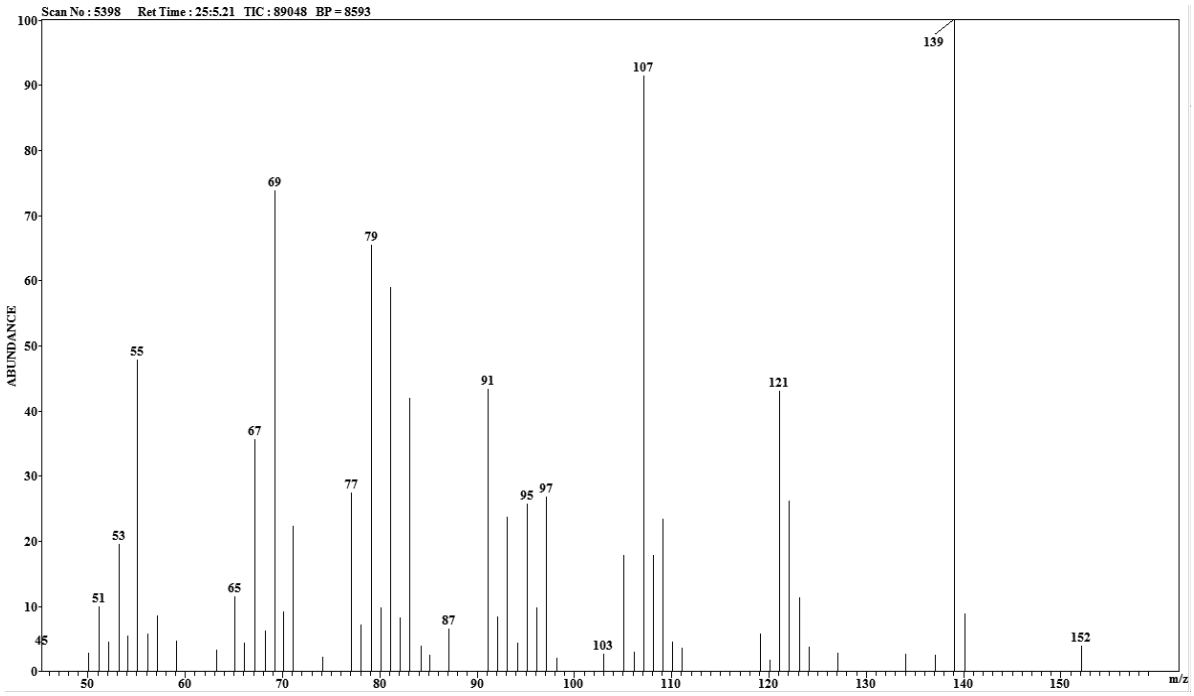

Figure B.9 Unknown 2 from the the *Artemisia vulgaris* essential oil (P11).

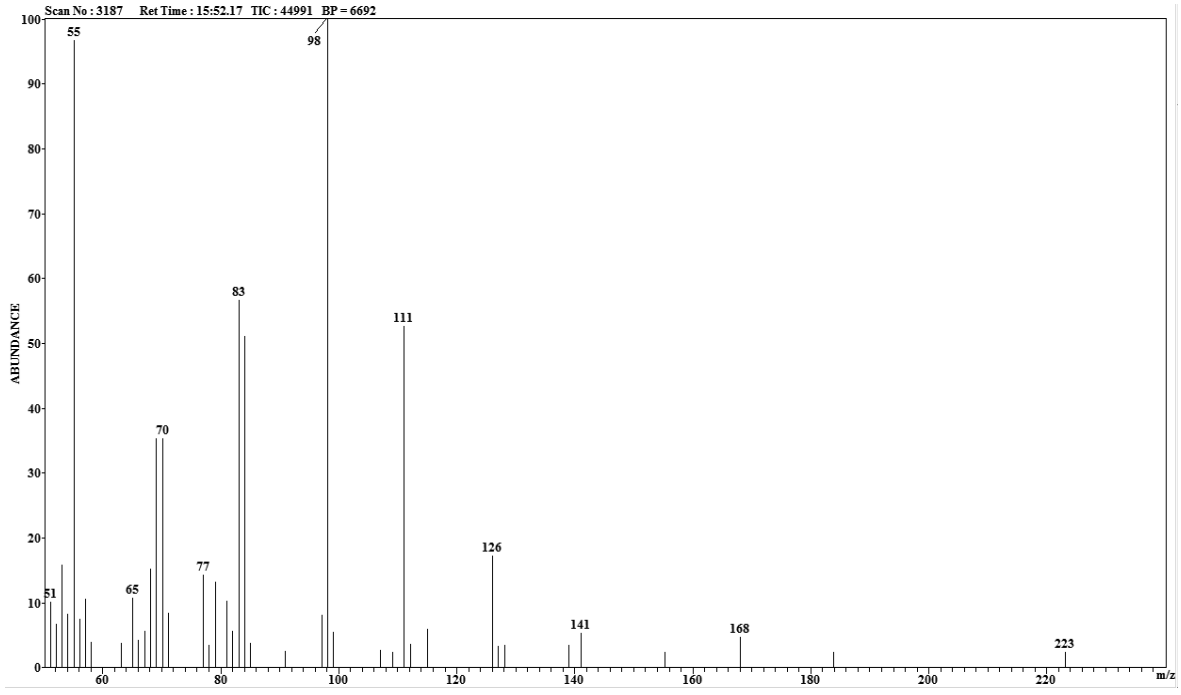

Figure B.10 Unknown 1 from the the *Artemisia indica* essential oil (P67).

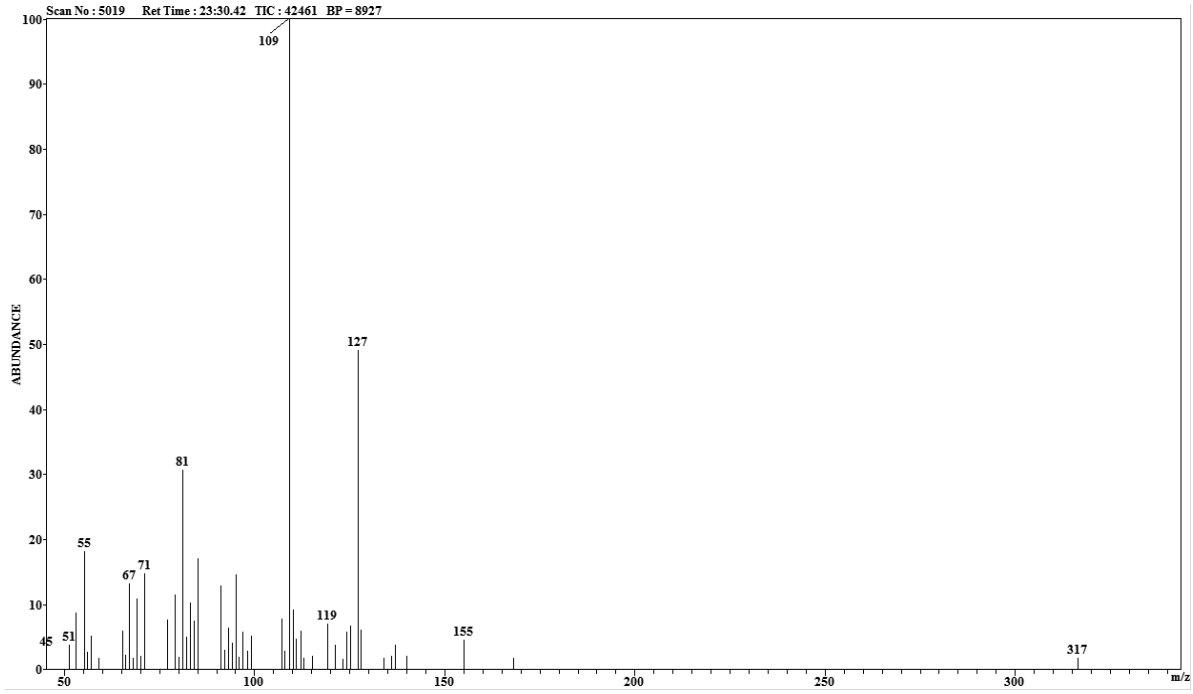

Figure B.11 Unknown 2 from the the *Artemisia indica* essential oil (P67).

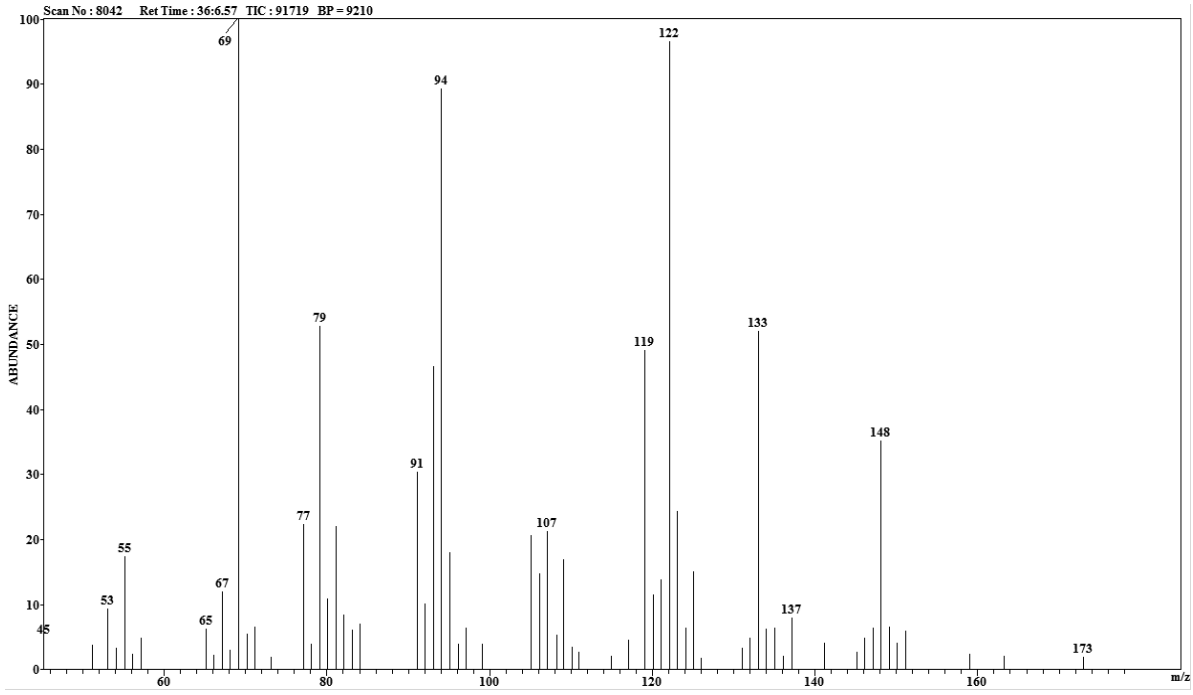

Figure B.12 Unknown 3 from the the *Artemisia indica* essential oil (P67).