Ethnobotany, phytochemistry and DNA metabarcoding studies on Indian traditional medicine

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I. **Gopalakrishnan Saroja Seethapathy**, Kaliamoorthy Ravikumar, Berit Smestad Paulsen, Hugo J. de Boer and Helle Wangensteen. (2018). Ethnobotany of dioecious species: Traditional knowledge on dioecious plants in India. *Journal of Ethnopharmacology*, 221: 56-64. doi: 10.1016/j.jep.2018.04.011

II. **Gopalakrishnan Saroja Seethapathy**, Christian Winther Wold, Kaliamoorthy Ravikumar, Hugo J. de Boer and Helle Wangensteen. (2019). Ethnopharmacology, biological activities, and chemical compounds of *Canarium strictum*: an important resin-yielding medicinal tree in India. *Manuscript*.

III. **Gopalakrishnan Saroja Seethapathy**, Margey Tadesse, Santhosh Kumar J. Urumarudappa, Srikanth V. Gunaga, Ramesh Vasudeva, Karl Egil Malterud, Ramanan Uma Shaanker, Hugo J. de Boer, Gudasalamani Ravikanth and Helle Wangensteen. (2018). Authentication of *Garcinia* fruits and food supplements using DNA barcoding and NMR spectroscopy. *Scientific Reports*, 8(1):10561. doi: 10.1038/s41598-018-28635-z

IV. **Gopalakrishnan Saroja Seethapathy**, Ancuta-Cristina Raclariu, Jarl Andreas Anmarkrud, Helle Wangensteen and Hugo J. de Boer. (2019). DNA metabarcoding authentication of Ayurvedic herbal products on the European market raises concerns of quality and fidelity. *Frontiers in Plant Science*, 10(68). doi: 10.3389/fpls.2019.00068

Summary

Medicinal plants form the basis of traditional medicine health systems and play an important role in meeting primary healthcare needs around the world. At the same time, traditional knowledge on medicinal plants is eroding and deteriorating due to ongoing cultural, ecological and socio-economical changes. This poses a serious threat to biodiversity-based cultural knowledge and can cause an attrition of leads for drug discovery. On the other hand, it is also important to validate traditional knowledge on plant use using scientific methods to promote or discourage their wider usage. Therefore, the first objective of the thesis is to document traditional knowledge on medicinal plants in Southern India, and validate traditional knowledge of selected medicinal plant using phytochemical methods and biological assays. **Paper I** aimed to document traditional knowledge on dioecious plants of India. Specifically it addressed the research questions: do folk healers have preference for plants of a specific gender? If so, what are those plants? In addition, do folk healers differentially utilize male and female plant of a particular species for food, medicines or timber? The study found that informants recognize the phenomenon of dioecy in plants, and reported gender preferences for a number of species with respect to uses as timber, food and medicine. **Paper II** aimed to document the medicinal uses of *Canarium strictum* Roxb. (Burseraceae) by folk healers in India, and to investigate the chemical constituents and biological activities of the resin and stem bark. Our results revealed the presence of α-amyrin and β-amyrin as the major compounds in the resin. Whereas gallic acid, methyl gallate, scopoletin, 3,3'-di-*O*-methylellagic acid 4-*O*-α-arabinofuranoside, elephantorrhizol (3,3',4',5,6,7,8-heptahydroxyflavan) and procyanidins were isolated from the bark methanol extract. It is noteworthy that the finding of elephantorrhizol in *C. strictum* is of chemotaxonomic interest as it is the first report from the family Burseraceae. Furthermore, radical scavenging and 15-lipoxygenase inhibitory activities were tested in resin and bark extracts, but no toxicity towards *Artemia salina* nauplii was found. Similarly, dose dependent inhibition of NO production was observed in resin and dichloromethane bark extracts.

Traditional medicine based herbal products have gained increasing popularity in developing countries as complementary therapies. However, herbal products are prone to contamination, adulteration and substitution, and this raises quality and safety concerns for the public. Therefore, the second part of thesis focused on molecular authentication of raw herbal drugs and marketed herbal products using NMR spectroscopic and DNA methods. The aim of **Paper III** was to assess

the adulteration of morphologically similar samples of *Garcinia* using DNA barcoding, and to quantify the content of (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone in raw herbal drugs and *Garcinia* food supplements using NMR spectroscopy. Our DNA barcoding study revealed that there was no adulteration in raw herbal drugs of *Garcinia*. Whereas, analysis of ten *Garcinia* food supplements revealed a large variation in the content of (−)-hydroxycitric acid content per capsule or tablet. **Paper IV** aimed to test the composition and fidelity of Ayurvedic products marketed in Europe using DNA metabarcoding. Our analysis revealed that the fidelity for single ingredient products was 67%, and the overall ingredient fidelity for multi ingredient products was 21%, and for all products 24%.

Abbreviations

ABS	Access and Benefit Sharing
ASE	Accelerated Solvent Extraction
AFLP	Amplified Fragment Length Polymorphism
API	Ayurvedic Pharmacopoeia of India
ARMS	Amplification Refractory Mutation System
CAM	Complementary and Alternative Medicine
CBD	Convention on Biological Diversity
CE	Capillary Electrophoresis
CTAB	Cetyl trimethyl ammonium bromide
DCM	Dichloromethane
DNA	Deoxyribonucleic acid
EFSA	European Food Safety Authority
EMA	European Medicines Agency
EtOAc	Ethyl acetate
EU	European Union
FDA	Food and Drug Administration
GC	Gas Chromatography
H_2O	Water
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer Chromatography
HTS	High-Throughput Sequencing
ITS	Internal Transcribed Spacer
MeOH	Methanol
MOTU	Molecular Operational Taxonomic Unit
MS	Mass Spectrometry

NIR	Near-Infrared Spectroscopy
NMR	Nuclear Magnetic Resonance
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
rDNA	Ribosomal DNA
rbcL	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit
RFLP	Restriction Fragment Length Polymorphism
SCAR	Sequence Characterized Amplified Region
SSR	Simple Sequence Repeat
TBGRI	Tropical Botanical Garden and Research Institute of India
TLC	Thin Layer Chromatography
TM	Traditional Medicine
WHO	World Health Organization

1) Introduction

1.1) Ethnobotany

The term Ethnobotany was suggested by John William Harshberger in 1896. Originally it was applied to the study of the utilitarian relationship between humans and the plant environment in primitive settings, and Harshberger (1896) noted that "*ethnobotany aids in elucidating the cultural position of the tribes who used the plants for food, shelter or clothing*". However, ethnobotany has been given various definitions over time, and has now evolved into a much broader meaning that covers not only a utilitarian relationship, but also relationships that embrace the symbolic, ecological and cognitive, as well as the human–plant relationship in a modern setting (Soejarto et al., 2005; Leonti, 2011). Ethnobotany was defined by Catherine M Cotton in 1996 as "*encompassing all studies which concern the mutual relationships between plants and traditional people*" (Cotton, 1996). Albuquerque et al. (2017) defines ethnobotany as "*the study of the direct interrelationship between people of extant cultures and the plants in their environment*". In simple terms ethnobotany refers "*to study of the interactions between people and plants*" (Martin, 2004). Under the utilitarian approach ethnobotanical studies allow the documentation of the use of plants and its associated traditional knowledge by ethnic communities (Berlin, 1992). Such documentation is an important part in understanding and analyzing elements of traditional knowledge, it serves as base line data for future research, and aids to preserve the traditional knowledge for both communities and future generations (Awas et al., 2010; Leonti, 2011; de Boer and Cotingting, 2014).

One of the sub-disciplines of ethnobotany is ethnomedicine, and ethnomedicine studies the plants used as medicine by people (Neumann and Lauro, 1982; Lee and Balick, 2001). Knowledge of traditional medicines are preserved mostly by oral tradition, but with the advent of writing systems, different cultures have documented their knowledge into pharmacopoeias or *materia medica* (Leonti, 2013). The first record of poppy uses dates back to 6000 B.C., cuneiform tablets from the Mesopotamian basin refers the medicinal properties of opium calling the "plant of joy" (Brook et al., 2017). Similarly, another written evidence on plant usage for the preparation of drugs dates back to 3000 B.C. and has been found on a Sumerian clay slab comprising twelve recipes for preparation of drugs referring to over 250 various plants (Petrovska, 2012). The Ebers Papyrus, an Egyptian medical papyrus, dates back to 1550 B.C. and contains about 700 formulas and remedies

(Petrovska, 2012; Rahman et al., 2018). The earliest documentation of Chinese *materia medica* dates back to around 1100 B.C. with several numbers of drug descriptions (Leung, 2006). Ayurveda is one such *materia medica* and it dates back to at least 200 B.C. in India (Chaudhury and Rafei, 2001; Jaiswal and Williams, 2017). The Sanskrit word "*Ayurveda*" consists of two words '*Ayu*' which means life and '*Veda*' which means knowledge or science. Thus "Ayurveda" in totality means 'Science of life'. The genesis of Ayurveda is believed to be based on four eminent compilations of knowledge (Vedas) called Yajur Veda, Rig Veda, Sam Veda and Atharva Veda (Jaiswal and Williams, 2017; Mukherjee et al., 2017). Maharishi Charaka is an Indian sage who compiled all aspects of Ayurvedic medicine as "Charaka Samhita", and this compilation is one of the first and the most important ancient authoritative writings on Ayurveda (1000 BC). A number of compilations like Nighantu Granthas, Madhava Nidana and Bhava Prakasha are from the contributions of various scholars (Jaiswal and Williams, 2017; Mukherjee et al., 2017). The *materia medica* of Ayurveda describes the uses of over 1,500 medicinal plants and 10,000 formulations (Joshi et al., 2017). However, there are many ethnic groups around the globe that continue to orally transmit their indigenous knowledge systems that are yet to be documented (Totelin, 2009).

The World Health Organization (WHO) defines Traditional Medicine (TM) as a medical system that "*includes diverse health practices, approaches, knowledge and beliefs incorporating plant, animal, and/or mineral based medicines, spiritual therapies, manual techniques and exercises, applied singly or in combination to maintain well-being, as well as to treat, diagnose or prevent illness*" (WHO, 2002). Based on this definition, traditional medicine and practices can be classified broadly into:

(a) codified systems of traditional medicine which have a systematic body of knowledge in the form of ancient scriptures such as Ayurveda, Siddha, Unani, Tibetan medicine and Traditional Chinese medicine (Ved and Goraya, 2007; Upadhya et al., 2014), or

(b) non-codified systems of traditional medicine or folk medicine in which the medicinal knowledge, skills and practices are passed on orally from generation to generation (Ved and Goraya, 2007; Upadhya et al., 2014).

While these two types of TMs are found in India, the non-codified systems of traditional medicine (folk medicine) are diverse and vary with geography, regional flora and culture, and these have often developed in accordance with the primary needs and locally available resources of a particular region (de Boer and Lamxay, 2009; Weckerle et al., 2009; de Boer et al., 2012). Each ethnic culture has its own relationship with the environment and a medical knowledge that uses specific medicinal species (Macía et al., 2005). However, the practice of TM is diminishing with time due to several factors including cultural, ecological and socio-economical changes, the abandonment and devaluation of traditional customs, particularly the influence of urbanization and influence of western lifestyles, and the increasing reliance on conventional medicine (Benz et al., 2000; Byg and Balslev, 2004; Srithi et al., 2009; Vandebroek and Balick, 2012).

1.1.1) Ethnopharmacology and traditional medicine

The term Ethnopharmacology was formally introduced in 1967 in the title of a book on hallucinogens: *Ethnopharmacological search for psychoactive drugs* (Efron, 1967). After the initial use of the term ethnopharmacology in the context of hallucinogenic plants, it was only used occasionally until 1979. Ethnopharmacology was established more definitively when the *Journal of Ethnopharmacology* was founded by Laurent Rivier and Jan Bruhn in 1979 (Rivier and Bruhn, 1979), and the scope ethnopharmacology was broadened to '*a multidisciplinary area of research concerned with the observation, description, and experimental investigation of indigenous drugs and their biological activity*' (Rivier and Bruhn, 1979). Though the term ethnopharmacology is relatively new, researchers in the past have dealt with traditional uses of pharmacologically active compounds in a comprehensive way so that their investigations would nowadays be classified as ethnopharmacological research (de Smet and Rivier, 1989; Heinrich and Jäger, 2015). For example William Withering (1741-1799) systematically studied the medicinal properties of foxglove (*Digitalis purpurea* L., Scrophulariaceae) which was traditionally used to treat dropsy. He used orally transmitted knowledge to develop a medicine that can be used by physicians. Prior to such studies, traditional medicine practices were more interested in the patient's welfare and less focused on the systematic study and chemical properties of the employed medicinal plants (Heinrich and Jäger, 2015). Ethnobotany and ethnopharmacology have often been seen as a tool for drug discovery (Heinrich et al., 2006). Ethnobotanical studies not only focus on medicinal plants, but also on other products derived from plants, such as foods, shelter, coloring agents, fiber plants,

poison, fertilizer, ornamentals and oil. Ethnopharmacological studies focus only on the study of indigenous medicines derived from natural resources with a special focus on the evaluation of such therapeutic uses by empirical science (e.g. pharmacological, phytochemical and toxicological). Ethnopharmacology is defined by Michael Heinrich as, "*a scientific approach to the study of the biological activities of any preparation used by humans, which have, in a very broad sense, either beneficial or toxic or other direct pharmacological effects*" (Heinrich, 2014). Ethnopharmacological studies and/or traditional medicines guided drug discovery have been the basis for modern science and several studies have highlighted the importance of ethnopharmacological studies for drug development (Patwardhan and Mashelkar, 2009; Graziose et al., 2010; Zhao et al., 2015). A noteworthy recent recognition of this drug discovery process is the 2015 Nobel Prize in Physiology or Medicine which was awarded to Youyou Tu for the discovery of artemisinin from *Artemisia annua* L., (Compositae). Artemisinin was isolated in 1972 using information from the well-documented Chinese compendium of *materia medica* written by Shizhen Li (1518 -1593) and Ge Hong's 'handbook of prescriptions for emergencies' from 340 AD to combat malaria (as reviewed in Kong and Tan, 2015; Su and Miller, 2015).

Ethnopharmacology has provided some very notable successes in discovering drug molecules, including artemisinin isolated in 1972, morphine isolated in 1804 (as reviewed in Brook et al., 2017), quinine isolated in 1820 (as reviewed in Willcox et al., 2004), atropine isolated in 1833 (as reviewed in Shutt and Bowes, 1979), podophyllotoxin isolated in 1880 (as reviewed in Cragg et al., 2011), ephedrine isolated in 1897 (as reviewed in Preuss and Bagchi, 2012), and vinblastine isolated in 1958 as reviewed in Raviña, 2011). These compounds, or their analogs and derivatives, are still in widespread use. Morphine is an alkaloid derived from *Papaver somniferum* L. (opium poppy), and is one of the best known examples of a drug discovered based on traditional medicine. The poppy plant has a long history of medicinal use as pain killer and as sedative, with morphine being a responsible compound for the pharmacological activity. The first record of poppy uses dates back to 6000 B.C., cuneiform tablets from the Mesopotamian basin refers the medicinal properties of opium calling the "plant of joy" (Brook et al., 2017). The *Ebers papyrus*, an Egyptian scroll from approximately 1500 B.C., lists opium, along with other medicinal plants such as garlic, mandrake and aloe. The Greek physician Hippocrates (460-370 BC) recommended whole poppy heads, not just opium, soaked in water to treat dropsy. However, the first ethnopharmacological study on poppy began in the 15th century AD, when Theophrastus Bombastus Von Hohenheim

developed a tincture of opium which he called *laudanum* (Brook et al., 2017). Later, Friedrich Wilhelm Adam Serturner isolated opium's active ingredient in 1805 and named the crystalline powder 'morphium'. With the help of J.L. Gay-Lussac, the name 'morphium' was changed to 'morphine'. Furthermore in 1819 C.F. Wilhelm Meissner classified morphine as the first compound known as an alkaloid (Brook et al., 2017). In 1925, Sir Robert Robinson uses the chemical principle of aromatization to rationalize the structure of morphine, and he was awarded the Nobel Prize in Chemistry in 1947 for his achievements in morphine research (Robinson and Sugasawa, 1931, 1932, 1933; Bentley, 1987). Apart from morphine, three other important alkaloids found in opium are codeine, noscapine and papaverine (Bernhoft et al., 2010; Brook et al., 2017).

Some of the successful ethnopharmacology lead drug compounds from Ayurvedic medicinal plants (Balachandran and Govindarajan, 2007) are andrographolide isolated from *Andrographis paniculata* (Burm. F.) Nees in 1911 (Chakravarti and Chakravarti, 1951; Smith et al., 1982), vasicine isolated from *Justicia adhatoda* L. (Syn. *Adhatoda vasica* Nees) in 1924 (Sen and Ghose, 1924), allicin isolated from *Allium sativum* L. in 1944 (Cavallito and Bailey, 1944), and reserpine isolated from *Rauvolfia serpentina* (L.) Benth. ex Kurz in 1952 (Müller et al., 1952; Curzon, 1990).

Case study: *Kani* berries in India

An interesting case study from India demonstrates how traditional medicine guided drug development has been a global model for recognizing the traditional knowledge and drug discovery. According to a World Intellectual Property Organization report (Gupta, 2004), during an ethnobotanical expedition in 1987 by the All India Coordinated Research Project on Ethnobiology, in which members of the *Kani* ethnic group were guiding researchers on an expedition organized by the Tropical Botanical Garden and Research Institute of India (TBGRI), the researchers noticed that the *Kani* were not getting tired, despite significant physical exertion and they were constantly chewing on some black berries. On inquiring with the *Kanis*, the researchers were given a few of the black berries to chew on, after which they felt revitalized. The researchers realized that the berries had properties that relieve fatigue (Chaturvedi, 2009). The plant was later identified as *arogyapacha* (*Trichopus zeylanicus* Gaertn.). The researchers from TBGRI collected the berries for laboratory research and after a decade of research they managed to isolate several active chemical compounds with beneficial properties (Chaturvedi, 2009). Incidentally, during their research it was discovered that the leaves were more beneficial than the berries in terms of

bioactivity (Gupta, 2004; Chaturvedi, 2009; Reddy and Lakshmikumaran, 2015). Subsequently, in 1994, the researchers at the TBGRI filed for patents and licensed the same to an Indian pharmaceutical company for USD 50,000 plus 2 % royalties on all sales. In 1997 the TBGRI assisted the *Kani* in setting up a trust to document their traditional knowledge that placed them in control of the first payment of USD 12,500 for development (Moran, 2000; Gupta, 2004; Reddy and Lakshmikumaran, 2015).

1.1.2) Dioecy in angiosperms and traditional medicine

Angiosperms (flowering plants) are the most widespread and diverse group of plants, and the major sexual systems of angiosperms are bisexual (~90 %), including those with hermaphroditic flowers and those that are monoecious (~5 %), i.e., with separate male and female flowers on the same individual (Charlesworth, 2002). A minority of plant species are 'sexually polymorphic', which are dioecious (having separate sexes containing either only male or female flowers in a plant), gynodioecious (having both hermaphrodite and female only flowers), androdioecious (hermaphrodite and male), and polygamodioecious (populations with bisexual individuals, male individuals, and female individuals) (Charlesworth, 2002). In general, the three most important components for the survival of a plant is the maintenance of vegetative growth, the struggle for existence with competitors (defense), and the reproduction. However, each of these components and their activities requires the expenditure of energy, which is in limited supply, and current investments in each one of these activities result in losses in the potential investments in the other (Obeso, 2002). Therefore, there is a trade-off at the physiological level of resource allocation to vegetative growth, defense or reproduction in plants. The evolution of dioecy has long intrigued evolutionary biologists (Renner, 2014), and there are a number of studies that have used dioecious plants to understand the cost of reproduction and resource trade-offs in plants (Obeso, 2002).

Trade-offs between allocation of resource to defense, growth and reproduction among genders of dioecious plants varies and has shown to affect the production and concentration of secondary metabolites (Obeso, 2002; Simpson, 2013; Milet-Pinheiro et al., 2015; Bajpai et al., 2016). Understanding the ecology of plant biodiversity has been highlighted as an important strategy for drug discovery (Coley et al., 2003), as well as ethnobotanical studies and/or traditional medicines for drug development (Patwardhan and Mashelkar, 2009). In India, it is estimated that 8,000 plants have medicinal usages. Considerable evidence for sex-biased herbivory and variation in secondary

metabolites in dioecious plants is available in scientific studies, but little is known about traditional concepts and preferences for dioecious plants, either male or female.

Tinospora cordifolia (Willd.) Miers) (Menispermaceae) is a widely distributed and commonly used dioecious medicinal plant used in Ayurvedic medicine in India for several therapeutic properties (Panchabhai et al., 2008). A significant variation in the concentration of alkaloids were reported between male and female plants of *T. cordifolia* (Bajpai et al., 2016), and an in vitro cytotoxicity evaluation revealed that the stem extracts of male *T. cordifolia* was more effective in inhibiting the growth of cancerous cell lines compared to the female plant stem extracts (Bajpai et al., 2017a; Bajpai et al., 2017b). On the contrary, female plant stem extract caused a significant up regulation in the pro-inflammatory and anti-inflammatory cytokines and activated the peritoneal exudate cells leading to significant higher release in reactive oxygen species and enhanced the in vitro lymphocyte proliferation more than male stem extract (Bajpai et al., 2017a; Bajpai et al., 2017b). These findings highlight the importance of studying the variation in secondary metabolites and bioactivity of male and female plants in dioecious species.

1.2) Herbal products and commercialization

Traditionally, assuring the quality and safety of traditional medicines was the responsibility of the traditional medicinal practitioner who collected and prepared the medicine in small amounts for curing diseases (Valiathan, 2006; Weston, 2009). WHO defines herbal medicine as (i) *the use is well-established and widely acknowledged, i.e., the use represents the accumulated experience of many practitioners over an extended period of time*, (ii) *the use of the herbal medicine, including dosage, indication, and administration route is well-established and documented*; and (iii) *the use is generally and currently regarded as safe* (WHO, 1998). However, in recent decades traditionally used herbal medicines have continued to become mainstream commodities driven by the health industry from craft-based tradition to globalized industry (Jagtenberg and Evans, 2003; Moahi, 2007). Opening up and interconnectedness of the world via globalization have opened up economic and internationalized trade markets worldwide (Jagtenberg and Evans, 2003; Moahi, 2007). Such globalization has paved the way for the herbal industry and its market demand across nations. Countries like India and China with a long history of traditional medicine are utilizing such opportunities of globalization and internationalized trade markets to promote their traditional medicinal products to improve their economy (Sen et al., 2011). Traded medicinal herbal products

are defined as "*finished, labelled pharmaceutical products in dosage forms that contain one or more of the following: powdered plant materials, extracts, purified extracts, or partially purified active substances isolated from plant materials*" (WHO, 1998).

Based on the historical and economic development in a country, a nation's traditional system of medicine is often complemented with conventional medicine. Conversely, in other regions, alongside conventional medicine different traditional systems of medicine and alternatives (complementary medicine) are established (Leonti, 2013). Complementary and alternative medicine (CAM) is referred to as a broad set of healthcare practices that are not part of that country's own tradition and are not integrated into the dominant healthcare system (WHO, 2013). Use of herbal medicine is one among the practices of CAM (Fischer et al., 2014) and the usage of TM based herbal medicine is increasing worldwide (WHO, 2013). According to the CAMbrella consortium in Europe (Fischer et al., 2014), over 100 million Europeans are TM & CAM users and the usage of herbal medicine is the most commonly reported CAM therapy in Europe (WHO, 2013; Fischer et al., 2014; Hegyi et al., 2015). Based on the National Health Interview Survey from 2002 to 2012, one-third of adults in USA used some form of CAM (Clarke et al., 2015). With the substantial growth, and increasing evidence for the usage of CAM in developed countries (WHO, 2013), the WHO traditional medicine strategy for the period of 2014-2023 aims to facilitate the integration of TM & CAM into the national health care systems of WHO member's states, and also aims to strengthen the quality assurance, safety, efficacy, and proper use of herbal medicines by various measures (WHO, 2013). Some of the reasons for the popularity of herbal products are in addition to the belief that herbal products are safe and have no side effects because they are natural, the desire for the personal therapeutic regime, as natural herbal products that can be freely combined with pharmaceutical drugs (Ernst, 2004; Byard et al., 2017). Also access to traditional medicines does not require a prescription and products can be purchased as over the counter products, prominently advertised in the popular media, marketed and distributed via channels, including pharmacies, natural herbal shops, and online retail stores (Ernst, 2004; Byard et al., 2017).

Ayurveda is one among the traditional medicines recognized as a CAM by WHO (WHO, 2013), and among the core primary healthcare options in India. A predominant proportion of the Indian population uses traditional medicines for their healthcare needs (Katoch et al., 2017; Srinivasan and Sugumar, 2017). Ayurvedic medicines are commonly used as crude materials or extracts for

therapeutic purposes (Joshi et al., 2017). In India, it is estimated that approximately 7,000 plants have medicinal usages in codified and non-codified Indian systems of medicine, of which approximately 1,178 plants are reported to be actively traded. The total commercial demand for herbal raw drugs in India for the year 2014-2015 is estimated to be 512,000 metric tons with a value of more than 1 billion USD (Goraya and Ved, 2017). India has over 8,000 licensed medicinal drug manufacturing units, and in order to bring uniformity among the manufacturers and to follow the same formula of ingredients, the Ayurvedic formulary and Ayurvedic Pharmacopoeia of India (API) was been compiled to implement the standard norms of the Drugs and Cosmetics Act (Mukherjee et al., 2012; Katoch et al., 2017).

1.2.1) Authenticity issues of herbal products

Herbal products as a commodity are not without safety or quality concerns, and the growing commercial interest in herbal products increases the incentive for adulteration and substitution in the medicinal plants market (Raclariu et al., 2018). An increasing awareness among consumers, and several media reports on herbal product adulteration and mislabeling are calling attention to the quality of traded herbal products that directly affect consumer safety (Ouarghidi et al., 2012; Walker and Applequist, 2012; Newmaster et al., 2013). The most important issues affecting the quality of the herbal products is adulteration (de Boer et al., 2015). Herbal product adulteration can be deliberate in order to maximize the profits, and deliberate adulteration leads to the use of alternate plant parts other than the parts used traditionally or totally other plant materials of inferior quality. For example, based on different pharmacopoeias, the roots of *Withania somnifera* (L.) Dunal (Indian ginseng) are considered to be a medicinally potent plant part due to the pharmacologically active withanolides (Mundkinajeddu et al., 2014). The root samples are supposed to be marketed based on the content of withanolides; however, the aerial parts of the plant also contain the marker compounds along with flavonoid glycosides specific to the aerial parts of the plant. As a result of the increase in the global herbal trade, the roots of these plants are often mixed with the aerial parts of *W. somnifera* and are then fraudulently marketed (Mundkinajeddu et al., 2014). Addition of synthetic substances to herbal products is also common under fraudulent adulteration (Calahan et al., 2016; Rocha et al., 2016). For instance, herbal products for sexual enhancement have been found to be adulterated and counterfeited with sildenafil, tadafil, vardenafil, and several other synthetic derivatives (Gratz et al., 2006; Balayssac et al., 2009; Balayssac et al.,

2012); slimming herbal products with sibutramine and fenfluramine (Balayssac et al., 2009; Vaysse et al., 2010; Monakhova et al., 2012) and body-building products with anabolic steroids (Klinsunthorn et al., 2011).

Herbal product adulteration is also often due to misidentification or substitution with allied congeneric species and geographically co-occurring species (Mitra and Kannan, 2007; de Boer et al., 2015). For example, phenotypically very similar *Phyllanthus* species and *Berberis* species that could easily be misidentified and mixed within herbal products, are collected from the wild by local farmers or collectors who often rely only on their experience in identifying the species, and the services of specialists like taxonomists are rarely used for authentication (Srirama et al., 2010; Srivastava and Rawat, 2013). A DNA barcoding study reported that 24 % of raw drugs obtained from herbal markets were adulterated with six other morphologically similar species (Srirama et al., 2010). In another study using microscopy, physicochemical parameters and high-performance thin-layer chromatography (HPTLC), it was reported that different species of *Berberis* were traded and adulterated in different herbal markets of India (Srivastava and Rawat, 2013). Species adulteration might also arise due to the same vernacular name being applied to different species in various indigenous systems of medicine, or incorrect use of scientific generic names for the raw drugs (Wu et al., 2007; Begum et al., 2014; Bennett and Balick, 2014; Rivera et al., 2014). In 1993 several cases of renal failure were reported in Belgium resulting from the consumption of a weight-loss supplement. This nephropathy was due to the misidentification of *Stephania tetrandra* S.Moore and *Aristolochia fangchi* Y.C.Wu ex L.D.Chow & S.M.Hwang which is rich in aristolochic acid. Apparently in traditional Chinese medicine both the herbs shared an identical vernacular name (Wu et al., 2007; Debelle et al., 2008).

The consequences of unreported ingredients and undeclared fillers used in herbal products may range from simple misleading of consumers on labelling to potential adverse drug reaction or poisoning due to toxic contaminants (Chan, 2003; Ernst, 2004; Gilbert, 2011). However, the pharmacovigilance of herbal products remains difficult because these products are sold over the counter, without any medical prescription, and under no legislative framework that traces and monitors the adverse reactions that may occur (de Boer et al., 2015).

1.2.2) Regulatory status of herbal products

According to WHO there is considerable variation from country to country in the quality control of herbal materials and products. This variation not only has an impact on public health, as contaminants in herbal medicines may represent avoidable risks for patients and consumers, but also has effects on international trade (WHO, 2007, 2013). Though regulation is complex, it is constantly developing. For example, until 2008 *Ginkgo biloba* L. was considered as food in the United Kingdom, while it was consistently regulated as a medical product in Germany, whereas in the USA it is a food supplement. However, in the United Kingdom and in many other European countries ginkgo is now generally regulated as a traditional herbal medical product (Heinrich, 2015). In Europe the herbal products fall under two main categories, primarily depending on their intended use (i) 'herbal medicines' that are regulated as medicinal products by the European Medicines Agency, and (ii) 'herbal food supplements' which are regulated under the provision of food legislation (European Union., 2002, 2004; Vlietinck et al., 2009).

Based on the European Directive 2004/24/EC criteria's herbal medicinal products can be classified into two categories: (i) well established medicinal herbal products; and (ii) traditional used herbal medicinal products (European Union., 2004). The European Directive 2004/24/EC provides a mechanism that allows manufacturers of herbal medicines to register medicinal herbal products based on a tradition of use (European Union., 2004). The traditional use is based on evidence that a corresponding herbal product is derived from the same botanical drug and prepared in a similar way, and has been used traditionally for at least 30 years including 15 in non-European countries and 15 years in Europe or more than 30 years in Europe. Evidence on biological assays or clinical trials are not required to prove the safety and efficacy of traditional herbal medicine, however proof that '*harmful in specified conditions of use and that the pharmacological effects and the efficacy of the medicinal product are plausible on the basis of longstanding use and experience*' are mandatory (European Union., 2004; Vlietinck et al., 2009; Jütte et al., 2017). The requirements for the well-established medicinal herbal products are the published scientific literature on recognized efficacy and safety (European Union., 2004; Calapai, 2008). The Committee on Herbal Medicinal Products is part of European Medicines Agency (EMA) and responsible for establishing monographs on therapeutic and safe use of medicinal products and to provide an inventory of safe herbal substances with a long history of use (Vlietinck et al., 2009; Jütte et al., 2017). However,

the pharmacovigilance of the marketed herbal medicines is the responsibility of manufacturers and suppliers (Raclariu, 2017).

In India, the Ayurvedic Pharmacopoeia of India (API) is a legalized document of the Government of India describing the quality, purity and strength of selected drugs that are manufactured, distributed, and sold by the licensed manufacturers in pan India (Joshi et al., 2017; Katoch et al., 2017). Once a Pharmacopoeia, or an article in it, has been approved by the Ayurvedic Pharmacopoeia Committee, it has the compliance with the quality prescribed therein becomes mandatory under the Drugs and Cosmetics Act 1940 (Joshi et al., 2017). The API is comprised of two parts; part-I is the Ayurvedic Formulary of India and comprises the monographs of selected formulations of natural origin, and part-II includes monographs on single drugs sourced from the schedule-1 books under the Drugs and Cosmetics Act, 1940 comprising of popular Ayurvedic classics of different period of time (API, 2001). Each monograph in API includes the drug title in Sanskrit along with drug definition regarding its integrity in scientific nomenclature and concise knowledge with respect to its source, occurrence, distribution and collection precautions. Synonymies in Sanskrit and also names in other Indian regional languages are listed (API, 2001; Joshi et al., 2017). The first edition of API has been published in 1978, and currently comprises 976 compound formulations of Ayurveda and 540 monographs of plant, animal and mineral (including metals) origin (Joshi et al., 2017).

1.2.3) Quality control methods

One of the core interests of modern pharmacognosy refers to the identification and authentication of drug substances and to the quality of the resulting herbal medicine (Trease and Evans. D., 2009; Heinrich and Anagnostou, 2017). Alongside the guidelines and regulations, the lack of appropriate analytical procedures and methods that vary largely between countries adversely complicate the monitoring and quality assessment of herbal products along the entire value chain (Bent, 2008; Booker et al., 2012; Heinrich, 2015). The quality of herbal products is directly reflected in safety and efficacy of that product, and one of the major challenges in herbal pharmacovigilance is to develop novel strategies and appropriate standards to exhaustively assess and monitor the quality of both raw materials and herbal products (Barnes, 2003; de Boer et al., 2015; Raclariu, 2017). Quality monitoring ensures that the products are of the appropriate quality required for their indented use in order to protect the integrity of public health (de Boer et al., 2015; Raclariu, 2017).

Currently the WHO's guidelines for assessing quality of herbal medicines include a series of procedures that are mainly to ensure the identity of the raw plant materials, and screening a specified marker compound, and the microbiological purity of the herbal product (WHO, 2007, 2011). Using authenticated raw material is the basic starting point in developing a herbal product (Trease and Evans. D., 2009). Most herbal monographs specify the use of macroscopic and microscopic characterization, phytochemistry based analysis of specific markers compounds, assays for toxic constituents such as heavy metals, and the use of different chromatographic approaches to detect adulteration (Raclariu, 2017). The herbal product manufacturers are supposed to use at least one appropriate test to determine the identity of the ingredients before plant ingredients are being used in the preparation of a herbal product (Pawar et al., 2016). However, it is important to note that the appropriate quality assurance methods can be applied based on the various stages of value chain and preceded on the basis of case-by-case evaluation, starting from the plant material harvest, storage, and to the finished herbal products (WHO, 2007).

1.2.3.1) Macroscopic and microscopic authentication

Both macroscopic and microscopic investigations are the classical botanical authentication and characterization techniques for whole plants, plant parts, and in some cases, the plant material that has been dried and powdered (Smillie and Khan, 2010; Khan and Smillie, 2012). In macroscopic technique the plant morphological characteristics are examined to aid in the authentication process. The examination includes the plant traits such as habit (e.g., woody/suffruticose/herbaceous), leaf shape, size, and morphology (e.g., leaf margins: entire, undulate, dentate, serrate, lobed, or pinnatifid); flower characters such as type of inflorescence (e.g., spike, raceme, panicle, cyme, corymb, helicoid cyme, head); floral morphology (e.g., epigynous, perigynous, hypogynous; stamen number and shape; number of carpels in ovary; number of seeds per carpel); root characteristics including surface texture, type (corm, bulb, rhizome, etc.), and tissue layering (banding patterns). As part of authentication, macroscopic techniques are applicable only when a plant still retains one or more of these above "key" characters, so it is possible to determine the identity of target plant species and adulterants that have similar morphology yet having a certain level dissimilarity in some morphological key characters (Applequist, 2006; Smillie and Khan, 2010; Khan and Smillie, 2012).

Microscopic approaches involve techniques such as fluorescence microscopy, scanning electron microscopy or standard light microscopy to analyze characteristics such as the presence or absence of trichomes, oil glands, particular cell types, seed morphology, pollen morphology and vascular traces (Joshi and Khan, 2005; Joshi et al., 2005). Microscopic techniques are specifically more useful than macroscopic techniques in specific cases such as while attempting to establish authenticity of the samples which are ground plant material. Under such condition most macroscopic characters are lost in such ground plant material (Joshi and Khan, 2005; Smillie and Khan, 2010; Khan and Smillie, 2012).

The capability of macroscopic and microscopic authentication techniques are hampered while analyzing the complicated multicomponent powdered samples, or when there is no cellular distinction between closely related genera, or where a material is processed beyond the ability to provide distinct morphological characterization (Joshi and Khan, 2005; Khan and Smillie, 2012). Under such circumstances, it is necessary to utilize alternative techniques in order to effectively identify and authenticate botanical samples (Khan and Smillie, 2012; Pawar et al., 2016). Another demerit of these techniques is that highly qualified individuals are required for both macroscopic and microscopic identification. The number of trained taxonomists are in decline in recent years, and a lack of interest in the area from the current generation of students has put this skill at a premium (Smith and Figueiredo, 2009).

1.2.3.2) Chromatographic and spectroscopic methods

Analytical techniques based on phytochemicals are the most reliable and applicable authentication methods that are routinely used for the quality control of raw plant materials and herbal products (Liang et al., 2004; Khan and Smillie, 2012). The basic analytical technique is chemical fingerprinting by Thin Layer Chromatography (TLC) (Wagner and Bladt, 1996). This method is based on the separation of compounds on a solid phase, after a sample is applied on the thin layer of adsorbent material such as silica gel, and a solvent (known as the mobile phase) is drawn up in the plate via capillary action. Compounds ascend on the TLC plate at different rates based on their chemical properties, and this creates a fingerprint for the compounds that are present in the sample when visualized under different conditions (Wagner and Bladt, 1996). TLC is a cost effective method and applicable for the preliminary screening of the compounds, and to establish initial identification and quality control of raw plant materials (Liang et al., 2004). TLC can also be used

as a semi quantitative technique (Liang et al., 2004). High Performance Thin Layer Chromatography (HPTLC) is an advancement of TLC method, used in quality control of the raw plant materials and the herbal products (Khan and Smillie, 2012). HPTLC is both a quantitative and qualitative method with automation in different steps, along with development chambers, digital imagery, and densitometry capabilities to analyze the qualitative and quantitative data (Khan and Smillie, 2012).

High Performance Liquid Chromatography (HPLC) is both a qualitative and a quantitative method and uses well characterized marker compounds for the quality control of the raw plant materials and the herbal products (Lazarowych and Pekos, 1998; Liang et al., 2004; Fan et al., 2006). However in order to establish identity of the plant material, hyphenated HPLC methods, such as HPLC-MS or HPLC-NMR, are often used (Liang et al., 2004). Yet, HPLC is suitable for the development of the fingerprints for raw plant material and herbal products (Liang et al., 2004). Gas chromatography (GC) is a highly sensitive method used for fingerprint analysis of volatile chemical compounds (Liang et al., 2004). The advantage of GC clearly lies in its high sensitivity of detection of volatile chemical compounds, the extraction of the volatile oil is relatively straightforward and can be standardized, and the components can be identified using hyphenated GC–MS analysis (Liang et al., 2004; Ong, 2004; Zeng et al., 2007).

In order to use, adapt and validate any of these analytical fingerprint methods, a sufficient quantity of the selected marker compounds that are readily available is required (Smillie and Khan, 2010; Khan and Smillie, 2012). The lack of commercially available standard markers for many compounds limits the application of these analytical chemical methods for authentication of the raw plant materials and the herbal products (Smillie and Khan, 2010; Khan and Smillie, 2012). However, fingerprinting methods and phytochemical identity techniques have been developed and combined as hyphenated techniques (combination of chromatographic and spectroscopic methods) such as GC-MS, HPLC-MS and CE-MS to vastly improve the authentication process of herbal products (Patel et al., 2010; Heyman and Meyer, 2012; Khan and Smillie, 2012).

The combination of phytochemical identity methods and chemometric analysis have also been proposed in order to cope with the major confinement of relying on prior established species-specific marker compounds (Khan and Smillie, 2012; Booker et al., 2016). For instance, Nuclear Magnetic Resonance (NMR) based chemometric profiling has been emerged as another

methodology for the evaluation of botanical extracts (Heyman and Meyer, 2012). ^{1}H NMR is a robust, reliable and non-destructive method that may be used to simultaneously detect, identify and quantify chemical compounds in a plant sample due to the relatively high sensitivity and widespread occurrence of protons in plant metabolites (Kim et al., 2010; Heyman and Meyer, 2012). The spectroscopic evaluation of ^{1}H NMR of herbal extracts provides a complete secondary metabolite profile, however, the metabolites need to be solved in the suitable solvent medium. Thus NMR is a valuable companion for quality control of raw herbal material and products. However, a major delimitation of ^{1}H NMR is considerable overlap in the complex spectra of plant extracts (van der Kooy et al., 2009; Heyman and Meyer, 2012).

1.2.3.3) DNA based identification

Genetic markers are Deoxyribonucleic acid (DNA) sequences with a physical location on a chromosome. DNA sequences are unique and specific to individual species and molecular markers utilize these genetic variations in DNA sequences to identify individuals or species. One of the advantages of DNA based identification methods is that DNA markers are least affected by age, environmental factors and physiological conditions of the plant samples. Even though, DNA markers do not correspond to the chemical profile, they are not tissue specific and thus can be detected at any stage of development, with a small amount of sample, in any physical form (Zhang et al., 2007). Broadly DNA based identification methods can be classified into:

(i) Hybridization based methods include Restriction Fragment Length Polymorphism (RFLP) (Botstein et al., 1980) and variable number tandem repeats (Nakamura et al., 1987). RFLP is considered to be one of the first developments in the field of DNA markers and are based on the principle of genetic variation caused by mutation, insertion or deletion in restriction enzyme binding and cleavage sites (Ganie et al., 2015). The digested fragments vary in size, have to be separated using southern blot analysis and accordingly visualized by hybridization to specific probes which could be homologous or heterologous in nature (Ganie et al., 2015). Labelled probes such as random genomic clones, cDNA clones, probes for microsatellite and minisatellite sequences are hybridized to filters containing DNA, which have been digested with restriction enzymes (Neumann and Kumar, 2008). Polymorphisms are then detected by presence or absence of bands upon hybridization (Jeffreys et al., 1985; Litt and Luty, 1989). However, the major

disadvantages of RFLP are time consuming, the usage of radioactive substances, laborious and difficult to automate (Ganie et al., 2015).

(ii) Polymerase Chain Reaction (PCR) methods involve in vitro amplification of particular loci of DNA using a specific or arbitrary oligonucleotide primers. The arbitrary primer methods include Random Amplified Polymorphic DNA (RAPD), arbitrarily primed PCR and DNA amplification fingerprinting (Joshi et al., 2004). Specific primer based polymorphism detection system includes inter simple sequence repeats polymorphism where a terminally anchored primer specific to a particular Simple Sequence Repeat (SSR) is used to amplify the DNA between two opposed SSRs of the same type. Polymorphism occurs whenever one of the SSRs has a deletion or insertion that modifies the distance between the repeats (Joshi et al., 2004; Passinho-Soares et al., 2006). Another approach known as Amplified Fragment Length Polymorphism (AFLP) is a technique that is based on the detection of genomic restriction fragments by PCR amplification. Adaptors are ligated to the ends of restriction fragments followed by amplification with adaptor homologous primers. AFLP has the capacity to detect thousands of independent loci and can be used for DNAs of any origin or complexity (Passinho-Soares et al., 2006). Sequence characterization of amplified regions (SCAR), and Amplification Refractory Mutation System (ARMS) are some of the most important marker for authentication of medicinal plants (Zhu et al., 2004; Kiran et al., 2010). SCAR markers can be designed using a specific gene such as nrITS region or a random DNA fragment in the genome of an organism such as the AFLP, RAPD and ISSR DNA fragment linked to a trait of interest (Kiran et al., 2010; Ganie et al., 2015). The designed SCAR primers sequence-specific and are used to identify the target species from a pool of related species by the presence of a single, distinct and bright band in the desired sample (Kiran et al., 2010). Similarly, ARMS is based on the use of sequence-specific PCR primers that will amplify the test DNA only when the target DNA allele is contained within the sample and will not amplify the non-target DNA allele (Zhu et al., 2004). However, the disadvantages of markers such as RAPD and AFLP are less reproducible, and require high molecular weight DNA. Whereas, the demerit of SCAR and ARMS markers is the need for prior sequence data to design the PCR primers (Zhu et al., 2004; Ganie et al., 2015).

(iii) Sequencing based methods uses the genetic variations caused by transversion, insertion or deletion that can be directly assessed and information on a defined locus can be obtained. DNA barcoding is one of the sequencing based methods that aids in the identification of biological

organisms. The term DNA barcoding was coined by Hebert et al. (2003) and can be defined as use of short nuclear or organelle DNA sequences for the identification of organisms. Since it was first proposed, the technique has been found to be very useful in fingerprinting and identification of species to a remarkable 98 to 100 per cent accuracy in many organisms (mostly in Kingdom Animalia) including birds (Hebert et al., 2004b), fish (Ward et al., 2005), butterflies (Burns et al., 2007), insects (Ojha et al., 2014), reptiles (Khedkar et al., 2016), and amphibians (Biju et al., 2014). Further, DNA barcoding has helped in clarifying the taxonomic position of 'apparent species complex' by revealing several cryptic species within a 'single' species described through conventional taxonomy (Hebert et al., 2004a). In the last one decade, the technique has been found to be very useful in not only fingerprinting and identification of species but has extended beyond and currently used in rapid biodiversity assessment studies (Valentini et al., 2009), biomonitoring of pests and diseases (Hajibabaei et al., 2011), in identification of prey species consumed by the predators (Peters et al., 2015), in forensic studies especially in investigating illegal trade of endangered species and their products (Dubey et al., 2011), and in detecting poisonous plants (Bruni et al., 2010). DNA barcoding has also been used in authentication of herbal products (Newmaster et al., 2013), in identification of mislabelled food products (Carvalho et al., 2015), and also as an educational tool to assess biodiversity in school campuses (Naaum et al., 2014). These examples point to a number of applications of the DNA barcoding technique from fundamental research on biodiversity to enforcement of food laws, from teaching to quarantine and phyto-sanitary laws and protection of wildlife (Bonants et al., 2010; Cawthorn et al., 2012; Yan et al., 2013; Liu et al., 2014).

Crucial to all DNA barcoding studies is the choice of an ideal DNA barcode region. An ideal DNA barcode is the one that allows unambiguous species identification by having sufficient sequence variation among species (inter-specific) and low intra-specific variation, and yet be conserved to be present across the > 400 million years of evolutionary divergence of land plants (Chase et al., 2007). Mitochondrial DNA in animals is highly conserved in terms of gene content and order with a high rate of nucleotide substitution. Whereas, higher plants exhibit frequent rearrangements in genomes, transfer of genes to the nuclear genome, and incorporation of foreign genes, and nucleotide substitution rates are much slower in plants compared to animals (Cowan et al., 2006; Vijayan and Tsou, 2010). Therefore, a number of chloroplastic candidate gene regions have been suggested as a barcode for plants, [coding regions *accD*, *matK*, *ndhJ*, *rpoB2*, *rpoC1*, and *ycf5*, *rbcL*;

trnL intron, UPA (Universal Plastid Amplicon), *rpoB*] and non-coding spacers (Taberlet et al., 2006; Kress and Erickson, 2007; Lahaye et al., 2008; Newmaster et al., 2008; Hollingsworth et al., 2009). In the absence of suitable single locus DNA barcode region for land plants, the Consortium for the Barcode of Life has proposed combination of two locus (*rbcL* + *matK*) as the standard plant barcode for land plants after screening a number of different chloroplast regions such as *atpF–atpH* spacer, *matK* gene, *rbcL* gene, *rpoB* gene, *rpoC1* gene, *psbK–psbI* spacer, and *trnH–psbA* spacer based on major criteria's such as universality, easy amplification, sequence quality, coverage and species discrimination ability (Hollingsworth et al., 2011). Several studies have suggested a combination of *trnH–psbA, matK, rbcl* and *nrITS* as the most potential DNA barcodes (de Vere et al., 2012; Zimmermann et al., 2013; Yan et al., 2015). A single DNA barcode usually have the species discriminatory power of about 75-85 %, and a combination of multi-locus DNA barcode yield the discriminatory power >95 % in most taxa (Chen et al., 2010; Burgess et al., 2011).

Apart from several applications of the DNA barcoding, more specifically it has been proved to be useful method in dealing with authenticity issues of medicinal plants and herbal products (de Boer et al., 2015; Parveen et al., 2016; Heinrich and Anagnostou, 2017; Sgamma et al., 2017). Several investigations using DNA barcoding method to identify and authenticate various herbal products have reported varying degree of adulteration and substitution. For example, using ITS2 DNA barcode region Shi et al. (2017) reported 98 % (28 out of 38 samples) of adulteration in the traditional Chinese medicine herbal product 'Baitouweng' which supposed to contain the authentic species *Pulsatilla chinensis* (Bge.) Regel., Ghorbani et al. (2017) reported 26 % of adulteration (18 out of 68 samples) in raw plant materials purchased from the herbal markets of Iran. Similarly, 6 % of saw palmetto, 16 % of gingko, and 25 % of black cohosh herbal dietary supplements were adulterated in the samples purchased in New York (Baker, 2012; Little and Jeanson, 2013; Little, 2014). Likewise a number of studies have utilized DNA barcoding to audit the quality of herbal products (de Boer et al., 2015; Sgamma et al., 2017; Raclariu et al., 2018). It is not worthy that the conventional DNA barcoding exclusively applicable for the authentication of single ingredient herbal products, and one of the major limitations with DNA barcoding is the inability of traditional Sanger sequencing technique in detecting DNA in herbal products containing more than one species such as polyherbal products (Ivanova et al., 2016; Wilkinson et al., 2017). Several comprehensive reviews on DNA-based authentication of botanicals have highlighted the merits

and demerits of Sanger sequencing based DNA barcoding and its application in quality control of herbal products (de Boer et al., 2015; Parveen et al., 2016; Sgamma et al., 2017).

The rapid development of high-throughput sequencing (HTS) methods offers new opportunities for the identification and quality control of herbal products using the DNA barcoding approach. DNA metabarcoding is a combination of DNA barcoding and high-throughput DNA sequencing methods. It offers several key advantages over conventional DNA barcoding such as mass-amplification and sequencing of barcodes from complex mixtures of multiple species, analyzes of samples with varying levels of DNA degradation, products containing fillers or contaminants, and superior sensitivity of the method (Taberlet et al., 2012; Staats et al., 2016). The two most widely used sequencing platforms are Illumina and Ion Torrent for the quality control of herbal products (Speranskaya et al., 2018). Several studies have utilized this approach in authenticating herbal products. For example, (Raclariu et al., 2017a; 2017b) have evaluated 78 herbal products containing *Hypericum perforatum* and 16 herbal products containing *Veronica officinalis* using High-throughput sequencing based DNA metabarcoding, and revealed that DNA metabarcoding detected the species of interest in 68 % and 15 % of the products, respectively. Though, HTS has several advantages in the identification and quality control of herbal products. One of the limitations of DNA metabarcoding is that it depends on the data integrity of the reference sequences database and some species are hard to clearly distinguish by means of HTS and DNA barcoding. Therefore it is recommended to use the integrative approach involving both chemical profiling and DNA-based barcoding methods for identification of herbal products (Raclariu et al., 2018; Speranskaya et al., 2018; Xu et al., 2018).

All the above mentioned methods have different roles in quality control and authentication, and the appropriateness of applying such methods are based on the various stages of value chains i.e., from harvest of a plant to herbal product to consumers. Figure 1 illustrates the methods that can be used during various stages of value chain for quality control and authentication.
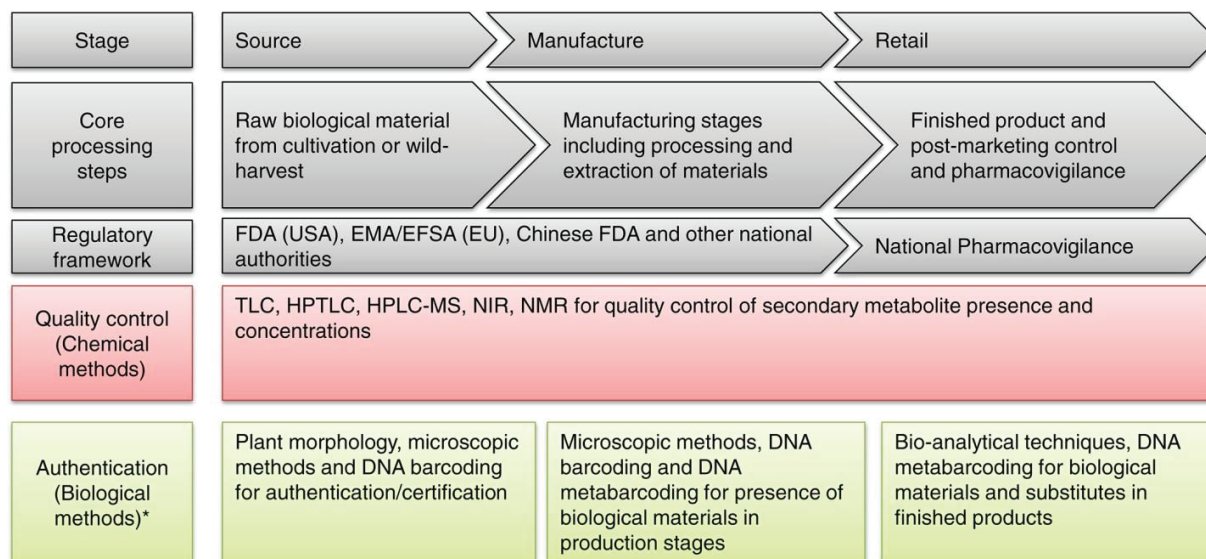
Figure 1. Various stages involved in the production of regulated or unregulated herbal products and the roles of different quality control (chemical and biological) methods. * Quality control methods are also commonly used for authentication. Permission to use the figure is obtained from Raclariu et al. (2018).

2) Aim of the thesis

The overall aim of the thesis is to provide an insight into the traditional knowledge of dioecious medicinal plants in India, validate the traditional knowledge on *Canarium strictum* by utilizing phytochemical methods and biological assays and to authenticate herbal products using analytical chemical and DNA methods.

The aim of **Paper I** was to document the traditional knowledge on dioecious plants of India. Specifically this paper tried to answer these research questions: do folk healers have preference for plant genders? If so, what are those plants? And document those plants. Other addressed aspects were: do folk healers differentially utilize male and female plant of a particular species for food, medicines or timber? Furthermore this study addresses folk healers' perceptions of what are considered to be male and female plants in their community and traditions. Overall, the concept of plant gender in Indian systems of medicine are studied.

The aim of **Paper II** was to document the medicinal uses of *Canarium strictum* (Burseraceae) by Indian folk healers. Furthermore to investigate the constituents of the resin and stem bark, and the radical scavenging and antioxidant properties of the resin and stem bark extracts. In addition, the effects of the resin and stem bark extracts on the NO production in lipopolysaccharide stimulated murine dendritic D2SC/I cells were investigated.

The aim of **Paper III** was to find out whether the herbal raw drugs of *Garcinia* species and herbal products said to be based on this plant are substituted and/or adulterated with morphologically similar species of *Garcinia*. In this study DNA barcoding is used to authenticate herbal raw drugs of *Garcinia* species, and NMR spectroscopy to be used to identify and quantify the principle organic acids, (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone in *Garcinia* raw herbal drugs and in food supplements that are labelled to contain *Garcinia* extracts.

The aim of **Paper IV** is to authenticate and detect species diversity in Ayurvedic herbal products in Europe using DNA metabarcoding. More specifically, it tests the presence of labelled species in herbal products, and aims to detect adulteration and substitution, and/or presence of other unlabelled plant species.

3) Material and methods

The materials and different methods used in this thesis are presented in detail in the Papers from I to IV. In this section therefore only an overview of different methods and materials will be presented.

3.1) International legislative framework

Prior to conducting any ethnobotanical or ethnopharmacological studies, it is mandatory that the researchers are aware of Convention on Biological Diversity (CBD), which is a multilateral treaty comprising international laws and agreements relating to all ecosystems, species and genetic resources (CBD, 1992, 2000). CBD has three main goals: (i) the conservation of biodiversity; (ii) sustainable use of the components of biodiversity; and (iii) sharing the benefits arising from the commercial and other utilization of genetic resources in a fair and equitable way (CBD, 2000). Equitable sharing is a prerequisite for achieving the two first objectives. Without 'Access' there will be fewer benefits to share, and without 'benefit sharing' there will be fewer resources conserved for future use. 'Prior informed consent' and 'mutually agreed terms' are meant to guide access to genetic resource (Koester, 1997; Rosendal, 2006). Genetic resources refer to genetic material of actual or potential value. It may be any material of plant, animal, microbial or other origin containing functional units of heredity (Rosendal, 2006). Genetic resources constitute as an important input factors to biotechnology companies for bioprospecting; the screening of biodiversity and related traditional knowledge in search for commercially valuable genetic and bio-chemical resources (Rosendal, 2006). CBD also provides legal protection to genetic resources associated with traditional knowledge and the benefits arising from its utilization (CBD, 2011). Therefore, being an ethnobotanical or ethnopharmacological researcher, it is necessary to be aware of the third goal of CBD which deals with access and benefit sharing of genetic resources, and supplementary agreement to the CBD 'The Nagoya Protocol'.

The Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization (ABS) to the Convention on Biological Diversity is a supplementary agreement to the Convention on Biological Diversity (CBD, 2011). It provides a transparent legal framework for the effective implementation of the objective. In terms of access to genetic resources, benefit-sharing and compliance, the Nagoya Protocol consists of several core

obligations, and some important obligations are to 'establish clear rules and procedures for prior informed consent and mutually agreed terms', and the 'utilization includes research and development on the genetic or biochemical composition of genetic resources, as well as subsequent applications and commercialization' (CBD, 2011).

3.2) Ethnobotanical field studies

In order to conduct the ethnobotanical studies on dioecious plants in India, an application form for access to biological resources and associated traditional knowledge was submitted to the National Biodiversity Authority (NBA); a statutory autonomous body under the Ministry of Environment and Forests, Government of India (http://nbaindia.org/). Permission was granted to access the traditional knowledge of Indian plants. The Access and Benefit-sharing Clearing-House Unique Identifier is ABSCH-IRCC-IN-237734-1.

The ethnobotanical and ethnopharmacological studies were conducted during the period of March to August 2016, and February 2018. In total 50 folk healers were interviewed using semi-structured questionnaires and the study participants were selected using the snowball sampling method (Berlin and Berlin, 2005). Prior to the study, the purpose of the study was explained to the folk healers and the prior informed consent to conduct the study was requested and agreed. A detailed description of the study protocol, and the semi-structured questionnaire used in the study can be found in the method sections and appendix section of Paper I and II.

3.2) Material collection

The resin and stem bark of *Canarium strictum* Roxb. (Paper II) were collected from a tree in Kolli Hills, India, in 2016. *C. strictum* is a polygamodioecious species. The tree was tapped for the oozing of resin prior to three days of collection.

For Paper III ten herbal products labeled as containing either *Garcinia gummi-gutta* (L.) Roxb. or *Garcinia indica* (Thouars) Choisy (Clusiaceae) with hydroxycitric acid were purchased from pharmacies and via e-commerce, and 21 raw herbal drugs being traded as *Garcinia* species were collected from different herbal markets of South India. The lists of samples including label information can be found in the supplementary information of Paper III.

For Paper IV a total of 79 Ayurvedic herbal products either food supplements or herbal medicines were randomly purchased via e-commerce and pharmacies from Europe. The lists of samples including label information can be found in the supplementary information of Paper IV.

The herbal products analyzed in Paper III and IV were imported into Norway for scientific analyses under Norwegian Medicines Agency license no. 16/04551–2. Voucher specimens of plant materials used in this study are deposited in the herbarium of Ashoka Trust for Research in Ecology and the Environment, and in FRLH-Herbarium and Raw Drug Repository of The Institute of Trans-Disciplinary Health Sciences and Technology, India. All the plant nomenclature in this study follows The Plant List (The Plant List, 2013 http://www.theplantlist.org),

3.3) Extraction, isolation and characterization of chemical compounds

The stem bark and resin of *C. strictum* (Paper II) were air dried and powdered, and then extracted with dichloromethane (DCM) followed by methanol using an Accelerated Solvent Extraction (ASE) instrument (ASE350 Solvent Extractor).

The resin DCM crude extract was applied to a normal phase silica column and chromatographed with a gradient of ethyl acetate/dichloromethane (10–100% EtOAc) to yield fractions of compounds. The stem bark methanol extract was purified by using Sephadex LH-20 column material and eluted with a gradient of H_2O/MeOH (35-100% MeOH) and H_2O/acetone (70% acetone). The obtained fractions were each separately applied to a VersaPak C18 column with a gradient of H_2O/acetonitrile (5-100% acetonitrile) followed by H_2O/acetone (70% acetone). In parallel to the fractionation work, analytical TLC (normal phase silica plate or reverse phase C_{18}-plates), analytical HPLC coupled to a C_{18}-column and a diode array detector, and/or NMR was used to guide in method development and select fractions for further purification. Selected fractions were purified by preparative HPLC (gradient of 0.1% trifluoroacetic acid in water and acetonitrile) to obtain pure compounds for identification and structure elucidation. 1D and 2D NMR spectroscopy was conducted on a Bruker AVII400 or Bruker AVI600 instrument. The detailed methodology for antioxidant activity, NO production in lipopolysaccharide stimulated murine dendritic D2SC/I cells and toxicity assay are provided in Paper II.

For Paper III, *Garcinia* raw drugs were freeze-dried for 24 hours due to the high hygroscopic nature of *Garcinia* fruits, and pulverized in a commercial blender. *Garcinia* fruit powders were extracted

with Milli-Q water using an ASE instrument. Similarly, the extraction of *Garcinia* food supplements were carried out using the same methodology as for the *Garcinia* water extracts. Prior to the extraction of *Garcinia* food supplements, the capsules were opened and their contents removed, whereas tablets were ground to a powder. ^{1}H NMR spectra of *Garcinia* water extracts and food supplement extracts were acquired on a Bruker AVII400 instrument. For quantification reference solvent solution was prepared containing 2.0 mg/ml maleic acid (internal standard) and 0.1% 3-(trimethylsilyl) propionic 2,2,3,3-d$_4$ acid sodium salt in deuterium oxide.

3.4) DNA barcoding methods

For the DNA barcoding study (Paper III), a reference database was compiled for 11 authentic *Garcinia* species with 2–5 individuals in each species using nrITS, *psbA-trnH* and *rbcL* sequences. DNA was extracted from fresh as well as silica dried plant material using the Cetyl trimethyl ammonium bromide (CTAB) method optimized for *Garcinia* species (Asish et al., 2010), and the details of the wild collected *Garcinia* species voucher specimens, geographic origin, and National Center for Biotechnology Information GenBank accessions can be found in the supplementary information provided in Paper III. Similarly, DNA from raw herbal drugs of *Garcinia* traded as Kodampuli and Kokum were isolated using the same using the CTAB method optimized for *Garcini*a species (Asish et al., 2010) and nrITS sequence was used to establish the identity of *Garcinia* raw herbal drugs.

DNA metabarcoding method was utilized to authenticate herbal products in Paper IV. A detailed description of the metabarcoding protocol can be found in the method sections of Paper IV. Seventy nine herbal products labelled to contain a total of 159 plant species belonging to 132 genera and 60 families. Conventional CTAB DNA extraction method was used to isolate DNA from the herbal products (Doyle and Doyle, 1987), and the markers nrITS1 and nrITS2 were used as barcodes to identify the presence of labelled species, and to simultaneously detect substitutes and adulterants as well as to check the presence of any off labelled species in the product.

4) Summary of results

Paper I: Ethnobotany of dioecious species: Traditional knowledge on dioecious plants in India

More than 15,000 angiosperm species are dioecious. However, little is known about the ethnobotany of dioecious species and whether preferences exist for a specific gender, e.g., in food, medicine or timber. Therefore the specific objective of this study was (a) to study and document whether Indian folk healers have preference for plant genders, and their knowledge on dioecious species and uses. (b) to understand the concept of plant gender in Ayurveda and whether Ayurvedic literature includes any references to gender preferences. In order to conduct the study, lists of dioecious plants used in Indian systems of medicine and folk medicine were compiled. Ethnobotanical data were collected from 40 folk healers of Malayali communities in Kolli Hills, Servarayan Hills, and Sittlingi Valley of Eastern Ghats, Tamil Nadu India. Folk healers' perceptions and awareness of dioecious plants and any preferences of use for specific genders of dioecious species were documented using semi-structured interviews. In addition, twenty Ayurvedic doctors were interviewed to gain insight into the concept of plant gender in Ayurveda.

Our results revealed that Indian systems of medicine viz., Ayurveda, Siddha, Unani, and Sowa-Rigpa, and folk medicine, contain 5–7 % dioecious species, and this estimate is congruent with the number of dioecious species in flowering plants in general. Folk healers recognized the phenomenon of dioecy in 31 out of 40 species, and reported gender preferences for 13 species with respect to uses as timber, food and medicine. Among folk healers' different plant traits such as plant size, fruit size, and visibility of fruits determines the perception of a plant being a male or female. For example, informants were unaware of dioecy for two shrubs (*Dodonaea angustifolia* L.f., *Dodonaea viscosa* (L.) Jacq.), two climbers (*Cocculus hirsutus* (L.) W.Theob., *Cyclea peltata* (Lam.) Hook.f. & Thomson), and two lianas (*Asparagus racemosus* Willd., *Cissampelos pareira* L.). They are all sourced from the wild in the study area, but the useful parts of these plants are not fruits or seeds. On the contrary, informants were aware of dioecy for *Celastrus paniculatus* Willd. (liana), and *Embelia tsjeriam-cottam* (Roem. & Schult.) A.DC. (shrub), because the seeds are used as medicines from these plants, and informants were aware of a plant that did not produce seeds.

It was also observed that folk healers recognize a particular plant species either as male or female based on different phenotypes, texture of different plant parts and morphological appearance of closely related species by providing gender specific vernacular names, and such plant species are not dioecious in nature. For example *Artocarpus hirsutus* Lam. (ayanipala; kattupala; peyppala) is considered to be male plant due to its property of more durable timber than *Artocarpus heterophyllu*s Lam. (palamaram; narpala), which is a female plant.

Ayurvedic classical literature provides no straightforward evidence on gender preferences in preparation of medicines or treatment of illness; however it contains details about reproductive morphology and sexual differentiation of plants. From this study it is evident that people have traditional knowledge on plant gender and preferential usages towards one gender.

Paper II: Ethnopharmacology, biological activities and chemical compounds of *Canarium strictum*: an important resin-yielding medicinal tree in India

Canarium strictum Roxb. (Burseraceae) is a resin yielding, polygamodioecious tree distributed across parts of India, Myanmar and China. The resin is used for rheumatism, asthma, venereal disease and chronic cutaneous diseases; the bark is used as mosquito repellent. Despite the widespread use of this plant in traditional systems of Indian medicine, no systematic surveys of its use among healers or phytochemical studies on the stem bark have hitherto been performed. Therefore, the aim of this paper was to document the medicinal uses of *C. strictum* by Indian folk healers. Furthermore, to investigate the constituents of the resin and stem bark, and the radical scavenging and antioxidant properties, the effects of NO production in lipopolysaccharide stimulated murine dendritic D2SC/I cells.

Ethnopharmacological data were obtained from ten folk healers in parts of Western Ghats, India. The resin and stem bark were extracted with DCM followed by MeOH by using an Accelerated Solvent Extraction instrument. The extracts were fractionated using different chromatographic methods, and isolated compounds were identified by NMR spectroscopy.

Our results revealed that the healers employed resin, and the reasons for the use were related to cold, airways diseases and rheumatoid arthritis. α-Amyrin and β-amyrin were identified as the major constituents in the resin. Compounds isolated from the stem bark MeOH extract were identified as procyanidins, gallic acid, methyl gallate, scopoletin, 3,3'-di-*O*-methylellagic acid 4-*O*-α-arabinofuranoside and elephantorrhizol (3,3',4',5,6,7,8-heptahydroxyflavan). The finding of elephantorrhizol in *C. strictum* is of chemotaxonomic interest, and the first report for the family Burseraceae. Furthermore, phloroglucinol degradation of procyanidins indicated the presence of catechin as the terminal unit and epicatechin as the extender units.

Radical scavenging and 15-lipoxygenase inhibitory activity, and dose dependent inhibition of NO production were observed in resin and bark extracts. No toxicity towards *Artemia salina* nauplii was found.

Paper III: Authentication of *Garcinia* fruits and food supplements using DNA barcoding and NMR spectroscopy

Garcinia L. (Clusiaceae) fruits are widely used in traditional medicine, and the fruits of several *Garcinia* species are locally and commercially harvested from the wild. The fruits are a rich source of (−)-hydroxycitric acid, a compound that has gained considerable attention as an anti-obesity agent. Commercial *Garcinia* based products are popular as food supplements worldwide, and the trade in *Garcinia* dried fruit rinds and its value-added products are constantly increasing. Increasing demand and limited supply of medicinal plants have resulted in widespread adulteration of a number of medicinal plants. Therefore, the specific objective of this study was to assess the adulteration of morphologically similar samples of *Garcinia* using DNA barcoding, and to quantify the content of (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone in raw herbal drugs and Garcinia food supplements using NMR spectroscopy.

A DNA barcode library for 41 individuals comprising of eleven different species was established using taxonomically authenticated samples referred to as biological reference material. DNA barcoding was carried out for these 41 samples using three DNA barcode regions, nrITS, and plastid markers *psbA-trnH* and *rbcL*. Twenty-one major herbal markets of South India were visited and data for all known vernacular and trade names of *Garcinia* species were obtained from the vendors, and raw drug samples were collected. For NMR quantification, one-gram aliquots of fruit powder and food supplements were extracted using an Accelerated Solvent Extraction system with Milli-Q water, and ^{1}H NMR spectra of *Garcinia* water extracts and food supplement extracts were acquired for quantitative analysis on a Bruker AVII400 instrument equipped with a 5 mm BBOF probe and operating at a frequency of 400 MHz at 298 K, and with number of scans = 128.

DNA barcoding results revealed that *nrITS* is a suitable DNA barcode region for *Garcinia* species by exhibiting the high mean interspecific genetic distance, and low intraspecific genetic distance followed by the plastid makers *psbA-trnH* and *rbcL*. Similarly, nrITS and *psbA-trnH* illustrated the clear grouping within *Garcinia* species with well supported bootstrap values, whereas *rbcL* on the other hand showed poor resolution in delineating the *Garcinia* species. Analyses of nrITS sequences of *G. gummi-gutta* (Kodampuli) and *G. indica* (Kokum) along with BRM barcode library revealed that there was no adulteration in the species, and also that the morphological

characters of fruits aided in the authenticity verification of collected raw drug samples from the market.

^{1}H NMR signals of (-)-hydroxycitric acid, (-)-hydroxycitric acid lactone and the internal standard maleic acid were identified in water extracts of *Garcinia* and used for quantification. The ^{1}H NMR signals for H-1 in (-)-hydroxycitric acid appear as two closely spaced doublets at δ 3.07 ppm (d, J = 16.6 Hz) and 3.16 ppm (d, J = 16.6 Hz), while H-3 appears as a singlet at δ 4.45 ppm (Figure 2). In the same spectrum the H-4 protons in (-)-hydroxycitric acid lactone give rise to two doublets at δ 2.92 ppm (d, J = 18.0 Hz) and 3.29 ppm (d, J = 18.0 Hz), H-2 gives a singlet at δ 5.00 ppm (Figure 2). The two vicinal protons of the internal standard maleic acid gave rise to the singlet at δ 6.37 ppm (Figure 2). A slight variation in the characteristic signals of (-)-hydroxycitric acid and (-)-hydroxycitric acid lactone within triplicates of each sample and between samples was observed (relative standard deviation < 0.03). These variations in chemical shifts values were possibly due to minor pH differences or interactions with other molecules in the extracts. Further, the total content of (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone in *G. gummi-gutta* and *G. indica* was found, and it varied from 1.7% to 16.3%, and 3.5% to 20.7% respectively. Analysis of ten *Garcinia* food supplements revealed a large variation in the content of (−)-hydroxycitric acid, from 29 mg (4.6%) to 289 mg (50.6%) content per capsule or tablet. Only one product contained quantifiable amounts of (−)-hydroxycitric acid lactone, and this product was also labelled to contain crude drug. Furthermore the study demonstrates that DNA barcoding and NMR could be effectively used as a regulatory tool to authenticate *Garcinia* fruit rinds and food supplements.
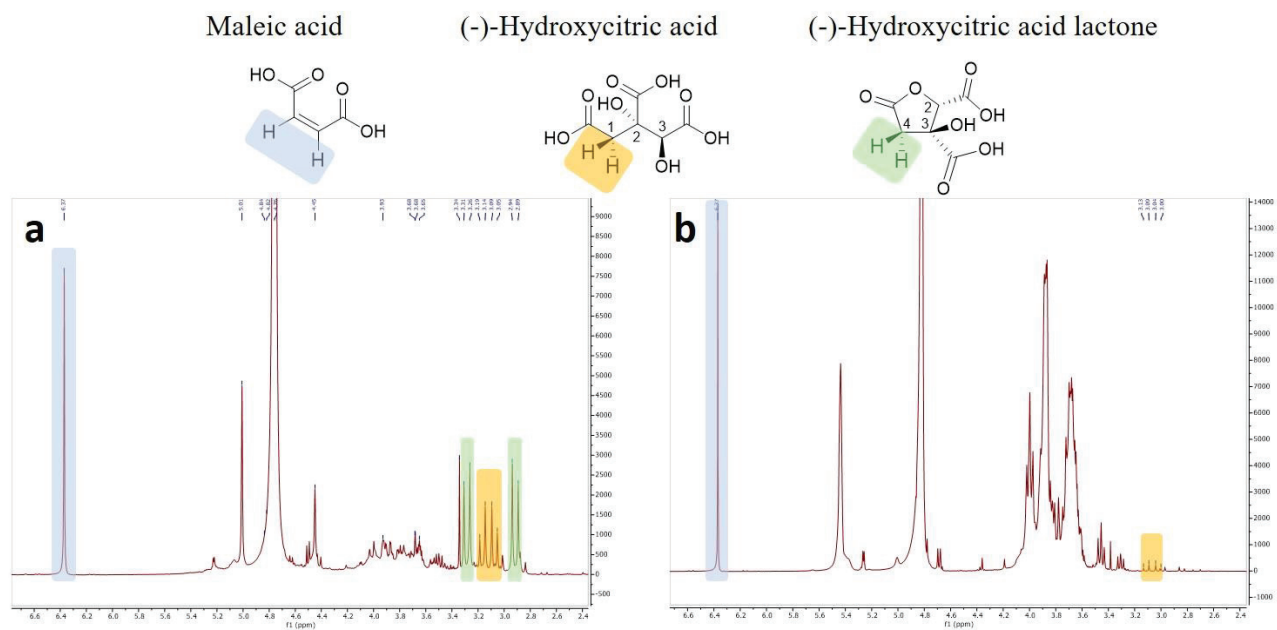
Figure 2. ^{1}H NMR spectrum of (**a**) *Garcinia gummi-gutta* water extract, and (**b**) water extract of *Garcinia gummi-gutta* food supplement (product 5). Orange and green labelled signals were used for quantification of (–)-hydroxycitric acid and (–)-hydroxycitric acid lactone, respectively, while blue labelled signals are from maleic acid (internal standard).

Paper IV: DNA metabarcoding authentication of Ayurvedic herbal products on the European market raises concerns of quality and fidelity

Ayurveda is popular and used worldwide in complementary and alternative healthcare and medical practices, but the growing commercial interest in Ayurveda based products has increased the incentive for adulteration and substitution within this herbal market. Consequently, the quality and safety of herbal products are affected. Therefore this study aimed to test the composition and fidelity of Ayurvedic products marketed in Europe using DNA metabarcoding (HTS using Ion Torrent), and to evaluate the ability of DNA metabarcoding to identify the presence of authentic species, any substitution and adulteration and/or presence of other off labeled plant species.

Seventy-nine Ayurvedic herbal products sold as tablets, capsules, powders and extracts were randomly purchased via e-commerce and pharmacies across Europe. After high throughput sequencing 35 products out of 79 (44%) yielded no Molecular Operational Taxonomic Units (MOTUs) in any of the replicates either for nrITS1 or nrITS2 that fulfilled our quality criteria, and they were excluded from the results and the further discussion. These products consisted of 13 tablets, 11 capsules, and 11 powders. The products that yielded MOTUs were represented by 17 tablets, 19 capsules, 5 powders and 3 extracts. A total of 188 different plant species belonging to 154 genera and 65 families were identified from the retained MOTUs using BLAST. Another quality selection criteria where a species was considered and validated as being present within the product only if it was detected in at least 2 out of the 3 replicates was applied. It resulted in another five additional products that failed to yield the same MOTU in any of the replicates which were discarded. The remaining 39 products resulted in a total of 97 plant species belonging to 40 families (62 species for nrITS1, and 60 species for nrITS2). The species detected for all the replicates for both ITS1 and ITS2, were merged for each sample for further analyses. Further analysis revealed that only two out of 12 single ingredient products contained only one species as labelled, eight out of 27 multiple ingredient products contained none of the species listed on the label, and the remaining 19 products contained 1 to 5 of the species listed on the label along with many other species not specified on the label. The fidelity for single ingredient products was 67%, the overall ingredient fidelity for multi ingredient products was 21%, and for all products 24%.

The DNA metabarcoding analysis also confirmed the presence of four threatened species, i.e., *Celastrus paniculatus* Willd., *Glycyrrhiza glabra* L., *Gymnema sylvestre* (Retz.) R.Br. ex Sm., and

Saraca asoca (Roxb.) Willd., whereas the remaining threatened species were not detected despite being included as labelled ingredients. The following species were found in over 20% of the products: *Withania somnifera* (L.) Dunal (3%), *Tribulus terrestris* L. (27%), *Convolvulus prostratus* Forssk. (23%), *Coriandrum sativum* L. (23%), *Ipomoea parasitica* (Kunth) G. Don (23%), *Ocimum basilicum* L. (23%) and *Senna alexandrina* Mill. (23%).

5) Discussion

In order to conduct any ethnobotanical or ethnopharmacological studies, researchers should be aware and learn more about ethical issues and research permission. These two are the single most important aspects that govern and regulate the genetic materials and its associated traditional knowledge. Until 1990, most ethnobiological research was not regulated on a global scale, and the collection of genetic material was governed by few restrictions like the Convention on International Trade in Endangered Species of Wild Flora and Fauna (Sands and Bedecarre, 1989; Weckerle et al., 2018). The import of genetic materials required a Phytosanitary Certificate that would certify that biological material was not carrying any disease agents (Weckerle et al., 2018). However, the Convention on Biological Diversity (CBD) coming into effect in 1993 (CBD, 2000), and the Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization (Nagoya Protocol) in 2014 (CBD, 2011, 2014), have changed this practice, and are implementing a legal framework and regulation on genetic materials and its associated traditional knowledge on a global scale (Weckerle et al., 2018).

The CBD assigned genetic resources as a property to national states, and the Nagoya Protocol clearly assigns the property rights of traditional knowledge to the respective knowledge holders. Any community work should be performed under the Nagoya Protocol on Access to Genetic Resources and Equitable distribution of benefits from their use. The right of use and ownership of any traditional knowledge of all informants remains with them. Except for scientific publication, use of any other information requires the additional consent of the traditional owners (CBD, 2011, 2014). Both these treaties have several implications for research ethics and permits towards ethnobiological research.

Prior to the ethnobotanical and ethnopharmacological data collection on dioecious plants (Paper I) and *C. strictum* (Paper II), ethical approval for these studies was obtained from the National Biodiversity Authority, Government of India (http://nbaindia.org/). While not all countries have ratified the Nagoya Protocol, India has ratified the protocol. Especially researchers in European Union are legally required to comply with the Nagoya Protocol, because the European Union has ratified it. As of January 2019, 114 countries have ratified the protocol. Researchers from the countries that have not ratified the protocol are still required to comply with Nagoya Protocol when it comes to the import of biological material and its associated knowledge. After obtaining the

ethical approval for this study, the ethnobotanical and ethnopharmacological surveys were conducted. The purpose of the study was explained to the informants and the consent to conduct the study was requested and agreed in written format. Weckerle et al. (2018) have highlighted that signing the papers is simply not common for the folk healers or participants, and a request for written consent may create distrust among participants. Therefore, it was recommended to provide a statement on what kind of consent they obtained and to state if they followed a specific code of ethics. For ethnobiological research, the current standard is the International Society of Ethnobiology Code of Ethics (ISE, 2006).

In our study (Paper I), a significant knowledge gap was identified. It was estimated that angiosperms include 352,000 species worldwide, and an enormous effort is being made to document and systematically study the traditional uses of flowering plants. However, it is noteworthy that angiosperms have different sexual systems, and reproductive physiology of each sexual systems demands varying level of resources (Charlesworth, 2002; Obeso, 2002). Dioecy is one such sexual system of angiosperms and documenting traditional uses of dioecious plants based on their gender was lacking in ethnobotanical literature. Therefore, this study (Paper I) aimed to contribute to the scientific community interested on ethnobotanical research. This study involved the listing of dioecious species that occur in India followed by ethnobotanical data collection in parts of the Eastern Ghats in Tamil Nadu, India. A list of dioecious species occurring in India were derived from 15,600 dioecious angiosperm species compiled by Renner (2014), and the occurrence of 5–7 % dioecious medicinal plants in Indian systems of medicines in general is congruent with the diversification rate of dioecious species in flowering plants (Käfer et al., 2014; Renner, 2014).

Though considerable evidence for sex-biased herbivory and variation in secondary metabolites in dioecious plants is available in scientific studies, little is known about traditional concepts and preferences for dioecious plants, either male or female. Several studies on folk classification of plants, and ethno-ecology reported the traditional knowledge associated with dioecious plants, and the importance of how the different genders of plant species are named and classified in local languages (Berlin et al., 1973; Khasbagan, 2008; Bjora et al., 2015). In our study, snowball sampling method was used to collect ethnobotanical data for 40 dioecious plants. Snowball sampling method was employed in order to find informants who have a rich knowledge on traditional usage of plants. In order to document the informants' knowledge on dioecy, examples

such as papaya and palm trees were used to make them understand the phenomenon of dioecy, coconut trees and pumpkins were explained for monoecy, and goose berries for bisexual plants before initiating the interview process. The study area (Kolli Hills, Servarayan Hills and Sittilingi Valley) was chosen due to the diverse natural vegetation, and the main ethnic group in the study area is the *Malayali* community.

The research findings show that the informants have knowledge about the existence of the dioecious nature of many plants, and informants were aware of 31 out of 40 dioecious plants. However, the informant's perception on what is male and female plants are highly dependent on a species-specific plant characteristic traits. For example, informants were unaware of dioecious plans such as *Dodonaea angustifolia* L.f., *Dodonaea viscosa* (L.) Jacq., *Cocculus hirsutus* (L.) W.Theob., *Cyclea peltata* (Lam.) Hook.f. & Thomson, *Asparagus racemosus* Willd. and *Cissampelos pareira* L.. On the other hand, informants were aware of dioecy for *Celastrus paniculatus* Willd. and *Embelia tsjeriam-cottam* (Roem. & Schult.) A.DC. this is due to whether the useful part of the plants is fruits/seeds or other parts. Several studies have also shown that human utilization of a plant is highly influenced by its species traits (Díaz et al., 2011; Díaz et al., 2013). This has also been shown by Cámara-Leret et al. (2017) who demonstrated that people preferentially use large, widespread species rather than small, narrow ranged species, and that different traits are linked to different uses. Few other factors that might have affected the informants perception of a plant being dioecy or not is sex ratio and plasticity in sex expression of a particular plant (Mcarthur, 1977; Borges et al., 1997; Pathak and Shukla, 2004; Geetha et al., 2007; Sharma et al., 2010; Field et al., 2013; Renner, 2014).

Our results also highlights that the male tree timbers are preferred for construction purposes such as houses, huts and furniture because it is believed that male plants have expected size and more durable timber than female trees. Such preference on one gender in timber could be explained with plant resource allocation theory that the male plants comparatively allocate more resource to vegetative growth than the female plants. On the other hand, informants also consider that the male plants of Myristica dactyloides Gaertn. are not beneficiary for them and selectively chosen for firewood. Similar information were documented for Carica papaya L., the informants do not prefer the male plants to be grown in their garden since it yields no fruits. Such selective logging has been shown to endanger the plant species (Martínez-Garza and Howe, 2003). Further, any anthropogenic

activity that modifies the male–female distance, sex ratio, plant size and pollinator abundance or behavior could affect the long-term viability of dioecious plants, and endangers the species (Somanathan and Borges, 2000).

The phytochemical study (Paper II) aimed to document the medicinal uses of *C. strictum* and investigate the phytochemical contents of resin and stem bark, and the radical scavenging and antioxidant properties, and the effects of NO production in lipopolysaccharide stimulated murine dendritic D2SC/I cells. The present ethnopharmacological study revealed that the folk healers mostly utilized the resins of *C. strictum* for various medicinal usages. Among the documented usage common cold, runny nose and sneezing were the most commonly mentioned indications. In addition, healers reported that resin is burnt in in house to produce smoke, which in turn act as insect repellent. Similarly, *Canarium schweinfurthii* resin also reported to act as insect repellent (Youmsi et al. (2017). The phytochemical analysis showed that α-amyrin and β-amyrin were the major compounds present in the resin. Whereas procyanidins, gallic acid, methyl gallate, scopoletin, 3,3'-di-*O*-methylellagic acid 4-*O*-α-arabinofuranoside, and elephantorrhizol (3,3',4',5,6,7,8-heptahydroxyflavan) were isolated from the bark MeOH extract. It is noteworthy that the finding of elephantorrhizol in *C. strictum* is the first report for the family Burseraceae, and it has chemotaxonomic interest. In addition 3,3'-Di-*O*-methylellagic acid 4-*O*-α-arabinofuranoside is also a rare natural product and first report from Burseraceae, and it has been previously reported four plant species such as *Cornus capitata* Wall., *Cornus kousa* F.Buerger ex Hance, *Euphorbia humifusa* Willd., *Euscaphis japonica* (Thunb.) Kanitz, (Tanaka et al., 2001; Tanaka et al., 2003; Lee et al., 2007; Deng et al., 2008). However, ellagic acid derivatives have been reported from other *Canarium* species (as reviewed in Mogana and Wiart, 2011). Scopoletin and gallic acids are also known from *Canarium*. Radical scavenging and 15-lipoxygenase inhibitory activity were observed in resin and bark extracts, whereas no toxicity towards *A. salina* nauplii was found. The bark MeOH crude extract showed higher activity in the antioxidant assays, than the methanol and DCM extracts of resin. This finding could be explained by the presence of a high content of polyphenols in the bark. On the other hand, 15-LO inhibition of MeOH and DCM bark extracts showed relatively high activity compared with the positive control quercetin. The 15-LO inhibitory activity of the MeOH bark extract is probably due to the procyanidins (Bräunlich et al., 2013). Methanol and DCM extracts of resin shows a dose dependent anti-inflammatory effect by reducing levels of NO, without being toxic to the cells. Whereas, DCM bark extract also showed significant

inhibition of NO. Similarly, several studies have reported the anti-inflammatory properties of *Canarium* species (Mogana et al., 2013; Kuo et al., 2016).

Several studies have highlighted the importance of gender in phytochemical research and its impact on pharmacological properties of a species (Simpson, 2013). With this evidence that the medicinal dioecious species harness a potential to be studied comparatively for their chemical composition between male and female plants and the pharmacological activities, and also provides a platform to document ethno-ecological knowledge, and traditional knowledge of dioecious plants with special reference to its gender.

One of the parameters of pharmacognosy is identification and authentication of medicinal plant drugs and monitoring the quality of the resulting herbal medicines (Heinrich and Anagnostou, 2017). Complex herbal formulations pose unique challenges to the established quality control methods that include identity, purity and analyzing specific constituents within the herbal products. Also the synergistic action of polyherbal products is fundamental to the practice of Ayurveda and the use of such specific combinations of medicinal plants is expected to result in an enhanced outcome. To ensure the quality, safety and efficacy of such polyherbal products each of the plants included must be identified.

The pharmacognosy studies (Paper III and IV) aimed to contribute to all the challenging efforts of the scientific community towards finding and proposing improved alternative and complimenting quality control methods for the traded raw herbal drugs and marketed herbal products. These studies involved the use of NMR spectroscopy together with DNA barcoding, and DNA metabarcoding to assess 89 complex herbal products sold in five different countries, including three European countries. These studies involved top selling herbs such *Garcinia gummi-gutta*, *Garcinia indica* (Paper III), and a wide variety of highly traded Indian medicinal plants such as *Piper* spp.*, Phyllanthus* spp.*, Ocimum* spp., *Sida* spp., and *Withania somnifera* (Indian ginseng) (Paper IV).

NMR spectroscopy was chosen as an analytical method for this study (Paper III) considering its advantage of being robust, reliable and non-destructive. In Paper III, ^{1}H NMR quantification was used to identify and quantify the principle organic acids, (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone in *Garcinia* raw herbal drugs, and in food supplements that claim to contain *Garcinia* extracts respectively.

Quantitative ^{1}H NMR revealed varying amounts of (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone in dried *Kodampuli* and *Kokum* raw drugs. The total content of (−)-hydroxycitric acid, and (−)-hydroxycitric acid lactone varied between 1.7% to 16.3%, and 3.5% to 20.7%, respectively, in the two species. Several other studies using HPLC methods have also reported the content of (−)-hydroxycitric acid in *Garcinia* species (Jayaprakasha and Sakariah, 1998, 2002; Jena et al., 2002). However, a challenge with HPLC is to obtain a good resolution between the organic acid and its lactone in *Garcinia* extracts. There are examples showing no discrimination between (−) - hydroxycitric acid and the lactone or methods that suffer from poor resolution (Jayaprakasha and Sakariah, 1998, 2000, 2002). Another challenge is the low specificity at 210 nm, therefore sample preparation may be needed to remove co−eluting interfering substances (Jayaprakasha and Sakariah, 1998, 2000, 2002). Gas chromatography, and capillary zone electrophoresis have previously been used to quantify the hydroxycitric acid and hydroxycitric acid lactone content in *Garcinia* fruits and food supplements (Lowenstein and Brunengraber, 1981; Jayaprakasha and Sakariah, 2002; Muensritharam et al., 2008). Comparatively quantitative ^{1}H NMR employed in our study has the advantage of being rapid (less than 8 minutes needed for 128 scans) with no necessity for additional cleanup of extracts or derivatization. NMR makes it possible to detect several organic molecules in the same sample, as long as they are soluble in the NMR solvent, and is also highly reproducible with little instrument-instrument variation (Kruk et al., 2017).

The overlaps between the total content of (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone in *Garcinia* species suggest that these organic acids cannot not be used as marker compounds to distinguish between *Garcinia* species. The variation in the concentration of the organic acids in *Kodampuli* and *Kokum* raw drugs might be due to seasonal and geographic variations, and concentration of organic acids can also vary based on the harvest time, as it has been shown in other fruits (Raffo et al., 2004; Liu et al., 2006). Similarly, seasonal and geographic variations in chemical composition have been reported for a number of medicinal plants and also chemical variability along the value chains, harvesting pressure, harvest time, collection of immature plant parts etc. have been reported to influence the composition and concentration of plant constituents (Pandey and Shackleton, 2012; Booker et al., 2014).

After validating the quantitative ^{1}H NMR method developed to quantify the organic acids in *Garcinia* raw drugs, the method was applied to authenticate the validity of *Garcinia* food

supplements claiming to contain *Garcinia* extracts. Analysis of ten *Garcinia* food supplements revealed that five products contained both (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone, including only one product contained quantifiable amounts of (−)-hydroxycitric acid lactone. Whereas (−)-hydroxycitric acid lactone was missing or only present in trace amounts in the remaining products. The absence of (−)-hydroxycitric acid lactone in the supplements with *Garcinia* extracts might be due to the extraction method employed for the herbal products (Majeed et al., 1998). Previous quality control studies on *Garcinia* products have reported both adulteration and no adulteration (Jayaprakasha and Sakariah, 2000; Muensritharam et al., 2008; Jamila et al., 2016).

^{1}H NMR can be useful in qualitative and quantitative analysis of constituents in medicinal plants and herbal products (Hachem et al., 2016). This study has shown that quantitative NMR can accurately detect the presence or absence of targeted plant metabolites, and is also as a fast and sensitive enough method for the quantification of organic acids and lactone in raw herbal drugs and food supplements. However, one of the disadvantages of the quantitative NMR method is its inability in detecting infrageneric species and its substitution, and in providing information about other plant ingredients present in the products. This limits the applicability of quantitative NMR in quality control of complex herbal products.

The efficacy of DNA barcoding in dealing with authenticity issues of medicinal plants and herbal products has been discussed in various studies (Smillie and Khan, 2010; de Boer et al., 2015; Parveen et al., 2016; Sgamma et al., 2017). In Paper III, DNA barcoding was used as an analytical approach in herbal raw drug authentication of *Garcinia* raw herbal drugs in combination with ^{1}H NMR. Such combination of DNA barcoding with spectroscopic methods for authentication of herbal medicines increases the resolution in species identification and analysis of mixtures (Santhosh Kumar et al., 2016). nrITS marker was utilized to authenticate the raw drugs traded as *Kodampuli* and *Kokum,* and the analyses of nrITS sequences of *Kodampuli* and *Kokum* revealed that there was no adulteration in the species. In addition, the morphological characters of fruits aided to identify the collected raw drug samples from the market. This was also apparently due to the absence of taxonomic complexities in fruit morphological characters of *Kodampuli* and *Kokum* (Parthasarathy and Nandakishore, 2014).

DNA barcoding was utilized to test the authenticity of *Garcinia* fruits traded in the Indian herbal markets. On the other hand, it was not applicable to use the same method to authenticate *Garcinia* herbal products due to its major limitation of Sanger sequencing based DNA barcoding. Sanger sequencing has the inability to distinguish constituent species in multi-ingredient herbal products. One of the prerequisites for the DNA-based molecular analysis of herbal products is the isolation of good quality DNA. In our study, the yield of isolated DNA from the *Garcinia* food supplements ranged from 0.05 to 1 ng/ml, and therefore DNA metabarcoding was not feasible. Plant DNA can also be removed or degraded during the manufacturing process of herbal products: extensive heat treatment, irradiation, ultraviolet light exposure, filtration and extraction will affect DNA yield and integrity (de Boer et al., 2015).

The efficacy of DNA metabarcoding to investigate species diversity of several sorts of samples has been discussed in various studies (Taberlet et al., 2012; Staats et al., 2016). In our study (Paper IV) DNA metabarcoding was used as an analytical approach to identify the presence of all the labelled species in 79 Ayurvedic herbal products. Out of 79 products analyzed, 35 products (44 %) have not yielded MOTUs in any of the replicates either for nrITS1 or nrITS2 that fulfilled our quality criteria, and they were excluded from the results and the further discussion. The success rate in generating raw sequences reads from the herbal products, and the number of products from which MOTUs could be identified after applying strict trimming and filtering quality criteria are reported to vary in several studies. For example, 53% success rate was obtained for dietary products derived from *Echinacea*, *Ginkgo*, *Hypericum*, *Trigonella* and *Veronica* (Ivanova et al., 2016), and 100 % success rate for individual unprocessed crude traditional Chinese medicine products (Cheng et al., 2014). These differences are likely due to the different quality and type of raw materials, but also due to the effect of different variables in the experimental and data processing that can be optimized to improve the quality of the results.

DNA metabarcoding detected considerable incongruences between the detected species and those listed on the product label. In ten out of twelve single ingredient products that were labeled as containing only one species, we detected multiple species, from which six contained the species labeled on the product together with other species, whereas four products did not contain the species listed on the product label but contained several other non-listed species. Out of 27 successfully analyzed multiple ingredient products, 8 (29.6%) products contained none of the

species listed on the label, and the remaining 19 products contained between one to five species listed on the label along with many other species not specified on the product label. The fidelity rate for single ingredient products was 67% (8 out of 12), and the overall ingredient fidelity (detected species from product label/total number of species on label) for multi ingredient products was 21%, and for all products 24%.

The discrepancies between the species detected using DNA metabarcoding and those listed on the products require a careful consideration of possible contamination, and the fact that DNA metabarcoding is a highly sensitive detection method (Raclariu, 2017; Raclariu et al., 2018). Even small traces of pollen grain or plant dust left in production equipment may be revealed from the analysis. It is therefore important that the herbal product authentication using DNA metabarcoding focuses on the presence and/or absence of target species. Alarms need not to be raised over trace contamination from other species, plausible present in the cultivation, transport or production chain (Raclariu, 2017; Raclariu et al., 2018). However this requires a case based analysis including the experimental steps (e.g., sample preparation, library preparation, and HTS) and post-bioinformatics analysis that may provide false positive or false negative results (Robasky et al., 2014; Ficetola et al., 2015). The large number of non-listed plant species may thus be expected as contamination bias, amplification bias, sequencing errors or false-positive taxonomic identifications due to error-prone barcode sequences reference databases (Robasky et al., 2014). On the contrary, the overlooked species can be explained by false negative detections, usually determined by the availability and integrity of plant DNA that can be removed or highly degraded during harvesting, drying, storage, transportation and processing (e.g., mode of extraction, exposure to ultraviolet light, heat or pressure) (Novak et al., 2007). In addition, primer fit issues or primer-template mismatches (Piñol et al., 2015), stochasticity due to low DNA concentration or incomplete barcode sequences reference databases (Giguet-Covex et al., 2014). The use of replicates may considerably reduce these risk of missing the present taxa at the expense of substantially increasing sequencing costs and time (Ficetola et al., 2015). Nevertheless, the bioinformatics post processing of the sequencing data may help to reduce the uncertainty of the results (Robasky et al., 2014). In paper IV, efforts were made to reduce the amount of sequencing errors, which were known to affect the Ion torrent sequencing platform (Loman et al., 2012; Salipante et al., 2014).

In the context of the quality control of herbal products, DNA barcoding and DNA metabarcoding do not provide any quantitative nor qualitative information about the active plant metabolites in the raw plant materials or in herbal products, thus it narrows its applicability only to identification and authentication procedures. Whereas, quantitative NMR method is accurate in identifying and quantifying the presence of targeted chemical compounds, but limited ability in identifying the targeted species. The results of this study Paper III and IV reveal that there is a need for a better quality control of herbal products. A novel analytical approach should eventually use a combination of innovative high throughput methods that complement the recommended standard methods.

6) Concluding remarks and perspectives

At the start of this PhD research, the aim was to focus on documenting and comparatively studying the medicinal plants used by various ethnic groups in different ecological and cultural traditions in India, and validate the traditional knowledge using scientific methods such as phytochemical characterization of plants and associated biological activities. Simultaneously, the aim was to employ DNA barcoding and high-throughput sequencing methods for identification of medicinal plants in trade, and medicinal herbal products. However during literature searches, the existence of a significant knowledge gap in ethnobotanical and ethnopharmacological literature was identified, i.e., substantial ethnobotanical knowledge that has been documented lacks the documentation of plant usages based on their sexual systems. Therefore, the present study started with the documentation of traditional knowledge and use of dioecious species by folk healers in Eastern Ghats of India. The majority of the healers had knowledge on genders of plants species, and the results from this study revealed that the folk healers have traditional knowledge on gender of plants and preferential usages towards one gender for some species. With this evidence, we propose that researchers conducting ethnobotanical and ethnopharmacological studies should consider documenting traditional knowledge on sexual systems of plants, and test the existence of gender specific usages in their conceptual framework and hypothesis testing. The incorporation of such concepts could provide new dimensions of scientific knowledge with potential implications to conservation biology, chemical ecology, ethnoecology and drug discovery. The presence of the bioactive compounds might give give a rationale for the wide spread usage of the resin in India. Furthermore, the identified compounds can be utilized to comparatively study the phytochemical profile of male and female plants of *Canarium strictum*.

Developments in pharmacognosy analytical methods are leading towards finding new systematic approaches for monitoring the safety of traded medicinal plants and marketed herbal products. Hence, the second phase of the PhD research focused on comprehensive evaluation of quantitative ^{1}H NMR, DNA barcoding and DNA metabarcoding as a viable approach for resolving current limitations in authentication of herbal products in order to improve the quality control of traded/marketed herbal products. The results revealed that different analytical methods have its own merits and demerits, its specific strength lies, while applying appropriately in the value chain of herbal products. These analytical methods, quantitative ^{1}H NMR, DNA barcoding and DNA

metabarcoding have the potential to be applied in the context of quality control of both well and poorly regulated supply systems. However, standardization of protocols is necessary before these methods can be implemented as routine analytical approaches and approved by the competent authorities for use in regulatory procedures.

In the context of pharmacognosy and pharmacovigilance, a combination of analytical methods is compulsory for comprehensive authentication and quality control of raw material and resulting products.

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Ethnobotany of dioecious species: Traditional knowledge on dioecious plants in India

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ABSTRACT

Ethnopharmacological relevance: More than 15,000 angiosperm species are dioecious, i.e., having distinct male and female individual plants. The allocation of resources between male and female plants is different, and also variation in secondary metabolites and sex-biased herbivory is reported among dioecious plants. However, little is known about the ethnobotany of dioecious species and whether preferences exist for a specific gender, e.g., in food, medicine or timber.

Aim of the study: The aim of this study was: 1) to study whether Indian folk healers have preference for plant genders, and to document their knowledge and use of dioecious species; 2) to understand the concept of plant gender in Indian systems of medicine and folk medicine, and whether Ayurvedic literature includes any references to gender preference.

Materials and methods: Lists of dioecious plants used in Indian systems of medicine and folk medicine were compiled. Ethnobotanical data was collected on perceptions and awareness of dioecious plants, and preferences of use for specific genders of dioecious species using semi-structured interviews with folk healers in Tamil Nadu, India. In addition, twenty Ayurvedic doctors were interviewed to gain insight into the concept of plant gender in Ayurveda.

Results: Indian systems of medicine contain 5–7% dioecious species, and this estimate is congruent with the number of dioecious species in flowering plants in general. Informants recognized the phenomenon of dioecy in 31 out of 40 species, and reported gender preferences for 13 species with respect to uses as timber, food and medicine. Among informants different plant traits such as plant size, fruit size, and visibility of fruits determines the perception of a plant being a male or female. Ayurvedic classical literature provides no straightforward evidence on gender preferences in preparation of medicines or treatment of illness, however it contains details about reproductive morphology and sexual differentiation of plants.

Conclusions: A knowledge gap exists in ethnobotanical and ethnopharmacological literature on traditional knowledge of dioecious plants. From this explorative study it is evident that people have traditional knowledge on plant gender and preferential usages towards one gender. Based on this, we propose that researchers conducting ethnobotanical and ethnopharmacological studies should consider documenting traditional knowledge on sexual systems of plants, and test the existence of gender specific usages in their conceptual framework and hypothesis testing. Incorporating such concepts could provide new dimensions of scientific knowledge with potential implications to conservation biology, chemical ecology, ethnoecology and drug discovery.

1. Introduction

Enormous efforts are being made to document and systematically study the traditional uses of plants. Dioecy, where species have separate female and male plants, is widespread among flowering plants, and an estimated 6% of species are dioecious (Renner, 2014). Resource allocation, including trade-offs between allocation to defense, growth and reproduction, is different between genders of dioecious plants (Obeso,

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2002). Several studies have shown that differences in reproductive demands between the genders of dioecious plants cause gender physiology divergence that in turn affects the production and concentration of secondary metabolites (Bajpai et al., 2016; Milet-Pinheiro et al., 2015; Simpson, 2013). Herbivory has been suggested as a selective pressure that has resulted in the evolution of dioecy (Bawa, 1980), and studies have utilized the plant resource allocation theory (Levins, 1968) to understand the patterns of plant-herbivore interaction (Obeso, 2002), herbivore plant gender preferences (Hjalten, 1992), plant browsers (Danell et al., 1991), folivores (Maldonado-López et al., 2014), pollinators (Milet-Pinheiro et al., 2015), and gall formers (Wolfe, 1997). Cornelissen and Stiling (2005) reviewed the evidence of sex-biased herbivory in dioecious plants, and found that male plants exhibited significantly higher number of herbivores and herbivory in terms of plant damage compared to female plants, and showed that male plants exhibited significantly lower concentrations of secondary compounds and other defenses than female plants. However, there are also examples of the opposite, e.g., the male plants leaves of *Rhamnus alpinus* L. and *Juniperus macrocarpa* Sm. exhibited a higher concentration of anthraquinones, phenolics and terpenoids respectively than those of females, which contrasts with the resource allocation theory (Banuelos et al., 2004; Massei et al., 2006). Hence it is evident that resource allocation might have a profound effect on the composition and concentration of secondary compounds between individuals of dioecious species (Simpson, 2013).

Simpson, 2013 has highlighted the importance of gender in phytochemical research and its impact on pharmacological properties of a species. For example in the dioecious species *Cannabis sativa* L., the female plants are used for marijuana, whereas the male plants are preferred for fiber (Fetterman et al., 1971). In *Dodonaea polyandra* Merr. & L.M.Perry (Sapindaceae), labdane diterpenoids have been reported as major phytoconstituents, whereas female individuals contain clerodane diterpenoids (Simpson, 2013; Simpson et al., 2011, 2012). Similarly, a significant variation in the concentration of alkaloids was shown for the dioecious medicinal plant *Tinospora cordifolia* (Willd.) Miers (Menispermaceae). The mean abundances of magnoflorine, jatrorrhizine and oblongine were significantly higher in male plants while mean abundances of tetrahydropalmatine, norcoclaurine and reticuline were significantly higher in female plants (Bajpai et al., 2016). It has been suggested that female plants of *T. cordifolia* might be preferred for therapeutic use due to the higher accumulation of secondary metabolites and higher antioxidant activity (Choudhry et al., 2014).

The 15,600 dioecious angiosperms occur in 987 genera (6%) and 175 families (38%), with a number of families being entirely dioecious, e.g., Menispermaceae, Moraceae, Myristicaceae, and Putranjivaceae (Renner, 2014). Many of these dioecious species are well documented for their medicinal values (de Boer and Cotingting, 2014). In India, it is estimated that 8000 plants have medicinal usages. Some of these are codified in traditional pharmacopoeias, i.e., Ayurveda, Siddha, Unani, and Sowa-Rigpa, whereas others are part of oral traditions in different biocultural groups. Considerable evidence for sex-biased herbivory and variation in secondary metabolites in dioecious plants is available in scientific studies, but little is known about traditional concepts and preferences for dioecious plants, either male or female. Few studies on folk classification of plants and ethno-ecology report traditional knowledge associated with dioecy, and the importance of how the different genders of plant species are named and classified in local languages and how this reflects perceptions of the environment (Berlin et al., 1973), cultural values of biodiversity (Bjora et al., 2015), and ecological characteristics (Khasbagan, 2008). Bernstein et al. (1997) used plot survey inventories in Brunei to show that their informants were able to accurately predict the gender of dioecious plants. In Northern Morocco, it was reported that the vernacular taxonomy is congruent with the biological classification of the dioecious species *Ficus carica* L. among three communities inhabited in three socio-geographic regions who speaks Arabic, Berber, and both Arabic and Berber,

respectively (Hmimsa et al., 2012).

Several studies have highlighted the importance of understanding the ecology of plant biodiversity as a strategy for drug discovery (Coley et al., 2003), as well as ethnobotanical studies and/or traditional medicines for drug development (Patwardhan and Mashelkar, 2009). At the same time, erosion and deterioration of traditional knowledge threatens biocultural diversity and limits resilience in healthcare choices for local communities, which also can cause a loss in leads for drug discovery (de Boer and Cotingting, 2014; Srithi et al., 2009). In the context of ongoing cultural, ecological, and socio-economical changes, particularly the influence of urbanization and influence of western lifestyles, the increasing reliance on biomedical healthcare, the devaluation of traditional practices, and diminishing cultural cohesion are weakening the frequency and scope of traditional plant use and this poses a serious threat to biodiversity-based cultural knowledge (Srithi et al., 2009; Vandebroek and Balick, 2012). Documenting the use of plants by ethnic communities is an important part in understanding and analyzing elements of traditional medicines, and also a way to perpetuate knowledge at risk of being lost (de Boer and Cotingting, 2014).

The aim of this study was: 1a) to document traditional knowledge on dioecious plants among folk healers and 1b) to understand whether folk healers have preference for plant genders in food, medicines or timber; 2) to understand folk healers' perceptions of what are considered to be male and female plants in their community and traditions; and 3) to understand the concept of plant gender in Indian systems of medicine and folk medicine, and whether Ayurvedic literature contains any references to plant gender and preferences.

2. Methodology

2.1. Selection of Indian dioecious plants

Dioecious species in India were derived from the list of 15,600 dioecious angiosperms compiled by Renner (2014) by limiting to species occurring in India. Dioecious species in codified and non-codified Indian traditional medicine were mined from the Indian Medicinal Plant Database, National Medicinal Plants Board, Government of India, and full lists are provided in Supplementary Data S1. Nomenclature follows The Plant List (The Plant List, 2013 http://www.theplantlist. org) and Angiosperm Phylogeny Group IV (Byng et al., 2016). The ethnobotanical study focused on 40 dioecious plants in 30 genera and 20 families (Table 1), which occurred in the study area (see below) and were reported to be used in traditional medicine in previous studies.

2.2. Study area for ethnobotanical prospection

The present survey was conducted in the Kolli Hills (11.105°N, 78.150°E), Servarayan Hills (11.455°N, 78.175°E), and Sittlingi Valley (11.543°N, 78.365°E), all of which are part of the Eastern Ghats in Tamil Nadu, India. The natural vegetation of the study area is categorized into shola (tropical montane forest), evergreen, semi-evergreen, deciduous, scrub, and plantation (Jayakumar et al., 2002). The main ethnic group in the study is the *Malayali* communities (lit. malai = hill, ali = dwells and/or malai = hill, alu = person), one of 36 scheduled tribal communities in Tamil Nadu. The *Malayali* is spread along the contiguous hills of the Eastern Ghats from Pachamalai, Kollimalai, Sitheri, Palamalai, Javvadhu to the Servarayan Hills (Xavier et al., 2015). The major livelihood and local economy of these *Malayali* communities are cattle farming, agriculture, fuel-wood and collection of non-timber forest products such as herbal medicines, honey and some edible fruits and tubers (Xavier et al., 2015).

2.3. Ethnobotanical data collection

Forty folk healers aged 40 to 80 (33 males and 7 females) were interviewed in 2016, and their knowledge documented using a semi-

Table 1

Details of dioecious plants studied in Kolli Hills (Namakkal district), Sittingi Valley (Dharmapuri district) and Servarayan Hills (Salem district) of Tamil Nadu, India with preferences towards gender and uses.

Species; Family; Voucher	Tamil name	Habit	Plants recognized as dioecious (%)			Gender preference	Specific use[a]
			Y	N	Uncertain		
Anamirta cocculus (L.) Wight & Arn.; Menispermaceae; Cl 776	*Kakamari, Nancukkottai*	Liana	35	58	8	Yes	O: Fruits and seeds are poisonous which are used to poison fishes[1,9,17,38] (Tag et al., 2005) R: ♂ and ♀ leaves are used to do black magic[1,4,7,13,14,31]
Aphanamixis polystachya (Wall.) R.Parker; Meliaceae; Cl 777	*Cemmaram, Civappurmaram*	Tree	33	58	10	Yes	M: Leaves are used to cure skin diseases, and stomach pain[2,3,18,24,32] (Sen et al., 2011) R: Flowers are collected for fragrance, in which ♀ flowers tend to contain more fragrance, and occasionally offered in rituals[2,8,13,29]
Asparagus racemosus Willd.; Asparagaceae; Cl 778	*Tannirvittankizhangu*	Liana	0	88	13	No	M: Tubers are used to cure white discharge, stomach pain, reduces body heat, rejuvinative, and enhances lactation[1–40] (Bopana and Saxena, 2007)
Bischofia javanica Blume; Phyllanthaceae; Cl 779	*Romaviruksha pattai, Milachadayan*	Tree	35	58	8	No	M: Stem bark is used to cure body ache, stomach ulcers, mouth ulcers and inflammatory conditions[4,9,13,17,26,30,31,36]
Borassus flabellifer L.; Arecaceae; Cl 780	*Panai maram*	Tree	100	0	0	Yes	R: Stem and leaves are used to black magic in terms of removing fear[1,4,7,9,13,14,31,36] F: ♂ plant toddy is more vibrant than ♀ [1,3,13,17,22,25,27,38,40]. Fruits and tuberous seedlings are edible[1–40] (Davis and Johnson, 1987)
Canarium strictum Roxb.; Burseraceae; Cl 781	*karukunkiliyam*	Tree	68	15	18	Yes	M: Fruits and roots are used as diuretic, and antidiabetic[1,11,23,31] (Davis and Johnson, 1987) M: Resin is used as anti-inflammatory and to cure skin diseases, against poisonous bites[1–10, 23,33,36,37] O: ♀ tree yields more resin than ♂ plant[1–10, 31,35,37]. (Namsa et al., 2009).
Carica papaya L.; Caricaceae; Cl 782	*Pappali pazham*	Tree	100	0	0	No	F: Fruits are edible[1–40] (Krishna et al., 2008) M: Latex are used to control tooth ache, fruits used as rejuvenative and pregnancy abortive agent[1,4,15,21,24,27,29,34] (Krishna et al., 2008)
Cassine glauca (Rottb.) Kuntze; Celastraceae; Cl 783	*Karuvali*	Tree	83	0	18	No	M: Leaves and stem are used against dysentery, for wound healing, against poisonous bites, headache, fever[5,11,12,13,21,23, 26,29] (Moin et al., 2014)
Celastrus paniculatus Willd.; Celastraceae; Cl 784	*Valuluvai*	Liana	70	23	8	No	M: Seeds are used in mental problems, joint pain, arthritis, skin diseases, wound healing[1,5,10,14,18,20,31,36] (Rajkumar et al., 2007)
Cissampelos pareira L.; Menispermaceae; Cl 785	*Vattattiruppi*	Liana	0	73	28	No	M: Root and whole plant are used as appetizer, antidiarrhoeal, anthelmintics, antiulcer, and to cure digestive complaints.[1,3,7,13,16,19,21,27,34,36] (Amresh et al., 2007)
Coccinia grandis (L.) Voigt; Cucurbitaceae; Cl 786	*Kovai, Kovaikkay*	Vine	0	60	40	No	F: Fruits are edible and used as vegetable [1–40] (Addis et al., 2009)
Cocculus hirsutus (L.) W.Theob.; Menispermaceae; Cl 787	*Kattukkoti*	Climber	0	83	18	No	M: Leaves and roots are used to cure skin diseases, skin irritation, and stomach ache[1,4,7,9,14,15,29,30,34] (Patil et al., 2014)
Cyclea peltata (Lam.) Hook.f. & Thomson; Menispermaceae; Cl 788	*Malaithangi, Vattattiruppi*	Climber	0	78	23	No	M: Leaves and roots are used to cure poisonous bites, indigestion, stomach pain, boils and blisters[2,5,7,8,14,25,28,34] (Xavier et al., 2015)
Dioscorea alata L.; Dioscoreaceae; Cl 789	*Vettilai-valli*	Vine	68	0	33	No	F: Cooked tuber is used as food, and rejuvenative [1–40] (Kumar et al., 2017)
D. bulbifera L.; Dioscoreaceae; Cl 790	*Verrilai valli*	Vine	60	0	40	No	
D. esculenta (Lour.) Burkill; Dioscoreaceae; Cl 791	*Mucilam valli*	Vine	65	0	35	No	
D. hispida Dennst; Dioscoreaceae; Cl 792	*Kavalakodi*	Vine	63	0	38	No	F: Fruits are edible[12,13,19,22,29,30,32,35] (Mallavadhani et al., 1998; Rauf et al., 2017)
D. oppositifolia L.; Dioscoreaceae; Cl 793	*Maruvalli*	Vine	68	0	33	No	
D. pentaphylla L.; Dioscoreaceae; Cl 794	*Kattuvalli kalangu*	Vine	78	0	23	No	
Diospyros ebenum J.Koenig ex Retz; Ebenaceae; Cl 795	*Karunkali*	Tree	100	0	0	No	F: Fruits are edible[12,13,19,22,29,30,32,35] (Mallavadhani et al., 1998; Rauf et al., 2017)
D. melanoxylon Roxb; Ebenaceae; Cl 796	*Kattupala*	Tree	100	0	0	No	F: Fruits are edible [12,13,19,22,29,30,32,35] (Mallavadhani et al., 1998; Rauf et al., 2017) M: Leaves are used to cure stomach pain[7,11,25,28] (Mallavadhani et al., 1998; Rauf et al., 2017). Leaves are used to as regional cigarette for psychoactive effects [21,23,27,33] (Rathore, 1972)
D. montana Roxb.; Ebenaceae; Cl 797;	*Vakkanai, Vakkanathi*	Tree	100	0	0	No	F: Fruits are edible[12,13,19,22,29,30,32,35] (Mallavadhani et al., 1998; Rauf et al., 2017) M: Bark and stem are used to cure fractured bones, act as anticoagulant, and to relieve body pain[11,18,19,21,23,25] (Mallavadhani et al., 1998; Rauf et al., 2017)
Dodonaea angustifolia L.f.; Sapindaceae; Cl 798	*Virali*	Shrub	0	93	8	No	M: Leaves are used as wound healing, relieves body pain, anti-inflammatory. Pregnancy abortive agent, cleanse the womb[1,2,5,8,11,14,17,18,21,24,26, 37,38] (Getie et al., 2003; van Heerden et al., 2000)
D. viscosa (L.) Jacq; Sapindaceae; Cl 799	*Velari*	Shrub	0	93	8	No	

Table 1 (*continued*)

Species; Family; Voucher	Tamil name	Habit	Plants recognized as dioecious (%)			Gender preference	Specific use[a]
			Y	N	Uncertain		
Drypetes sepiaria (Wight & Arn.) Pax & K.Hoffm.; Putranjivaceae; Cl 800	*Kalvirai*	Tree	100	0	0	No	F: Fruits are edible[11–30] (Arinathan et al., 2007)
Embelia tsjeriam-cottam (Roem. & Schult.) A.DC.; Primulaceae; Cl 801	*Vaivilangam*	Shrub	78	0	23	No	M: Seeds are anthelmintic[1,4,5,9,16,19,21,27,34] (Venkatasubramanian et al., 2013)
Euphorbia tirucalli L.; Euphorbiaceae; Cl 802	*Tirukakalli*	Tree	0	100	0	No	M: Latex is used to cure neural dysfunction, joint pains, skin disease, and act as neural stimuli[2,3,4,5,11,13,17] (Bani et al., 2007)
Ficus hispida L.f.; Moraceae; Cl 803	*Peiatthi*	Tree	18	65	18	No	M: Fruits are eaten to cure male impotent and also to increase fertility [1,8,12,18,24,30,35] (Lansky et al., 2008)
Hydnocarpus pentandrus (Buch.-Ham.) Oken; Achariaceae; Cl 804	*Neeradimuthu*	Tree	70	23	8	No	F: Fruits are edible[3,8,32,35] (Sahoo et al., 2014) M: Leaves and seeds are used to cure skin diseases, chest pains, joint pains[1,33,34,37,38] (Sahoo et al., 2014)
Lannea coromandelica (Houtt.) Merr.; Anacardiaceae; Cl 805	*Odiyamaram*	Tree	58	35	8	No	M: Leaves and stem bark are used to cure fever, dysentery, and anti-inflammatory[7,9,15,28,33]
Mallotus philippensis (Lam.) Müll.Arg.; Euphorbiaceae; Cl 806	*Kamala, Manjanathi*	Tree	53	13	35	No	M: Leaves and stem bark are used to cure stomach ache. Fruits are used as antidiabetic[3,6,12,15,20,32,35,38,39]
Momordica dioica Roxb. ex Willd.;Cucurbitaceae; Cl 807	*Pakarkoti, Pakarkai*	Climber	23	73	5	No	F: Fruits are used as vegetable [1–40] (Talukdar and Hossain, 2014) M: Fruits are used as antidiabetic [1–40] (Talukdar and Hossain, 2014)
Myristica dactyloides Gaertn.;Myristicaceae; Cl 808	*Jathikai*	Tree	68	20	13	No	F: Mace and kernel is used in food[1–40] (Swetha et al., 2017)
Phoenix loureiroi Kunth; Arecaceae; Cl 809	*Malai eecham*	Tree	100	0	0	Yes	F: Fruits are edible[1–40], ♂ plant toddy is more vibrant than ♀ plant toddy[1,3,5,13,22,25,27,35,38,40] (Haynes and McLaughlin, 2000; Rhouma et al., 2010)
P. pusilla Gaertn.; Arecaceae; Cl 810	*Icham*	Tree	100	0	0	Yes	
P. sylvestris (L.) Roxb.; Arecaceae; Cl 81	*Icham*	Tree	100	0	0	Yes	
Piper betle L.; Piperaceae; Cl 812	*Vetrilai*	Climber	100	0	0	Yes	F: ♀ leaves are preferred[10,11,12,14,20,26,33,36] M: ♂ plant leaves are preferred to increase male potency, and ♀ plant leaves are prescribed to male and ♂ leaves to act as sexual stimuli[1,2,8,13,25,27,29,34,35,37,39,40]. (Sarkar et al., 2000) R: ♀ leaves are preferred in rituals[2,8,29,39]
Semecarpus anacardium L.f.; Anacardiaceae; Cl 813	*Senkottai*	Tree	78	0	23	No	M: Seeds are poisonous, cures muscle spasm, skin diseases, and act as wound healing[9,32,33,38] (Vijayalakshmi et al., 2000)
Streblus asper Lour.; Moraceae; Cl 814	*Kuttipilaa*	Tree	0	48	53	No	M: Leaves and stem bark are used to cure urinary infections[8,13] (Rastogi et al., 2006)
Tinospora cordifolia (Willd.) Miers; Menispermaceae; Cl 815	*Cintilikkoti*	Liana	38	0	63	Yes	M: Stem acts as antidiabetic, immunomodulatory[1,9,12,15,40], ♂ and ♀ roots are used in combination to treat menstrual disorders[2,8,16,19,21,39]. (Grover et al., 2000)

[a] Superscript numbers are the identifiers of informants as shown in Supplementary Data S3; F: Food, M: Medicine, O: Others, R: Ritual.

structured questionnaire aided by props consisting of live specimens and photo galleries of the selected 40 dioecious species (Table 1). Study participants were selected using the snowball sampling method (Berlin and Berlin, 2005), and we particularly focused on local people who are older than 40 years, regularly use plants for medicinal purposes, fuel-wood and non-timber forest product collectors, and plant harvesters. Sampling was initiated through the indication of community leaders. The semi-structured questionnaire assessed the informants' perception of dioecious plants, awareness about dioecious plants and, if aware, is their preference for choosing a specific gender of dioecious plants (Supplementary Data S2). Additional information such as the folk healers' perspective on gender in plants and its roles in their traditions were also recorded. The interviews were conducted in the informants' native language *Tamil*. Following the interviews, the plants mentioned during the interviews were collected and confirmed for identification. Prior to the ethnobotanical survey, the purpose of the study was explained to the informants and the consent to conduct the study was requested and agreed. The documented medicinal plants were collected and pressed for herbarium vouchers, and identified with the help of valid references. All collected specimens were vouchered and deposited in the FRLH-Herbarium and Raw Drug Repository of The Institute of Trans-Disciplinary Health Sciences and Technology, India (Table 1). Prior to the ethnobotanical data collection, ethical approval for this study was obtained from the National Biodiversity Authority, Government of India.

2.4. Plant gender and Ayurveda

Twenty Ayurvedic doctors, who were formally educated and qualified to practice Ayurveda, were interviewed using a semi structured questionnaire in order to gain insight into the concept of plant gender in Ayurveda and its literature. Before initiating the interview process, it was explained to the doctors that the biological classification of plants classifies plant gender on the basis of their floral sexual characters i.e. the presence or absence of the androecium and gynoecium.

3. Results and discussion

3.1. Indian dioecious plants

Among dioecious species sex ratios deviate from the mean, and species with a male bias are associated with long-lived growth forms (e.g., trees), biotic seed dispersal and fleshy fruits, whereas female bias is associated with herbaceous species, and abiotic pollen dispersal (Field et al., 2013). Plasticity in sex expression has also been reported for a number of species (Borges et al., 1997; Geetha et al., 2007; Mcarthur, 1977; Renner, 2014). In this study, out of 40 dioecious plants used in the ethnobotanical data collection, 31 plants belong to genera or families that are either strictly or completely dioecious (cf. *Dioscorea* and Menispermaceae). The sex ratio of these species is not well studied in the study area, but for example, *Mallotus philippensis* has been shown to be male-biased under low light environments and female-biased under more light environments in India (Pathak and Shukla, 2004). Biased sex ratios and plasticity in sex expression of a given dioecious plants might have a significant effect on informants observation and classification of a plant as male and female.

Supplementary Data S1 shows the list of dioecious plants that are documented for its medicinal values in folk medicine, Ayurveda, Siddha, Unani, and Sowa-Rigpa and it was found that 5–7% of medicinal plants in Indian systems of medicines are dioecious plants, and this estimate is congruent with the diversification rate of dioecious species in flowering plants (Käfer et al., 2014; Renner, 2014). Based on this, we propose that these lists of species harness a potential to be studied comparatively for their chemical composition between male and female plants and the pharmacological activities, and also provides a platform to document ethno-ecological knowledge, and traditional knowledge of

dioecious plants with special reference to its gender.

3.2. Traditional knowledge and plant gender preference

To elicit knowledge on dioecy, informants were explained the phenomenon of dioecy in flowering plants, as correct knowledge was decisive for the outcome of the survey. They were informed that male and female plants exist separately as individual plants, that male plants only bear flowers that will not yield fruits and seeds, whereas female plants bear flowers, fruits, and viable seeds. The existence of monoecious and bisexual plants was explained as well, and they were explained that if the same plant bears male and female flowers it is monoecious, and if the same flower contains both reproductive organs it is bisexual. Plants such as papaya, palm trees, coconut trees, pumpkin, and goose berries were given as examples to explain the reproductive systems of flowering plants before initiating the interview process. Table 1 shows the details of 40 dioecious plants used in the ethnobotanical study, and it was found that the informants were aware of existence of the dioecious nature of many plants. Out of 40 plants used in the study, informants recognized the phenomenon of dioecy in 31 species (Table 1), and no significant variation was found between the 33 male and 7 female informants about their knowledge on the existence of dioecious species and the number of dioecious species reported for usages. Therefore male and female informants were considered as one category of informants for further analysis (Supplementary Data S3). However, Table 2 shows a significant variation among the age groups of informants. The informants below the age 50 had less knowledge on dioecious species, and used less number of dioecious species. On the other hand, a linear growth was observed between the age groups for preferring any one gender of dioecious plants, while using the plants which suggests that the age older informants had better perception on gender of plants and their unique uses (Table 2).

A number of studies has documented the lack of traditional knowledge among younger people, and this has been attributed to the expansion of modern education, cultural change, and the influences of modernization (Srithi et al., 2009; Voeks and Leony, 2004). As a result of changing realities, traditional knowledge of medicinal plants that was once embedded in numerous indigenous cultures, is rapidly disappearing. It has been suggested that to avoid the loss of this intellectual heritage, it is necessary to either keep it alive, or at least to document and describe the traditional use of plants (Bussmann and Sharon, 2006).

Table 3 shows the overview of informants awareness, gender preference and the habit of the dioecious plants. Since, fruits being informed and considered as the main identity to distinguish male and female plants among the informants, it was observed that the visibility of fruit size, plant size and plant traits based uses of a particular plant determines the perception of a plant being male or female. For example, informants were unaware of dioecy for two shrubs (*Dodonaea angustifolia*, *Dodonaea viscosa*), two climbers (*Cocculus hirsutus*, *Cyclea peltata*), and two lianas (*Asparagus racemosus*, *Cissampelos pareira*) they are all sourced from the wild in the study area, but the useful part of these

Table 2
Total average of dioecious plants recognized, used, and preferred for its gender by different age group of informants.

Informant age cohorts	Total number of informants	Species recognized as dioecious by informants	Average no. of species reported for usages	Average no. of species preferred for gender
41–50	17	19.35	21.47	2.71
51–60	10	24.2	23.1	3.5
61–70	6	23.5	21.17	4.17
71–80	7	24.14	22.14	5.71

Table 3
Overview of dioecious plants grouped on their habit, and the informants awareness and gender preference.

Habit	Dioecious species	Species reported as dioecious by informants	Species preferred for specific gender by informants
Tree	21	19	10
Vine	7	6	0
Liana	5	3	2
Climber	4	2	1
Shrub	3	1	0

plants are not fruits or seeds. On the contrary, informants were aware of dioecy for *Celastrus paniculatus* (liana), and *Emebelia tsjeriam-cottam* (shrub), because the seeds are used as medicines from this plants, and informants were aware of a plant that did not produced seeds. Plant-based ecosystem services are crucial for satisfying human needs, and human utilization of a plant is highly influenced by its species traits. For example, humans have selected plant species with traits that maximize crop yield, such as large fruits or height, or large grain size (Díaz et al., 2013, 2011). Cámara-Leret et al. (2017) tested the relationship between plant traits and its perceived value by people through an inter-disciplinary perspective on the linkages between ecosystem services, human needs and species' traits. It was demonstrated that people preferentially use large, widespread species rather than small, narrow-ranged species, and that different traits are linked to different uses. For example, one would expect a species to possess traits that satisfy human basic needs such as food and health. Such traits are plant size, constantly high yielding subsistence, widespread and cost effective to gather, and in contrast a species trait have strong link to easy availability and weaker link to plant size for medicinal usages (Cámara-Leret et al., 2017).

3.3. Gender preference in food and medicinal usages

Table 1 shows the usages of plants under the categories of plants being utilized as food, medicine, rituals, and a category of others. Among the 40 dioecious species, informants have gender preference for 13 species (10 trees, 2 lianas, and 1 shrub), and it was found that the informants have better knowledge about the toddy (palm wine) prepared out of male and female palm trees (*Borassus flabellifer* and *Phoenix* species). Toddy is a traditional alcoholic drink prepared by the fermentation of sap or exudate collected by slicing off the tip of unopened flowers of palm trees (Davis and Johnson, 1987). Informants reported that male palms yield comparatively less toddy than female palms, and the former are in higher demand among consumers because it is believed to be more potent. In this study, we observed that this knowledge is particular to elder informants, the reason for this was that in mid-20th century in India due to the increasing demand of toddy's, it was reported that the toddy often was adulterated with chemical substances, such as chloral hydrate and diazepam, and the adulteration had detrimental health consequences for toddy consumers (Rao et al., 2004). Therefore, the consumption and sale of toddy were prohibited from time to time in India, and the production of Indian-made foreign liquor such as whisky and brandy was promoted through industrialization (Mahal, 2000).

It has been reported that harvesters in Nilgiri Biosphere Reserve, India were aware of male and female trees of *Canarium strictum*, and that resin yielding trees were female trees (Varghese and Ticktin, 2008). A similar case was observed in *Canarium strictum* in Kolli Hills and Servarayan Hills where the informants reported that male trees produce less resin than the female trees, and when inquired further about the quality variation between the two gender the informants did not comment on any quality variation in male and female plant resins, but informed about a general variation that based on the dryness of the

resin that the fragrance it produces varies. For example, resin composition of male and female trees of *Austrocedrus chilensis* (D. Don) Florin & Boutelje (Cupressaceae) is reported to vary between genders and during different seasons of the year (Olate et al., 2014).

Interestingly, for medicinal usages informants reported a gender preference for *Piper betle* and *Tinospora cordifolia*, and the usage was rather complex and dependent on spiritual beliefs and medication. For example, informants believe that the *Piper betle* leaves of any one gender can be used to balance the hormonal imbalance of people with transgender sign. i.e., if a man is showing a sign of woman, prescribing a male leaf extract along with goat or sheep milk may cure the illness and vice versa. Similarly male plant leaves are prescribed to woman, and female plant leaves are prescribed to men with the purpose to act both as a sexual stimuli and to foster a good relationship between men and women. The informants reported male leaves as harder to chew than the female leaves, therefore female leaves are prepared to make paan (paan is a combination of betle leaves with areca nut or tobacco, chewed for its stimulant and psychoactive effects). However, when enquired about the taxonomic identity of male and female *Piper betle* leaves, it was found that informants segregate male and female leaves based on the venation pattern and number of veins in a leaf, i.e., the harder the venation pattern, and a minimum of 5 veins in a leaf is believed to be male leaf, and the softer venation pattern and less than 5 veins in a leaf is a female leaf. In India, despite its availability in the wild, *Piper betle* is vegetatively propagated for cultivation and no flowering is observed in the subtropics due to the lack of inductive photoperiods. The female plants rarely produce any flower or fruit in the Indian climate (Bajpai et al., 2012; Guha, 2006). Despite the absence of flower and fruiting to identify male and female *Piper betle*, sexual dimorphism for leaf character was reported in terms of length and breadth ratio of leaves. Male leaves are reported to be narrowly ovate with 1.84 ± 0.21 length: breadth ratio and female leaves are cordate or ovate to round leaves with 1.26 ± 0.13 length: breadth ratio. Leaves of the female plants are mostly pungent and male plants are less pungent (Krishnamurthy et al., 2008). However the congruency between folk healers identification, and biological identification of male and female *Piper betle* is yet to be documented. Jing and Coley, 1990 have reported that male and female trees of *Acer negundo* (Aceraceae) could be distinguished from one another solely based on leaf characters, and the largest difference between the sexes was the toughness of leaves. Leaves from female trees were on average tougher than those from male trees, and suggests that male trees commonly suffered greater herbivory than females due to toughness of leaves. Sexual dimorphism in vegetative growth for several dioecious plants were also reported (Jing and Coley, 1990).

3.4. Timber plants and gender preference

Table 4 shows the plants used for its timber and preferential gender usages. Out of 21 tree species used in the study, informants have reported 9 species for various construction purposes, and among these 6 species are preferred based on the gender (Table 4). Timber of male palm trees (*Borassus flabellifer* and *Phoenix* species) and *Drypetes sepiaria* is preferred for construction purposes such as houses, huts and furnitures because it is believed that male plants have expected size and more durable timber than female trees. On the contrary, female plant timber of *Diospyros ebenum* is preferred over male plants and it is believed that carving in male plant timber is tough. Informants preference on one gender in timber could be explained with plant resource allocation theory that the male plants comparatively allocates more resource to vegetative growth than the female plants (Obeso, 2002). Obeso (1997) have reported that mean annual tree-ring width of *Ilex aquifolium* L. was greater in males than in females for a 30-year period and that the male plants grew more than females. Similarly, male trees of *Bursera morelensis* Ramírez, and *Dacryodes excelsa* Vahl were significantly taller and larger than female trees (Forero-Montaña et al.,

Table 4
Timber species with preferential gender usage.

Species	Preferential gender usages[a]
Borassus flabellifer L.	♂ timber is preferred for construction [4,7,11,16,19,21,27,30,34]
Diospyros ebenum J.Koenig ex Retz.	♀ timber is preferred for construction [4,7,11,18,25,28,30,32,35,38]
Drypetes sepiaria (Wight & Arn.) Pax & K.Hoffm.	♂ timber is preferred for construction [13,15,16,18,22,23,26]
Lannea coromandelica (Houtt.) Merr.	♂ is preferred to make wooden vessels (mara-kuduvai) to farm animals[12,13,15]. ♂ preferred to be grown in farm land in order to avoid seedings [2,6,7,9,14,17,22,28,33,35]
Myristica dactyloides Gaertn.	♂ preferably cut for firewood [1,2,3,7,10]
Phoenix loureiroi Kunth	♂ timber is preferred for construction [5,6,12,13,17,24,28,33,40]
P. pusilla Gaertn.	
P. sylvestris (L.) Roxb.	

[a] Superscript numbers are the identifiers of informants as shown in Supplementary Data S3.

2010; Pavón and de de Luna Ramírez, 2008). On the contrary male and female trees belonging to 16 species of Myristicaceae and *Cecropia schreberiana* Miq. showed no differences in annual growth rates implying that females can compensate the higher cost of reproduction (Forero-Montaña et al., 2010; Queenborough et al., 2007).

On the other hand informants reported that male plants of *Myristica dactyloides* are selectively chosen for fire wood considering that it has no other benefits for them. Similar information was documented for *Carica papaya* that the informants do not prefer the male plants to be grown in their garden since it yields no fruits to them. Selective logging is reported to be the far most common management strategies to exploit commercial timber trees in tropical regions (Putz et al., 2012), and woody plants are especially vulnerable due to selective logging, given their economic value as timber and their long regeneration time (Martínez-Garza and Howe, 2003). Thus, selective logging and economic value increases the threat to dioecious taxa because of an underlying correlation between woodiness and dioecy (Martínez-Garza and Howe, 2003). Among the threatened plants included in the IUCN Red List of Threatened Species, woody growth habit of dioecious species is contributing to the higher risk of extinction (Vamosi and Vamosi, 2005). Any anthropogenic activity that modifies the male–female distance, sex ratio, plant size and pollinator abundance or behavior could affect the long-term viability of dioecious plants, and endangers the species (Somanathan and Borges, 2000).

Apart from dioecious plants, informants had knowledge about the occurrence of monoecious plants (i.e., with separate male and female flowers on the same individual plant), especially about *Cocos nucifera* (coconut), and *Cucurbita* species (pumpkins). Informants aware of male flowers in coconut tree and pumpkins which will not bear fruits, and few informants have informed that male coconut flowers can be used as medicine to increase fertility for both men and women. On the other hand, the female informants specified their tradition of using male flowers of pumpkins as an ornamental.

3.5. Vernacular names and plant gender

In this study, it was observed that based on different phenotypes, texture of different plant parts and morphological appearance of closely related species, people have the tendency to represent a particular plant species either as male or female by providing gender specific vernacular names, and such plant species are not dioecious. For example, the phenotypic variations in the flowers such as blue and white in *Clitoria ternatea* L., Leguminosae (*Shankapusphi*) is attributed to gender in Kolli Hills. They consider white flower phenotype as female (resembles the Indian female god Lakshmi) and blue flower phenotype as male (resembles the Indian male god Krishna). They prefer either one phenotype during the rituals and the choice of phenotype is based on the ritual process and whether the spiritual god is male or female. Similarly, *Mimosa pudica* L., Leguminosae (*thottasinungi*; *thottasuringi*) was also categorized into male and female based on the characteristic observations in movements in the pulvini of leaves, pinnae and pinnules

of the plants in response to touch. If the leaf movement of shrinkage starts from top to bottom basal end upon the touch, it is called male variety (*munsuringi*), and if the shrinkage starts from bottom to top, it is called female variety (*pinsuringi*). On the other hand, two closely related monoecious species in Moraceae, *Artocarpus hirsutus* Lam. (*ayanipala*; *kattupala*; *peyppala*) and *Artocarpus heterophyllus* Lam. (*palamaram*; *narpala*), are considered to be male and female plants respectively based on the fruit texture and timber quality. Informants reported that the fruit of *Artocarpus hirsutus* is watery and mushy in nature, whereas the fruit of *Artocarpus heterophyllus* is fibrous. In addition, it was reported that the timber of *Artocarpus hirsutus* was more durable than that of *Artocarpus heterophyllus*.

3.6. Plant gender and Ayurveda

The interaction with Ayurvedic doctors indicated that the Ayurvedic classical literature has no straight forward evidence on gender preference to prepare medicine or to treat illness. However the concept of plant gender is mentioned in Ayurvedic literatures such as *Charaka Samhita, Vrikshayurveda,* and *Rajanighantu*. For example, *Charaka Samhita* describes the morphological appearance, properties, and uses of a particular medicinal plant called *Kutaja*, and the plant is described as male plant (*Pum-Kutaja*) and female plant (*Stri-Kutaja*), and these two plants are decoded as *Holarrhena pubescens* Wall. ex G.Don (Syn. *Holarrhena antidysenterica* (Roth) Wall. ex A.DC.) and *Wrightia tinctoria* R.Br. both belonging to the Apocynaceae family (Samhita, 2001). These two species are not biologically dioecious. Therefore, it appears that the concept of gender differentiation in *Charaka Samhita* for *Kutaja* is not based on the floral sexual characters of the plants, rather based on the morphological appearance and properties of the plants. On the contrary, *Vrikshayurveda* and *Rajanighantu* describe the concept of reproductive morphology and sexual differentiation of plants (Prasad and Narayana, 2007; Sengupta, 2010). For example, *Rajanighantu* mentions the existence of male and female individuals of a dioecious species plant called *Ketaki* (*Pandanus odoratissimus* L.f. (Syn. of *Pandanus odorifer* (Forssk.) Kuntze) (Adkar and Bhaskar, 2014), but includes no indication on gender preferential usage.

4. Conclusions

During the last century, substantial ethnobotanical knowledge has been documented, and ethnobotanical studies have evolved to demonstrate the importance of traditional ecological knowledge to livelihoods around the globe, but also highlighted the rapid rate at which knowledge is being forgotten and lost. From this study, we identified the existence of a significant knowledge gap in ethnobotanical and ethnopharmacological literature on traditional knowledge of dioecious plants. Hence, an explorative study was conducted, and from this study it is evident that people have traditional knowledge on gender of plants and preferential usages towards one gender for some species. Based on this, we propose that researchers conducting an ethnobotanical and

ethnopharmacological study should consider documenting traditional knowledge on sexual systems of plants, and test the existence of gender specific usages in their conceptual framework and hypothesis testing. The incorporation of such concepts could provide new dimensions of scientific knowledge with potential implications to conservation biology, chemical ecology, ethnoecology and drug discovery.

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Author contributions

GSS, KR, BSP, HdB and HW synthesized the study concept and designed the study methodology. GSS with the guidance of KR conducted the field study and collected the data. GSS, KR, BSP, HdB, and HW analyzed and interpreted the data. GSS wrote the manuscript, and all authors have contributed to the preparation and finalization of the article. All authors have read and approved the final version of the manuscript.

Conflicts of interest

The authors have no conflicts of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2018.04.011.

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Graphical abstract
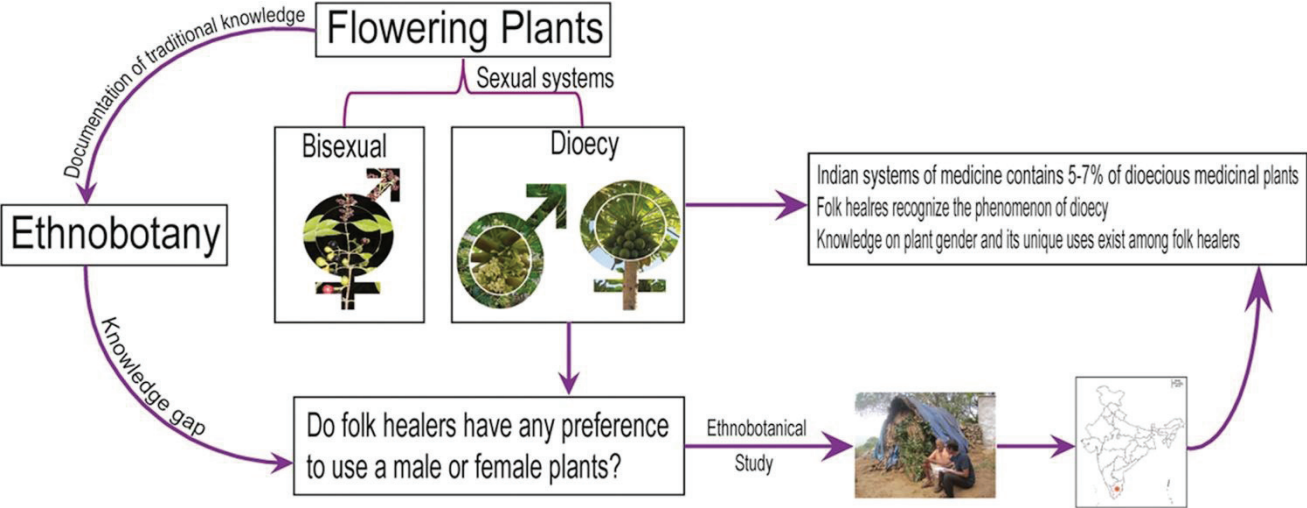
Ethnobotany of dioecious species: Traditional knowledge on dioecious plants in India.

Gopalakrishnan Saroja Seethapathy, Kaliamoorthy Ravikumar, Berit Smestad Paulsen, Hugo J. de Boer and Helle Wangensteen.

Supplementary Data

Supplementary Data S1. List of possible dioecious plants used in Indian systems of codified and non-codified medicine.

Supplementary Data S2. Study questionnaire on ethnobotany of dioecious plants.

Supplementary Data S3. Background details of informants, their knowledge on dioecious plants, and the category of preference for plant gender.

Dioecious plants	Family	Indian folk medicine	Ayurveda	Siddha	Unani	Sowa-Rigpa
Acalypha alnifolia Klein ex Willd.	Euphorbiaceae	+				
Acalypha hispida Burm.f.	Euphorbiaceae	+		+		
Actinodaphne angustifolia Nees	Lauraceae	+		+		
Actinodaphne dichotoma Florsk.	Lauraceae	+				
Actinodaphne hookeri Meisn.	Lauraceae	+		+		
Actinodaphne obovata (Nees) Blume	Lauraceae	+				
Aerva javanica (Burm.f.) Juss. ex Schult	Amaranthaceae	+	+	+		
Aerva lanata (L.) Juss.	Amaranthaceae	+	+	+		
Aerva tomentosa Forssk.	Amaranthaceae	+	+	+		
Agrostistachys indica Dalzell.	Euphorbiaceae	+				
Agrostistachys meeboldii Pax & K.Hoffm.	Euphorbiaceae	+				
Ailanthus altissima (Mill.) Swingle	Simaroubaceae	+				
Ailanthus excelsa Roxb.	Simaroubaceae	+	+	+		
Ailanthus glandulosa Desf.	Simaroubaceae	+				
Ailanthus triphysa (Dennst.) Alston	Simaroubaceae		+			
Alchornea rugosa (Lour.) Muell-Arg.	Euphorbiaceae	+				
Alchornea tiliaeafolia Muell-Arg.	Euphorbiaceae	+				
Amyris gileadensis L.	Burseraceae	+			+	
Anamirta cocculus (L.) Wight & Arn.	Menispermaceae	+	+	+	+	

Species	Family					
Antidesma acidum Retz.	Phyllanthaceae	+				
Antidesma alexiteria L.	Phyllanthaceae	+				
Antidesma bunius (L.)Spreng.	Phyllanthaceae	+		+		
Antidesma diandrum Roxb.	Phyllanthaceae	+				
Antidesma ghaesembilla Gaertn.	Phyllanthaceae	+				
Antidesma menasu (Tul) Mull.Arg.	Phyllanthaceae	+		+		
Antidesma zeylanicum Lam.	Phyllanthaceae	+				
Aphanamixis polystachya (Wall.) R. Parker	Meliaceae			+		
Aruncus dioicus (Walter) Fernald	Rosaceae	+				
Balanophora dioica R.BR.	Balanophoraceae	+				
Balanophora fungosa Subsp. indica (Arn.) Hans.	Balanophoraceae			+		
Balanophora involucrata Hook.f.	Balanophoraceae	+				
Balanophora polyandra Griff.	Balanophoraceae	+				
Baliospermum axillare Blum	Balanophoraceae	+	+	+	+	+
Baliospermum calycinum Muell.	Balanophoraceae		+	+		
Bauhinia malabarica Roxb.	Leguminosae	+	+	+		
Bauhinia racemosa Lam.	Leguminosae	+	+	+	+	
Bauhinia variegata L.	Leguminosae	+	+	+		
Bischofia javanica Blume	Phyllanthaceae	+		+		
Blyxa octandra (Roxb.) Planch. ex.Thwaites	Hydrocharitaceae		+			
Borassus flabellifer L.	Arecaceae	+	+	+	+	+
Borassus flabelliformis L.	Arecaceae	+	+	+	+	+
Bridelia crenulata Roxb.	Phyllanthaceae	+				
Canarium bengalense Roxb.	Burseraceae	+				
Canarium commune L.	Burseraceae	+				
Canarium euphyllum Kurz	Burseraceae	+		+		
Canarium resiniferum Bruce ex King	Burseraceae	+		+		

Species	Family					
Canarium strictum Roxb.	Burseraceae	+	+	+	+	
Canarium vulgare Leenh.	Burseraceae	+	+		+	
Cannabis indica L.	Cannabaceae	+	+	+	+	+
Cannabis sativa L.	Cannabaceae	+	+	+	+	+
Cannabis sativa L.	Cannabaceae					+
Carica candamarcensis Hook.f.	Caricaceae	+	+	+		
Carica papaya L.	Caricaceae	+	+	+	+	
Carica papaya L.	Caricaceae				+	
Cassine glauca (Rottb.) Kuntze	Celastraceae	+	+	+	+	
Ceratonia siliqua L.	Leguminosae	+				
Chamaerops ritchieana Griff.	Arecaceae	+		+		
Cissampelos pareira L.	Menispermaceae	+	+	+	+	+
Claoxylon indicum Hassk.	Euphorbiaceae	+		+		
Claoxylon polot (Burm.f.) Merr.	Euphorbiaceae	+		+		
Coccinia cordifolia (L.)Cogn.	Cucurbitaceae	+	+	+	+	+
Coccinia glauca Savi	Cucurbitaceae	+	+	+		
Coccinia grandis (L.) Voight	Cucurbitaceae	+	+	+	+	+
Coccinia indica Wight & Arn.	Cucurbitaceae	+	+	+	+	+
Coccoloba uvifera L.	Cucurbitaceae	+				
Cocculus cordifolius (Willd.) DC.	Menispermaceae	+	+	+	+	+
Cocculus hirsutus (L.) DIELS	Menispermaceae	+	+	+	+	
Cocculus laurifolius DC.	Menispermaceae	+				
Cocculus leaeba DC.	Menispermaceae	+				
Cocculus macrocarpus W. & A. PRODR.	Menispermaceae	+				
Cocculus pendulus (JR. & G. FORST) DIELS	Menispermaceae	+				
Cocculus suberosus DC.	Menispermaceae	+	+	+	+	
Cocculus villosus DC.	Menispermaceae	+	+	+	+	
Codiaeum variegatum (L.) Rumph. ex A.Juss.	Euphorbiaceae	+				

Species	Family					
Cordia dichotoma Forst. f.	Boraginaceae	+	+		+	+
Coscinium fenestratum (Gaertn.) Coleb.	Menispermaceae	+	+	+		+
Ctenolepis cerasiformis (Stocks) C.B.Clarke	Cucurbitaceae	+	+			+
Cyclea arnotii Miers.	Menispermaceae	+	+	+		
Cyclea bicristata Griff.	Menispermaceae	+				
Cyclea burmanni Arn. ex Wight	Menispermaceae	+	+	+		
Cyclea fissicalyx Dunn	Menispermaceae	+				
Cyclea peltata (Lam.) Hook. f. & Thomson	Menispermaceae	+	+	+		
Daemonorops draco Blume	Arecaceae		+	+	+	
Daphniphyllum himalayense (Benth.) Müll.Arg.	Daphniphyllaceae	+	+			
Daphniphyllum neilgherrense (Wight) K.Rosenthal	Daphniphyllaceae	+	+			
Datisca cannabina L.	Datiscaceae	+	+			
Debregeasia longifolia (Burm.f.) Wedd.	Urticaceae	+	+	+		
Dendrocnide sinuata (Blume) Chew	Urticaceae	+	+	+		
Dimorphocalyx glabellus Thwaites	Euphorbiaceae	+	+			
Dioscorea aculeata L.	Dioscoreaceae	+	+	+		
Dioscorea alata L.	Dioscoreaceae	+	+	+		
Dioscorea belophylla (Prain) Voigt ex Haines	Dioscoreaceae	+	+	+		
Dioscorea bulbifera L.	Dioscoreaceae	+	+	+	+	+
Dioscorea crispata Roxb.	Dioscoreaceae	+	+	+	+	+
Dioscorea daemona Roxb.	Dioscoreaceae	+	+			
Dioscorea deltoidea Wall. ex Kunth	Dioscoreaceae	+				

Species	Family				
Dioscorea esculanta (Lour.) Burkill	Dioscoreaceae	+		+	
Dioscorea glabra Roxb.	Dioscoreaceae	+	+	+	
Dioscorea globosa Roxb.	Dioscoreaceae	+	+	+	
Dioscorea hamiltonii Hook.f.	Dioscoreaceae	+	+		
Dioscorea hirsuta Dennst.	Dioscoreaceae	+	+		
Dioscorea hispida Dennst.	Dioscoreaceae	+	+		
Dioscorea oppositifolia L.	Dioscoreaceae	+	+	+	
Dioscorea pentaphylla L.	Dioscoreaceae	+	+	+	
Dioscorea prazeri Prain & Burkill	Dioscoreaceae		+		
Dioscorea puber Blume	Dioscoreaceae		+		
Dioscorea purpurea Roxb.	Dioscoreaceae	+	+	+	
Dioscorea rubella L.	Dioscoreaceae	+	+	+	
Dioscorea sativa L.	Dioscoreaceae	+	+	+	+
Dioscorea tomentosa J.Koenig ex Spreng.	Dioscoreaceae	+			
Dioscorea triphylla L.	Dioscoreaceae	+	+	+	
Dioscorea wallichii Hokk.f.	Dioscoreaceae	+			
Diospyros bourdilloni Brandis	Ebenaceae	+			
Diospyros buxifolia (Blume) Hiern	Ebenaceae	+	+		
Diospyros candolleana Wight	Ebenaceae	+	+	+	
Diospyros chloroxylon Roxb.	Ebenaceae	+		+	
Diospyros cordifolia Roxb.	Ebenaceae	+			
Diospyros ebenum Koenig	Ebenaceae	+	+	+	
Diospyros embryopteris Pers.	Ebenaceae	+	+	+	
Diospyros exsculpta Buch.-Ham.	Ebenaceae		+	+	+
Diospyros ferrea (Wildd.) Batch. F.	Ebenaceae	+		+	
Diospyros glutinosa Koenig	Ebenaceae		+		
Diospyros insignis Thwaites	Ebenaceae	+			
Diospyros kaki L.f.	Ebenaceae	+		+	

Species	Family					
Diospyros lancaefolia Roxb.	Ebenaceae	+				
Diospyros lotus L.	Ebenaceae	+				
Diospyros malabarica (Desr.) Kostel.	Ebenaceae	+	+	+	+	
Diospyros melanoxylon Roxb.	Ebenaceae	+	+	+	+	
Diospyros montana Roxb.	Ebenaceae	+	+	+		
Diospyros oocarpa Thwaites	Ebenaceae		+			
Diospyros paniculata Dalz.	Ebenaceae	+	+	+		
Diospyros peregrina (Gaertn.) Gurke	Ebenaceae	+	+	+	+	
Diospyros quaesita Thwaites	Ebenaceae	+				
Diospyros racemosa Roxb.	Ebenaceae	+				
Diospyros sylvatica Roxb.	Ebenaceae	+				
Diospyros tomentosa Roxb.	Ebenaceae		+	+		+
Diospyros toposia Buch.-Ham.	Ebenaceae	+				
Diploclisia glaucescens (Blume) Diels	Menispermaceae	+		+		
Dodonaea viscosa (L.) Jacq.	Sapindaceae	+	+	+		
Drypetes confertiflora (Hook. F.) Pax & K.Hoffm.	Putranjivaceae	+				
Drypetes macrophylla (Blume) Pax & K.Hoffm	Putranjivaceae	+				
Drypetes roxburghii (Wall.) Hurus	Putranjivaceae	+	+	+		+
Drypetes zeylanica (Thwaites) Hurus.	Putranjivaceae	+	+			
Embelia ribes Burm.f.	Primulaceae	+	+	+	+	+
Embelia subcoriacea (C.B.Clarke.) Mez.	Primulaceae	+				
Embelia tsjeriam-cottam (Roem. & Schult.) A.DC.	Primulaceae	+	+	+		
Eurya acuminata DC.	Pentaphylacaceae	+				

Species	Family				
Eurya japonica Thunb.	Pentaphylacaceae	+			
Eurya nitida Koth.	Pentaphylacaceae	+			
Excoecaria acerifolia Didr	Euphorbiaceae	+			
Excoecaria agallocha L.	Euphorbiaceae	+	+		
Excoecaria crenulata Wight	Euphorbiaceae	+			
Ficus exasperata Vahl	Moraceae	+	+	+	
Ficus fulva Reinw. ex Blume	Moraceae	+		+	
Ficus heterophylla L. f.	Moraceae	+	+	+	
Ficus hispida L.f.	Moraceae	+	+	+	+
Ficus oppositifolia Roxb.	Moraceae	+	+	+	+
Ficus tinctorea Forst. f. Subsp. parasitica (Willd.) Corner	Moraceae		+	+	
Ficus tinctoria Forst.f.	Moraceae	+		+	
Fraxinus excelsior L.	Oleaceae	+			
Galium elegans Wall. ex Roxb.	Rubiaceae	+			
Garcinia atroviridis Griff. ex T.Anderson	Clusiaceae	+			
Garcinia cowa Roxb. ex Choisy	Clusiaceae	+	+		
Garcinia echinocarpa Thwaites	Clusiaceae	+			
Garcinia gummi-gutta (L.) Roxb.	Clusiaceae	+	+	+	
Garcinia indica (Dup.) Choisy	Clusiaceae	+	+	+	+
Garcinia kydia Roxb.	Clusiaceae	+	+		
Garcinia morella (Gaertn.) Desr.	Clusiaceae	+	+	+	+
Garcinia pedunculata Roxb.	Clusiaceae				+
Garcinia purpurea G.Don.	Clusiaceae	+	+		+
Garcinia rubro-echinata Kosterm.	Clusiaceae	+			
Garcinia spicata Hook.f.	Clusiaceae	+			
Garcinia talbotii Raizada ex Santapau	Clusiaceae	+			
Garcinia travancorica Bedd.	Clusiaceae	+			
Garcinia wightii T.Anderson	Clusiaceae	+			

Species	Family				
Garcinia xanthochymus Hook.f. ex T.Anderson	Clusiaceae	+	+	+	
Girardinia heterophylla (Vahl) Decne.	Urticaceae	+			+
Givotia rottleriformis Griff.	Euphorbiaceae	+		+	
Gynocardia odorata R.BR.	Achariaceae	+	+		+
Gynostemma pentaphyllum (Thunb.) Makino	Cucurbitaceae	+	+		
Hagenia abyssinica (Bruce ex Steud.) J.F.Gmel.	Rosaceae	+			
Halophila ovalis (R.Br.) Hook.f.	Hydrocharitaceae	+			
Hippophae rhamnoides subsp. *salicifolia* (D. Don) Servettaz	Elaeagnaceae	+			
Hippophae salicifolia D.Don	Elaeagnaceae	+			
Hippophae tibetana Schltdl.	Elaeagnaceae	+			
Hodgsonia macrocarpa (Blume) Cogn.	Cucurbitaceae	+			
Homonoia retusa (Graham ex Wight) Müll.Arg.	Euphorbiaceae	+			
Homonoia riparia Lour.	Euphorbiaceae	+	+	+	
Horsfieldia glabra (Reinw. ex Blume) Warb.	Myristicaceae	+	+		
Horsfieldia irya (Gaertn.) Warb.	Myristicaceae	+			
Horsfieldia kingii (Hook.f.) Warb.	Myristicaceae	+			
Hydnocarpus alpina Wight	Achariaceae	+		+	
Hydnocarpus anthelminthica Pierre ex Gagnep.	Achariaceae	+			
Hydnocarpus castanea Hook.f. & Thomson	Achariaceae	+			
Hydnocarpus inebrians Vahl	Achariaceae	+	+	+	
Hydnocarpus kurzii (King) Warb.	Achariaceae	+	+	+	

Species	Family					
Hydnocarpus laurifolia (Dennst.) Steum.	Achariaceae	+	+	+		
Hydnocarpus macrocarpa Warb.	Achariaceae	+				
Hydnocarpus octandra Thwaites	Achariaceae	+				
Hydnocarpus pentandra (Buch.-Ham.) Oken	Achariaceae	+	+	+		
Hydnocarpus venenata Gaertn.	Achariaceae	+		+		
Hydnocarpus wightiana Blume	Achariaceae	+	+	+		
Hydrilla verticillata (L.f.) Royle	Hydrocharitaceae	+		+		
Hydrocharis dubia (Blume) Backer	Hydrocharitaceae	+				
Ilex denticulata Wall. ex Wight	Aquifoliaceae	+				
Ilex godajam Colebr. ex Hook.f.	Aquifoliaceae	+				
Ilex paraguariensis A.St.Hil.	Aquifoliaceae			+		
Ilex wightiana Wall. ex Wight	Aquifoliaceae	+				
Jateorhiza palmata (Lam.) Miers	Menispermaceae	+	+	+		
Knema angustifolia (Roxb.) Warb.	Myristicaceae	+				
Knema attenuata (Wall.) Warn.	Myristicaceae	+		+		
Knema erratica (Hook. f. & Thomson) J. Sinclair	Myristicaceae	+				
Knema linifolia (Roxb.) Warb.	Myristicaceae	+				
Laurus cassia Burm.f.	Lauraceae	+		+		
Laurus cinnamomum (L.)	Lauraceae	+	+	+		+
Laurus nobilis L.	Lauraceae	+			+	
Lindera caudata (Nees) Hook. f.	Lauraceae	+	+			
Lindera neesiana (Wall. ex Nees) Kurz	Lauraceae	+				
Lindera pulcherrima (Nees) Hook. f.	Lauraceae	+				
Litsea chinensis Lam.	Lauraceae	+	+	+	+	
Litsea citrata Blume	Lauraceae	+	+			
Litsea coriacea Hook.f.	Lauraceae	+				

Species	Family					
Litsea cubeba (Lour.) Pers.	Lauraceae	+	+			
Litsea glutinosa (Lour.) C.B. Rob.	Lauraceae	+	+	+	+	
Litsea lancifolia (Roxb. ex Nees) Fern.-Vill.	Lauraceae	+				
Litsea monopetala (Roxb.) Pers.	Lauraceae	+	+	+		
Litsea polyantha Juss.	Lauraceae	+	+	+		
Litsea quinqueflora (Dennst.) Suresh	Lauraceae	+				
Litsea sebifera Pers.	Lauraceae	+	+	+	+	
Litsea stocksii Hook.f.	Lauraceae	+				
Litsea zeylanica Nees & T. Nees	Lauraceae	+				
Lodoicea callypige Comm. ex J.St.Hil.	Arecaceae	+				
Lodoicea maldivica (J.F.Gmel.) Pers.	Arecaceae	+	+	+	+	
Lodoicea seychellarum Labill	Arecaceae	+	+	+	+	
Luffa echinata Roxb.	Cucurbitaceae	+	+	+		+
Macaranga denticulata (Blume) Müll.Arg.	Euphorbiaceae	+				
Macaranga indica W.	Euphorbiaceae	+		+		
Macaranga nicobarica N.P.Balakr. & Chakrab.	Euphorbiaceae	+				
Macaranga peltata (Roxb.) Muell.-Arb.	Euphorbiaceae	+		+		
Macaranga roxburghii Wight	Euphorbiaceae	+		+		
Macaranga tanarius (L.) Müll.Arg.	Euphorbiaceae	+				
Macaranga tetracoccus (Roxb.) Kurz	Euphorbiaceae	+				
Maclura pomifera (Raf.) C.K.Schneid.	Moraceae	+				
Mallotus ferrugineus (Roxb.)	Euphorbiaceae	+				

Species	Family					
Müll.Arg.						
Mallotus peltatus (Geiseler) Müll.Arg.	Euphorbiaceae	+				
Mallotus philippensis (Lam.) Muell.-Arg.	Euphorbiaceae	+	+	+	+	
Mallotus repandus (Willd.) Mull.-Arg.	Euphorbiaceae	+	+	+		
Mallotus rhamnifolius (Willd.) Müll.Arg.	Euphorbiaceae	+				
Mallotus tetracoccus (Roxb.) Kurz	Euphorbiaceae	+				
Maytenus senegalensis (Lam.) Exell	Celastraceae	+	+	+		
Menispermum cocculus L.	Menispermaceae	+	+	+	+	
Menispermum columba Roxb.	Menispermaceae	+	+	+		
Menispermum cordifolium Willd.	Menispermaceae	+	+	+	+	+
Menispermum glabrum Burm.f.	Menispermaceae	+				
Momordica cochinchinensis (Lour.) Spreng.	Cucurbitaceae	+	+	+		
Momordica dioica Roxb. ex Willd.	Cucurbitaceae	+	+	+	+	
Momordica umbellata (Klein ex Willd.) Roxb.	Cucurbitaceae	+				
Morus acidosa Griff.	Moraceae	+	+			
Morus alba L.	Moraceae	+	+	+	+	
Morus indica L.	Moraceae	+	+			
Myrica esculenta Buch.-Ham.	Myricaceae	+	+	+	+	
Myristica argentea Warb	Myristicaceae	+	+			
Myristica dactyloides Gaertn.	Myristicaceae	+	+	+		
Myristica fragrans Houtt.	Myristicaceae	+	+	+	+	+
Myristica laurifolia Spruce ex A.DC.	Myristicaceae	+	+			
Myristica malabarica Lam.	Myristicaceae	+	+	+		

Species	Family					
Myristica moschata Thunb.	Myristicaceae	+	+	+	+	+
Myristica officinalis L.f.	Myristicaceae	+	+	+	+	+
Natsiatum herpeticum Buch.-Ham. ex Arn.	Icacinaceae	+				
Neolitsea cassia (L.) Kosterm.	Lauraceae	+				
Neolitsea chinensis (Gamble) Chun	Lauraceae	+				
Neolitsea scrobiculata (Meissner) Gamble	Lauraceae	+		+		
Neolitsea umbrosa (Nees) Gamble	Lauraceae	+				
Neolitsea zeylanica (Nees & T. Nees) Merr.	Lauraceae	+				
Nepenthes distillatoria Auct. ex Steud.	Nepenthaceae	+				
Nepenthes khasiana Hook.f.	Nepenthaceae	+				
Osyris quadripartita Salzm. ex Decne.	Santalaceae	+				
Ottelia alismoides (L.) Pers.	Hydrocharitaceae	+				
Pachygone ovata (Poir.) Diels	Menispermaceae	+				
Pachygone zeylanica Santapau & Wagh	Menispermaceae	+				
Pandanus fascicularis Lam.	Pandanaceae	+	+	+	+	
Pandanus foetidus Roxb.	Pandanaceae	+	+			
Pandanus furcatus Roxb.	Pandanaceae	+	+			
Pandanus kaida Kurz.	Pandanaceae	+	+			
Pandanus odoratissimus L.f.	Pandanaceae	+	+	+	+	
Pandanus tectorius Parkinson ex Du Roi	Pandanaceae	+	+	+	+	
Pandanus thwaitesii Martelli	Pandanaceae	+				
Pandanus unipapillatus Dennst.	Pandanaceae	+	+			
Passiflora assamica Chakrav.	Passifloraceae	+				
Passiflora edulis Sims	Passifloraceae	+				

Passiflora foetida L.	Passifloraceae	+	+	+		
Passiflora perpera Mast.	Passifloraceae	+				
Passiflora quadrangularis L.	Passifloraceae	+				
Pericampylus glaucus (Lam.) Merr.	Menispermaceae	+				
Pericampylus incanus (Colebr.) Miers ex Hook. f. & Thomson	Menispermaceae	+				
Phoenix acaulis Roxb.	Arecaceae	+	+			
Phoenix asperulatus Hutch.	Arecaceae	+	+			
Phoenix dactylifera L.	Arecaceae	+	+	+	+	
Phoenix farinifera Roxb.	Arecaceae	+		+		
Phoenix humilis (L.) Cav.	Arecaceae	+	+			
Phoenix loureirii Kunth	Arecaceae	+		+		
Phoenix paludosa Roxb.	Arecaceae	+	+			
Phoenix pusilla Gaertn.	Arecaceae	+	+			
Phoenix sylvestris Roxb.	Arecaceae	+	+	+		+
Piper barberi Gamble.	Piperaceae	+	+			
Piper betle L.	Piperaceae	+	+	+	+	+
Piper betleoides C. DC.	Piperaceae	+	+			
Piper longum L.	Piperaceae	+	+	+		+
Piper nigrum L.	Piperaceae	+	+	+	+	+
Piper pedicellatum C. DC.	Piperaceae	+	+			
Piper sarmentosum Roxb.	Piperaceae	+	+			
Piper sylvaticum Roxb.	Piperaceae		+	+		
Piper thomsoni (C. DC.) Hook.f.	Piperaceae	+	+			
Piper wallichii (Miq.) Hand-Mazz	Piperaceae	+	+	+		+
Pipturus velutinus (Decne.) Wedd.	Urticaceae	+				
Poikilospermum suaveolens (Blume) Merr.	Urticaceae	+				
Polyscias fruticosa (L.) Harms	Araliaceae	+				
Populus alba L.	Salicaceae	+				

Populus caspica (Bornm.) Bornm.	Salicaceae	+			
Populus ciliata Wall. Ex Royle	Salicaceae	+			
Populus euphratica Oliv.	Salicaceae	+			
Populus nigra L.	Salicaceae	+			
Protium serratum (Wall. Ex Colebr.) Engl.	Burseraceae	+	+		
Pseuduvaria prainii (King) Merr.	Annonaceae	+			
Rhamnus dahuricus P. Lawson	Rhamnaceae	+			
Rhus chinensis Mill	Anacardiaceae	+		+	
Rhus hookeri K.C. Sahni & Bahadur	Anacardiaceae	+			
Rhus punjabensis J.L. Stewart ex Brandis	Anacardiaceae	+			
Rhus succedanea L.	Anacardiaceae	+	+	+	+
Rhus wallichii Hook.f.	Anacardiaceae	+			
Ribes orientale Desf.	Grossulariaceae	+			
Salix acmophylla Boiss.	Salicaceae	+			
Salix acutifolia Willd.	Salicaceae	+			
Salix alba L.	Salicaceae	+			
Salix babylonica L.	Salicaceae	+			
Salix caprea L.	Salicaceae	+	+	+	+
Salix daphnoides Vill.	Salicaceae	+			
Salix elegans Wall.	Salicaceae	+			
Salix pychnostachya Andersson.	Salicaceae	+			
Salix tetrasperma Roxb.	Salicaceae	+	+	+	+
Sarcostigma kleinii Wight & Arn.	Icacinaceae	+	+		
Sassafras albidum (Nutt.) Nees	Lauraceae	+			
Sassafras officinale Nees.	Lauraceae	+			
Schinus molle L.	Lauraceae	+			
Sclerocarya birrea (A.Rich.) Hochst.	Anacardiaceae	+			

Species	Family				
Scleropyrum wallichianum Arn.	Anacardiaceae	+			
Semecarpus anacardium L. F.	Anacardiaceae	+	+	+	+
Skimmia anquetilia N.P.Taylor & Airy Shaw	Rutaceae	+			
Skimmia arborescens T. Anderson ex Gamble	Rutaceae	+			
Skimmia laureola Franch.	Rutaceae	+			
Smilax aspera L.	Smilacaceae		+	+	
Smilax china L.	Smilacaceae	+	+	+	+
Smilax glabra Roxb.	Smilacaceae	+	+		
Smilax glaucophylla Klotzsch	Smilacaceae	+			
Smilax lanceifolia Roxb.	Smilacaceae		+		
Smilax macrophylla Poepp. ex A.DC.	Smilacaceae	+	+	+	
Smilax ocreata A.DC.	Smilacaceae	+			
Smilax ovalifolia Roxb.	Smilacaceae	+	+		
Smilax parvifolia Wall.	Smilacaceae	+			
Smilax perfoliata Lour.	Smilacaceae	+			
Smilax prolifera Roxb.	Smilacaceae	+			
Smilax pseudo-china Willd.	Smilacaceae	+		+	
Smilax wightii L.	Smilacaceae	+			
Smilax zeylanica L.	Smilacaceae	+	+	+	
Spinifex littoreus (Burm.f.) Merr.	Amaranthaceae	+	+	+	+
Spinacia oleracea L.	Poaceae	+		+	
Stephania glabra (Thung.) Miers.	Menispermaceae		+		
Stephania glandulifera Miers.	Menispermaceae	+			
Stephania hernandifolia (Willd.) Walp.	Menispermaceae	+	+	+	
Stephania japonica (Thunb.) Miers Var. discolour (Blume.) Forman	Menispermaceae	+	+	+	
Stephania japonica (Thunb.) Miers	Menispermaceae	+	+	+	

Species	Family					
Stephania japonica (Thunb.) Miers Var. japonica	Menispermaceae	+	+	+		
Stephania rotunda Lour	Menispermaceae	+				
Stephania wightii Dunn	Menispermaceae	+				
Streblus asper Lour	Moraceae	+	+	+		
Streblus taxoides (Roth) Kurz	Moraceae	+				
Suregada multiflora (A.Juss.) Baill.	Euphorbiaceae	+				
Tetrameles nudiflora R. BR.	Tetramelaceae	+		+		
Tetrastigma lanceolarium (Roxb.) Planch.	Vitaceae	+				
Tetrastigma leucostaphylum (Dennst.) Alston	Vitaceae	+		+		
Tetrastigma planicaule (Hook. f.) Gagnep.	Vitaceae	+				
Tetrastigma serrulata (Roxb.) Planch.	Vitaceae	+				
Thonningia axillaris (L.) Kuntze	Balanophoraceae	+		+		
Tiliacora acuminata Miers	Menispermaceae	+		+		
Tiliacora racemosa Colebr	Menispermaceae	+		+		
Tinospora andamanica Diels	Menispermaceae	+				
Tinospora cordifolia (Willd.) Miers	Menispermaceae	+	+	+	+	+
Tinospora crispa (L.) Hook. f. & Thomson	Menispermaceae	+				
Tinospora malabarica (Lam.) Hook. F. & Thoms	Menispermaceae	+	+	+		
Tinospora sinensis (Lour.) Merr.	Menispermaceae	+	+	+		
Tinospora tomentosa (Colebr.) Hook. f. & Thoms.	Menispermaceae	+	+	+		
Trachycarpus martianus (Wall. ex Mart.) H.Wendl.	Arecaceae	+				
Trewia nudiflora L.	Euphorbiaceae	+		+		

Species	Family					
Trewia polycarpa Benth.	Euphorbiaceae	+				
Trichosanthes bracteata (Lam.) Voigt	Cucurbitaceae	+	+	+	+	
Trichosanthes dioica Roxb.	Cucurbitaceae	+	+	+		
Trophis aspera Retz.	Moraceae	+	+	+		
Urtica ardens Link	Urticaceae	+				
Urtica dioica L.	Urticaceae		+			
Urtica hyperborea Jacq. ex Wedd.	Urticaceae	+				
Valeriana jatamansi Jones	Caprifoliaceae	+	+	+	+	+
Valeriana wallichii DC.	Caprifoliaceae	+	+	+	+	+
Vallisneria spiralis L.	Hydrocharitaceae	+		+		
Viscum album L.	Santalaceae	+	+		+	
Vitis heyneana Roem. & Schult.	Vitaceae	+		+		
Xylosma longifolium Clos	Salicaceae	+				
Zamonia indica L.	Cucurbitaceae	+	+			
Zanthoxylum acanthopodium DC.	Rutaceae		+	+	+	
Zanthoxylum armatum DC.	Rutaceae		+	+		+
Zanthoxylum rhetsa (Roxb.) DC.	Rutaceae	+	+	+		

+ indicates the presence of that particular species in different Indian traditional systems of medicine.

Supplementary Data S2. Study questionnaire on ethnobotany of dioecious plants.

Questionnaire deals with

Dioecious plants Identification, Preference and uses by Ethnic People

Form 1

Informants' consent for the participation in the study:

I…………………………………(name of informant) hereby give my full consent and conscious to participate in this study and declare that to the best of my knowledge the information that I have provided are true, accurate and complete. I am also aware that this conversation is being recorded; I have also been foretold that the information from this study will be shared with them   ( ) Prefer not to be recorded.

Date…………………………(Signature/Thumb impression of Informant)

1. Details of Location

1.   Code of the Community Based organization:_____

2.   Name of the Village:_____

3.   Name of the Panchayat:_____

2. Details of the interviewee

1.   Name:_____

2.   Gender: (M) (F)   Age:_____

3.   Tribe:_____

4.   Occupation:_____ & Education_____

3. Existing Knowledge on Medicinal Plants

1. Do you use locally available plants to treat/prevent any illnesses?

     Yes ( )   No ( )

2.) For how many years have you been practicing traditional medicine?

3.) Are you specialized in treating any particular illnesses?

_____

4. Knowledge on Dioecy Plants

1.) Have you observed a general phenomenon that plants can exist as male plant and female plant?
i.e., that a group of plants bear seeds/fruits and other from same species/kind do not.

Yes ( )   **No ( )**

2.) If yes, Could you list the species name? For example: Papaya, Panai, Jathikai,

2.1.) after writing the plant names, ask. Do you use these plants for any purposes?

2.1.1. If yes, fill in Table 1 general uses respective to their plant names

2.1.2) after writing the uses, ask. Would you prefer to use any one gender in the listed plants?

2.1.3) Ask for parts used, then availability, plant type and if there is anything else of importance for the species?

Table 1.

S.no	Plant names (Local)	General Uses[1] (M,F,T,A,H,O)	Gender[2] (M,F,B,N)	Parts used	Availability[3] (CL,CF,R,CR)	Plant type[4] (H,S,C,V,T)	Remarks
1							
2							
3							
4							
5							
6							
7							
8							
9							

1 M=Medicine; F=Food; T=Timber; A=Agriculture; H=Handcraft; O=Other-specify)

2 M=Male; F=Female, B=Both (both plants are equally potential), N=No (No consideration of dioecy)

3 CL=Common local; CF=Common Forest; R= Rare; VE=Very Rare

4 H= Herb; S=Shrub; C= Climber; V=Vines; T=Tree

5. Collection, uses and preparation methods for medicinal plants

1) Ask for information about the plants used for medicinal purposes in Table 2

Table 2

S.no	Parts collected and used	Collection strategies	Mode of preparing the medicines	Name of disease(s) treated/ Uses	Mode of administration and Dosage	Any special procedures (i.e.., a plant is poisonous or to improve efficacy)

6. Live specimens and photo guided interview

1.) If a person does not report any differentiation between male and female plants, then discuss using the plant photos or herbariums or collected live specimens!

Table 3

S.no	Plant names	Local Name	General Uses	Gender* (M,F,B,N)	Parts used	Availability** (CL,CF,R,CR)	Plant type*** (H,S,C,V,T)	Remarks

Supplementary Data S3. Background details of informants, their knowledge on dioecious plants, and the category of preference for plant gender.

Informants identity number	Gender and age of informants	Area of the study	Total number of species recognized as dioecious	Total number of dioecious species reported for usages	Total number of dioecious species preferred for its gender	Reported dioecious species for preference
1	Male, 75	Kolli Hills	25	28	8	*Anamirta cocculus* *Borassus flabellifer* *Canarium strictum* *Myristica dactyloides* *Phoenix loureiroi* *Phoenix pusilla* *Phoenix sylvestris* *Piper betle*
2	Female, 56	Kolli Hills	25	24	6	*Aphanamixis polystachya* *Canarium strictum* *Lannea coromandelica* *Myristica dactyloides* *Piper betle* *Tinospora cordifolia*
3	Male, 48	Kolli Hills	15	22	6	*Borassus flabellifer* *Canarium strictum* *Myristica dactyloides* *Phoenix loureiroi* *Phoenix pusilla* *Phoenix sylvestris*
4	Male, 70	Kolli Hills	22	22	4	*Anamirta cocculus* *Borassus flabellifer* *Canarium strictum* *Diospyros ebenum*
5	Male, 57	Kolli Hills	19	23	4	*Canarium strictum*

No.	Respondent	Location			Plants used
					Phoenix loureiroi Phoenix pusilla Phoenix sylvestris
6	Male, 45	Kolli Hills	14	19	5	Canarium strictum Lannea coromandelica Phoenix loureiroi Phoenix pusilla Phoenix sylvestris
7	Male, 50	Kolli Hills	19	24	6	Anamirta cocculus Borassus flabellifer Canarium strictum Diospyros ebenum Lannea coromandelica Myristica dactyloides
8	Female, 48	Kolli Hills	22	25	4	Aphanamixis polystachya Canarium strictum Piper betle Tinospora cordifolia
9	Female, 42	Kolli Hills	22	23	2	Canarium strictum Lannea coromandelica
10	Male, 47	Kolli Hills	15	18	3	Canarium strictum Myristica dactyloides Piper betle
11	Male, 55	Sittlingi Valley	25	24	3	Borassus flabellifer Diospyros ebenum Piper betle
12	Male, 42	Sittlingi Valley	18	26	5	Lannea coromandelica Phoenix loureiroi Phoenix pusilla Phoenix sylvestris Piper betle
13	Male, 80	Sittlingi Valley	26	28	9	Anamirta cocculus Aphanamixis polystachya

						Species
						Borassus flabellifer *Drypetes sepiaria* *Lannea coromandelica* *Phoenix loureiroi* *Phoenix pusilla* *Phoenix sylvestris* *Piper betle*
14	Male, 56	Sittlingi Valley	26	25	3	*Anamirta cocculus* *Lannea coromandelica* *Piper betle*
15	Male, 48	Sittlingi Valley	23	21	2	*Drypetes sepiaria* *Lannea coromandelica*
16	Male, 42	Sittlingi Valley	19	19	3	*Borassus flabellifer* *Drypetes sepiaria* *Tinospora cordifolia*
17	Male, 60	Sittlingi Valley	28	21	5	*Borassus flabellifer* *Lannea coromandelica* *Phoenix loureiroi* *Phoenix pusilla* *Phoenix sylvestris*
18	Male, 50	Sittlingi Valley	26	22	2	*Diospyros ebenum* *Drypetes sepiaria*
19	Male, 47	Sittlingi Valley	19	21	2	*Borassus flabellifer* *Tinospora cordifolia*
20	Male, 48	Sittlingi Valley	18	19	1	*Piper betle*
21	Male, 57	Sittlingi Valley	25	24	2	*Borassus flabellifer* *Tinospora cordifolia*
22	Male, 70	Sittlingi Valley	24	20	6	*Borassus flabellifer* *Drypetes sepiaria* *Lannea coromandelica* *Phoenix loureiroi* *Phoenix pusilla* *Phoenix sylvestris*

No.	Respondent	Location			No.	Plants used
23	Male, 61	Sittlingi Valley	23	20	2	*Canarium strictum* *Drypetes sepiaria*
24	Male, 40	Sittlingi Valley	18	20	3	*Phoenix loureiroi* *Phoenix pusilla* *Phoenix sylvestris*
25	Male, 80	Sittlingi Valley	25	21	6	*Borassus flabellifer* *Diospyros ebenum* *Phoenix loureiroi* *Phoenix pusilla* *Phoenix sylvestris* *Piper betle*
26	Male, 45	Sittlingi Valley	19	20	2	*Drypetes sepiaria* *Piper betle*
27	Male, 80	Sittlingi Valley	26	20	5	*Borassus flabellifer* *Phoenix loureiroi* *Phoenix pusilla* *Phoenix sylvestris* *Piper betle*
28	Male, 65	Sittlingi Valley	25	20	5	*Diospyros ebenum* *Lannea coromandelica* *Phoenix loureiroi* *Phoenix pusilla* *Phoenix sylvestris*
29	Female, 60	Sittlingi Valley	25	23	2	*Aphanamixis polystachy* *Piper betle*
30	Male, 58	Servarayan Hills	25	21	2	*Borassus flabellifer* *Diospyros ebenum*
31	Male, 40	Servarayan Hills	18	19	1	*Anamirta cocculus*
32	Male, 50	Servarayan Hills	23	23	1	*Diospyros ebenum*
33	Male, 60	Servarayan Hills	21	21	6	*Canarium strictum* *Lannea coromandelica*

						Phoenix loureiroi *Phoenix pusilla* *Phoenix sylvestris* *Piper betle*
34	Female, 60	Servarayan Hills	24	21	2	*Borassus flabellifer* *Piper betle*
35	Male, 65	Servarayan Hills	24	25	6	*Diospyros ebenum* *Lannea coromandelica* *Phoenix loureiroi* *Phoenix pusilla* *Phoenix sylvestris* *Piper betle*
36	Male, 42	Servarayan Hills	16	20	2	*Canarium strictum* *Piper betle*
37	Female, 65	Servarayan Hills	23	20	2	*Canarium strictum* *Piper betle*
38	Male, 77	Servarayan Hills	22	22	5	*Borassus flabellifer* *Diospyros ebenum* *Phoenix loureiroi* *Phoenix pusilla,* *Phoenix sylvestris*
39	Female, 75	Servarayan Hills	23	18	2	*Piper betle* *Tinospora cordifolia*
40	Male, 78	Servarayan Hills	22	17	5	*Borassus flabellifer* *Phoenix loureiroi* *Phoenix pusilla* *Phoenix sylvestris* *Piper betle*

III

SCIENTIFIC REPORTS

OPEN

Authentication of *Garcinia* fruits and food supplements using DNA barcoding and NMR spectroscopy

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Garcinia L. (Clusiaceae) fruits are a rich source of (−)-hydroxycitric acid, and this has gained considerable attention as an anti-obesity agent and a popular weight loss food supplement. In this study, we assessed adulteration of morphologically similar samples of *Garcinia* using DNA barcoding, and used NMR to quantify the content of (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone in raw herbal drugs and *Garcinia* food supplements. DNA barcoding revealed that mostly *G. gummi-gutta* (previously known as *G. cambogia*) and *G. indica* were traded in Indian herbal markets, and there was no adulteration. The content of (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone in the two species varied from 1.7% to 16.3%, and 3.5% to 20.7% respectively. Analysis of ten *Garcinia* food supplements revealed a large variation in the content of (−)-hydroxycitric acid, from 29 mg (4.6%) to 289 mg (50.6%) content per capsule or tablet. Only one product contained quantifiable amounts of (−)-hydroxycitric acid lactone. Furthermore the study demonstrates that DNA barcoding and NMR could be effectively used as a regulatory tool to authenticate *Garcinia* fruit rinds and food supplements.

Globalization in the trade of herbal products and an expanding commodity market have resulted in widespread consumption of medicinal plants as drugs, cosmetics and food supplements, both in developing and developed countries[1,2]. Quality, safety and efficacy of herbal medicines are key requirements for public health and a major concern for regulatory authorities[3]. In India, herbal raw materials for the herbal industry are predominately harvested from wild populations[4]. Unsustainable harvest of medicinal plants from the wild can lead to severe ecological consequences, including reduced plant populations, habitat destruction, loss of genetic diversity, and local extinction of species[5]. India is the second largest exporter of medicinal plants and exports mainly in the form of dried plant products[4]. Several reports suggest that only 10% of medicinal plants traded in India are being cultivated[2,4]. Growing commercial demands for raw drug products increases the incentive for adulteration and substitution in the medicinal plants trade, and such adulteration can threaten consumer health, damage consumer confidence, and generally lower the trade value of such products[1].

Garcinia L. (Clusiaceae) is a genus of evergreen polygamous trees and shrubs comprising 400 species with a pantropical distribution[6]. Many *Garcinia* species in tropical Asia, Southern Africa and Northern Australia are locally used, and the edible fruits are of interest worldwide, as well as having potential implication on the economy of local communities[7]. Thirty-five species occur in India, of which 17 species are reported from the Western Ghats[7]. *Garcinia* fruits are widely collected and commercially exploited for their medicinal value, and the fruit rinds of *Garcinia gummi-gutta* (L.) Roxb. (syn. *Garcinia cambogia* (Gaertn.) Desr.) are traditionally used to treat constipation, piles, rheumatism, edema, irregular menstruation and intestinal parasites, and are also used as a food flavoring agent and preservative[8]. The fruit rinds of *Garcinia indica* (Thouars) Choisy are traditionally used to treat rheumatic pains, bowel complaints, hemorrhoids, ulcers, inflammations, sores, dermatitis, dysentery, to prevent hyperhidrosis, and are an important culinary agent, as well[9]. In India, *G. gummi-gutta* and *G. indica* are

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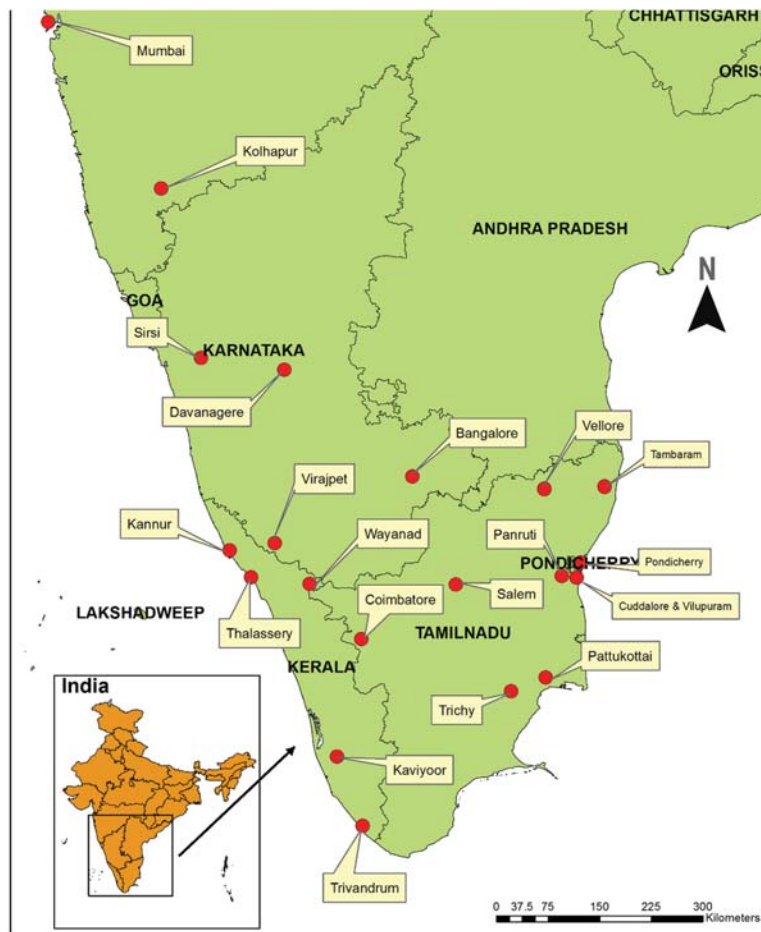
Figure 1. Places of collections of *Garcinia* raw drug samples in South India.

the predominant *Garcinia* species occurring in the Western Ghats, and these are locally traded as *Kodampuli* and *Kokum*, respectively (Fig. 1). These species are traded throughout India and exported to other countries as raw drugs, juices and extracts[7]. Several other species, such as *G. morella* (Gaertn.) Desr., *G. cowa* Roxb. ex Choisy, *G. mangostana* L., *G. kydia* Roxb., *G. lanceifolia* Roxb. and *G. pedunculata* Roxb. ex Buch.-Ham, are also traditionally used and traded for various culinary and medicinal purposes[7]. The trade analysis of *Garcinia* species suggests that the demand for the dried fruit rinds (raw drugs) and its value-added products are increasing[4,7].

Garcinia fruits are a rich source of (−)-hydroxycitric acid, anthocyanins and polyisoprenylated benzophenone derivatives like garcinol, camboginol, guttiferones and xanthochymol[10]. (−)-Hydroxycitric acid, often abbreviated HCA in commercial products and advertisements, has gained considerable attention as a promising anti-obesity agent, and commercial *Garcinia* extracts claiming a high content of (−)-hydroxycitric acid are popular food supplements worldwide[11]. In human and animal studies, (−)-hydroxycitric acid has been reported to effectively curb appetite, suppress food intake, increase the rates of hepatic glycogen synthesis, reduce fatty acid synthesis and lipogenesis and decrease body-weight gain[12]. However, contradictory results have been reported, suggesting that (−)-hydroxycitric acid can cause only short-term weight loss, the magnitude of the weight loss effect is small, and the clinical relevance is uncertain[13]. The safety related to the intake of extracts with a high content of (−)-hydroxycitric acid is also uncertain[14].

A common problem in herbal products highlighted in recent media reports and in scientific literature, is adulteration in high value raw drugs and finished products[15–17]. The underlying reasons for such adulterations may be due to confusion as morphologically similar material identified by vernacular names in different languages enters the chain of commercialization[18], lack of authentic raw plant material[19], and also adulteration for financial gain[20]. Adulteration may be considerable, given that there is no regulatory tool for quality control, no strict commercial testing for authentication of herbal products, and a lack of authentication along the value chains for highly traded medicinal plants[21].

Value chain research focuses on the nature of the relationships among the various participants involved in the chain and on the implications of these relationships for development[21]. The demand for medicinal herbs or herbal products is growing at the rate of 15–25% annually[21]. Surprisingly, only a limited number of studies exist with the focus on value chains for herbal medicines[22]. Understanding and establishing the value chains and trade network for herbal medicines may reflect the reasons for the variation in quality of a particular herbal drug.

Figure 2. (**a**) Raw drug samples collected as *Kodampuli* and *Kokum* from the raw drug markets of southern India. (**b**) *Garcinia gummi-gutta* fruit. (**c**) *Garcinia indica* fruit.

To authenticate herbal medicines, different tools for identification are available depending on the plant species and processes involved. These range from straightforward morphological or microscopic identification of plant parts to more advanced genetic or chemical approaches[23]. Among molecular identification tools, DNA barcoding has been successfully used in detecting and quantifying adulteration in raw herbal trade of a variety of medicinal plants. However, the application of the technique is often constrained by the inability to extract good quality DNA from raw herbal materials ranging from simple dried leaves to powdered plant parts, for subsequent PCR-based amplification of DNA barcode markers[19]. Alternatively, spectroscopic methods such as NMR can be used for the analysis of chemical compounds in complex mixtures such as plant extracts, pharmaceuticals and herbal preparations[24]. NMR spectroscopy is highly reproducible, robust and inherently quantitative without the need for prior chromatographic separation of multiple components. The technique has been used for detection, identification and quantitative determination of adulterants in weight loss food supplements[24,25]. Combining DNA barcoding with spectroscopic methods for authentication of herbal medicines increases the resolution in species identification and analysis of mixtures[19].

In this study, the specific objectives were: (1) to understand the value chain of *Garcinia* species based herbal products; (2) to assess the extent of adulteration in *Garcinia* species in the herbal raw drug trade of Southern India using DNA barcoding; (3) to quantify the principle organic acids, (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone, in *Garcinia* raw herbal drugs using NMR spectroscopy; and (4) to identify and quantify (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone in food supplements that claim to contain *Garcinia* extract, using NMR spectroscopy.

Materials and Methods

Raw drug trade analysis. Informal semi-quantitative interviews were conducted in villages of Sirsi (Fig. 1), which is one among the five forest divisions of Uttara Kannada district in Western Ghats of India. Total forest area of Sirsi is 1737 km^2, and the natural vegetation of Sirsi can be broadly divided into four different categories of forests, viz. tropical evergreen, semi-evergreen, moist deciduous and dry deciduous[26]. Twenty farmers were interviewed about the number of *Garcinia* species available in their region, how many species they collected for trade, and the amounts and which plant parts they collected. They were also asked if they used any post-harvest techniques to process the fruits and if they graded the fruits based on different qualities.

Collection of authenticated Biological Reference Material. Forty-one taxonomically validated herbarium vouchers from eleven species of *Garcinia* L. were collected from the Western Ghats and Northeast India, (Supplementary Table S1). The collected samples were identified using morphological keys in The Flora of India[27] as well as by Dr. Srikanth Gunaga, taxonomist at College of Forestry, University of Agricultural Sciences (Sirsi). Nomenclature follows The Plant List (The Plant List, 2013 http://www.theplantlist.org). For each of the species, herbarium specimens were prepared and deposited at the Herbarium of the Ashoka Trust for Research in Ecology and the Environment (ATREE), Bangalore (Supplementary Table S1). These taxonomically authenticated samples are referred to as Biological Reference Material (BRM). The BRM was used to amplify and sequence DNA barcodes for three regions: the nuclear ribosomal nrITS and the chloroplast markers *psbA-trnH* and *rbcL*.

Raw drug trade samples. Raw drug samples were obtained from major raw drug markets in Southern India (Figs 1 and 2). During interviews with vendors we elicited data for all known vernacular and trade names of *Garcinia* species (Supplementary Table S2), but we found that only *Kodampuli* (*G. gummi-gutta*) and *Kokum* (*G. indica*) were traded. One hundred grams of dried fruit rinds were purchased from each shop, totally 21 shops, and the obtained samples were vouchered as Herbal Authentication Service (HAS) with all the necessary details of shops and place of collection and deposited in the herbarium of ATREE, Bangalore (Supplementary Table S3). A chain of custody protocol ensured that samples were not mixed from the time of collection to DNA extraction

and NMR spectroscopic analysis. Although most of the raw drugs collected were dried fruit rinds, they had retained significant morphological features of the fruits.

Purchase of *Garcinia* herbal products. Ten herbal products that included either *G. gummi-gutta* or *G. indica* were purchased in pharmacies in India (5), Romania (1), and via e-commerce from United States (2), Norway (1), and Sweden (1). According to the label information, five products contained *G. gummi-gutta* (syn. *G. cambogia*), two products contained *G. indica*, and three products contained *G. gummi-gutta*, among other ingredients. An overview of the samples including label information, but not the producer/importer name, lot number, expiration date or any other information that could lead to the identification of that specific product is presented in Supplementary Table S4.

DNA barcoding and validation of trade samples. DNA was extracted from the leaf tissues of BRM (n = 11 species with 2–5 individuals in each species) using a previously described protocol[28]. Extracted DNA was quantified, and polymerase chain reactions were performed to amplify nrITS, *psbA-trnH* and *rbcL* using specific primers following standard protocols[19,29] (Supplementary Table S5). The amplified products of nrITS, *psbA-trnH* and *rbcL* were sequenced on an automated ABI 3100 Genetic Analyzer (Applied Biosystems, CA, United States) by Shrimpex Biotech, Chennai, India.

The obtained DNA sequence chromatograms were visualized and edited to remove ambiguous base calls and primer sequences using BioEdit v.5.0.6[30]. The sequences of nrITS, *psbA-trnH* and *rbcL* were deposited in NCBI GenBank and the accession numbers for all the BRM samples are given in Supplementary Table S1. Each species sequence was used as a query sequence in a BLASTn search. Along with the best match sequences in BLASTn search, the available DNA sequences for nrITS and *rbcL* of Indian *Garcinia* species were downloaded in FASTA format from GenBank and included in the analysis. No *psbA-trnH* sequences for these Indian *Garcinia* species were found (Supplementary Table S1). Interspecific and intraspecific genetic distances were calculated in PAUP* v.4.0b.10[31] using K2P model, as well as the number of parsimony characters per marker. A Maximum Likelihood tree was generated using RAxML[32] available through the Cipres Web Portal (http://www.phylo.org)[33]. RAxML analyses were run with default parameters for the three markers separately, and *Clusia* species (Clusiaceae) were chosen as outgroup. Further, in order to test the possibilities of concatenating the three markers, incongruence between the nuclear and plastid markers were tested using incongruence length difference test (ILD) in PAUP* v.4.0b.10[31], and also trees were visually evaluated whether there are any conflicting topologies in the gene trees, defined as ML bootstraps >85 on nodes.

In order to authenticate identities of the herbal raw drug samples procured as *Kodampuli* and *Kokum*, genomic DNA was isolated using a DNA extraction protocol optimized for *Garcinia* species[28] and amplified for nrITS and sequenced as described above. A single-locus Maximum Likelihood tree was constructed using both the herbal raw drug samples nrITS sequences and the nrITS BRM barcode library using RAxML[32] available through the Cipres Web Portal (http://www.phylo.org)[33] (Fig. 3).

Preparation of *Garcinia* fruit samples and food supplements for NMR analysis. Due to the highly hygroscopic nature of *Garcinia* fruits, the market samples of *Kodampuli* (n = 18) and *Kokum* (n = 6) were freeze-dried using an Alpha 1–4 LD plus laboratory freeze dryer (Martin Christ GmbH, Osterode am Harz, Germany) for 24 hours and then pulverized in a commercial blender (Waring Commercial, New Hartford, CT, United States). Aliquots of ca. 1 g, accurately weighed, of fruit powder were extracted with Milli-Q water at 120 °C using an Accelerated Solvent Extraction system (ASE350 Solvent Extractor, Dionex, Sunnyvale, CA, United States). Three grams of diatomaceous earth (Dionex) was mixed with the *Garcinia* fruit powder aliquots and loaded in 100 ml steel cartridges. The cartridges were fitted on to the system and exhaustive extraction was performed with three cycles. Preheating time was 5 min, static extraction per cycle was 5 min and the extraction was carried out under a pressure of ca. 1500 PSI (10 MPa). The extracts from each cycle was combined, and water was evaporated at 60 °C using a rotary evaporator (RV 10 Basic Rotavapor with vacuum controller, IKA-Werke GmbH & Co. KG, Staufen, Germany). Further the extract was dried by freeze-drying, yielding 462.2–772.3 mg (45.7–76.6%) of water extract.

For *Garcinia* food supplements analysis, the capsules were opened and their contents removed (7 capsules), whereas tablets were ground to powder (3 tablets). One to two grams of each food supplement was accurately weighed (Supplementary Table S4) and extracted (extraction yield was 0.8758–1.1854 g (53.8–98.3%)). Extraction and NMR analysis were carried out using the same methodology as for the *Garcinia* water extracts.

Apart from water extracts of fruit rinds and food supplements, methanol extracts of *Garcinia* food supplements and *Garcinia* fruit rind powder were prepared following a modified protocol[34] in order to understand whether polyisoprenylated xanthones can be used as marker compounds for identification. Briefly, aliquots of capsule contents, powdered tablets or fruit rind powder were weighed into 2 ml Eppendorf tubes. CD_3OD (1.5 ml) was added and the samples were vortexed for 1 min. The samples were then ultrasonicated for 20 minutes at room temperature before centrifugation at 1000 g for 30 minutes. To the supernatant was added 0.1% TFA and the acidified samples were transferred to NMR tubes for analysis.

NMR quantification of (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone. ^{1}H NMR spectra of *Garcinia* water extracts and food supplement extracts were acquired for quantitative analysis on a Bruker AVII400 (Bruker GmbH, Rheinstetten, Germany) instrument equipped with a 5 mm BBOF probe and operating at a frequency of 400 MHz at 298 K, and with number of scans = 128. A reference solvent solution was prepared containing 2.0 mg/ml maleic acid (internal reference) (Sigma-Aldrich, St. Louis, Missouri, United States) and 0.1% 3-(trimethylsilyl) propionic 2,2,3,3-d_4 acid sodium salt (TSP) (Sigma-Aldrich) in D_2O (Sigma-Aldrich). For NMR analysis, extracts were dissolved in 500 μl of the NMR reference solvent solution
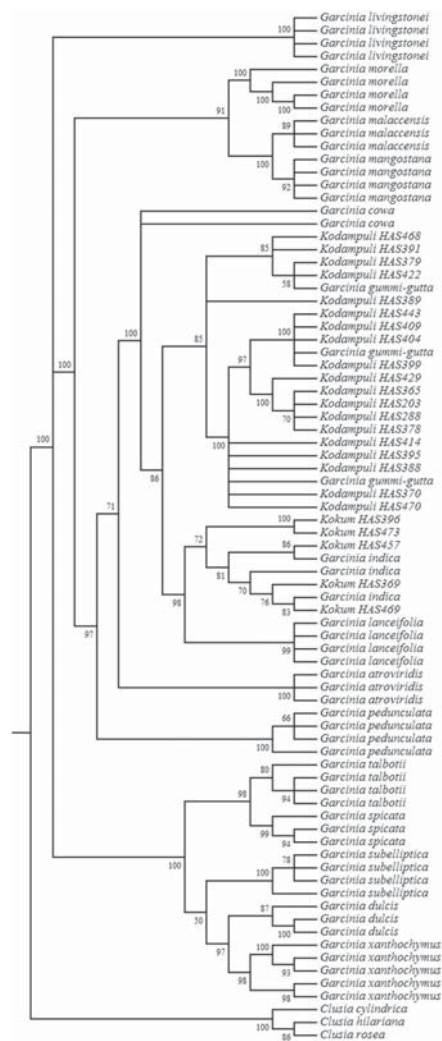
Figure 3. Maximum Likelihood tree (RAxML) of biological reference material of *Garcinia* species and *Garcinia* raw drug market samples using nrITS region.

under vortex agitation, giving a final concentration of 20 mg/ml of extract, and analyzed in triplicate. The pH of all *Garcinia* water extracts was measured to have a pH of ∼2 in the NMR solutions. For the *Garcinia* food supplement extracts, the NMR sample solutions were adjusted to a pH of ∼2 by adding trifluoroacetic acid-d (Sigma-Aldrich), which ensures that the carboxylic acid functional groups are in the protonated state and gives consistent shift values for (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone. Shift positions are relative to the TSP signal at δ 0.0 ppm.

The concentrations of (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone were measured by comparing the NMR peak areas of selected protons of (−)-hydroxycitric acid (δ ca. 3.17 ppm, H-3a) and (−)-hydroxycitric acid lactone (δ ca. 3.29 ppm, H-4a) with those of the internal reference (δ 6.37 ppm). The area of each peak is directly proportional to the number of corresponding nuclei. The percentages of (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone in the samples were calculated using the following equation, $P (Sample) = (I_{(a)} \times N_{(ir)} \times M_{(a)} \times m_{(ir)} \times P_{(ir)})/(I_{(ir)} \times N_{(a)} \times M_{(ir)} \times m_{(sample)})$, where $I_{(a)}$ and $I_{(ir)}$ are the areas of the analytes ((−)-hydroxycitric acid/(−)-hydroxycitric acid lactone) and the internal reference, $N_{(ir)}$ and $N_{(a)}$ are the number of protons contributing to the signal of the internal reference (2 protons) and the analytes (1 proton each for (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone), $M_{(a)}$ and $M_{(ir)}$ are the molecular weights of (−)-hydroxycitric acid (208.12 g/mol) and (−)-hydroxycitric acid lactone (190.11 g/mol) and the internal reference (116.07 g/mol), $m_{(ir)}$ and $m_{(sample)}$ is the mass of the internal reference (1 mg) and mass of the sample aliquot (10 mg), $P_{(ir)}$ is the purity of the internal reference (99.94%). The areas were measured by using the MNova v. 7.1.2 program (http://mestrelab.com/). In order to evaluate the NMR quantification method, test solutions containing *Garcinia* water extract in NMR reference solvent solution (0.18 to 40 mg/ml) were prepared and measured in triplicates. The S/N ratios were measured to determine Limit of Detection (LOD) and Limit of Quantification (LOQ)[35], and linearity over the concentration range was also calculated.

DNA regions	Reference PCR success (%)	Aligned sequence (bp)	Intraspecific distance (mean ± SD)	Interspecific distance (mean ± SD)	p-value	Parsimony-informative sites
ITS	100	581	0.037 ± 0.010	0.153 ± 0.085	<0.01	278
psbA-trnH	79	493	0.037 ± 0.010	0.129 ± 0.044	<0.01	198
rbcL	93	710	0.011 ± 0.005	0.023 ± 0.007	<0.01	70

Table 1. Evaluation of the three DNA barcode regions used for *Garcinia* species.

Data accessibility. GenBank (NCBI) [http://www.ncbi.nlm.nih.gov/genbank] Accession numbers of nucleotide sequences are listed in Supplementary Table S1.

Results and Discussion

Interviews with *Garcinia* collectors. The interviews with farmers from Western Ghats revealed that people predominantly collected *G. gummi-gutta* and *G. indica* from the wild, with quantities of *Garcinia* fruits up to 15 to 20 kg per day. Fruits are the traditionally used plant part of *Garcinia*, but during the interview we noticed that resin from the trees was also locally collected[36]. Apart from these two species, *G. morella* was collected for culinary purposes and domestic consumption. The other *Garcinia* species such as *G. mangostana* and *G. cowa*, which grow sparsely in the area, were locally traded based on their seasonal availability. Farmers commented that not all trees produce fruits, and distinguished between "male" and "female" trees of *Garcinia* species. The preliminary understanding from the survey is that there is limited added value for *Garcinia* trade at the farmers end. However, people do process the fruit post-harvest and tend to sell seedless fruits, which fetch a higher price than the fruits with seeds. In the case of *G. indica*, the juice is extracted from the fruits by several small and large-scale industries.

DNA barcoding of *Garcinia* raw drugs. In recent years, a number of medicinal plants have been shown to be adulterated with other low cost plant materials[15]. Contamination in herbal products presents a potential health risk for consumers[23]. In the present study, DNA barcoding was used to analyze the extent of adulteration in herbal raw drug trade of *Garcinia* species. The first step in analyzing the presence of adulteration was the construction of a taxonomically authenticated BRM DNA barcode library for *Garcinia* species. This was assembled in order to provide a reliable DNA library of reference samples, to authenticate the herbal raw drug samples. A single-locus approach was used to delineate nine species collected from Western Ghats and two species from Northeast India based on genetic divergence of three regions (i.e., nrITS, *psbA-trnH* and *rbcL*) using the Kimura 2-Parameter model (Table 1). *rbcL* and *psbA-trnH* exhibited the lowest mean interspecific distance (0.023 and 0.129, respectively); in contrast, nrITS exhibited the highest mean interspecific distance (0.153). Similarly, the mean intraspecific distance for nrITS was 0.037, which was significantly lower than the mean interspecific distance. In the case of *psbA-trnH* and *rbcL*, the mean intraspecific distance was 0.011 and 0.037 respectively, showing that *rbcL* was not a suitable marker because that the interspecific distance, 0.023 is lower than the intraspecific distance, 0.037, and that *psbA-trnH* was considerably better with a significant mean inter- and intraspecific genetic distance (0.129 and 0.037 respectively) (Table 1). nrITS had the most parsimony-informative characters with 278 characters. Similarly *psbA-trnH* and *rbcL* had 198 and 70 respectively (Table 1). The ILD test revealed significant incongruence between the nuclear and plastid markers (P > 0.01), and the visual inspection of phylogenetic trees suggests a topology conflict among the three markers, therefore the three matrices were not concatenated and single-locus trees were utilized for further analyses.

Authentication of herbal products has been a major challenge due to the fact that chemical studies have documented high content variability of active ingredients of medicinal plants due to geographic conditions and also variability among products from diverse manufacturers of supplements[16]. Conventional techniques such as organoleptic analysis and microscopy are also limited for species authentication in herbal products[37]. There are a number of studies that have assessed adulteration in medicinal plants in raw drug trade and also have identified ingredients added in finished herbal products using DNA barcoding[15,38–40].

However, a major limitation of Sanger DNA barcoding is the inability to distinguish constituent species in multi-ingredient herbal products[20,41]. Several comprehensive reviews on DNA-based authentication of botanicals have highlighted the merits and limitations of Sanger based DNA barcoding[23,37,42]. In this study, it is evident from the single-locus phylogenetic trees that nrITS and the *psbA-trnH* spacer are more suitable barcode regions for Indian *Garcinia* species than *rbcL* (Supplementary Figs S1, S2, and S3).

Tree-based methods are advantageous over sequence similarity based methods like BLAST that require a decision on a threshold at which a sequence is considered to belong to a certain taxon, which could be subjective and may be applicable to certain taxa but not to others[43]. In tree-based methods, a clear advantage is that no cut-off value is necessary if the query sequence is considered to belong to a certain taxon, and if it is found in the same clade consisting of a reference sequence for that particular taxon[38]. In addition distance based methods (e.g., BLAST) have a tendency to produce false positive identifications if the reference sequences are not available in databases, whereas taxonomic identification based on tree-based methods are not as sensitive to incomplete databases and avoid false positive identification[43]. Supplementary Figs S1 and S2 of nrITS and *psbA-trnH* illustrate the clear grouping within *Garcinia* species with well supported bootstrap values, whereas *rbcL* on the other hand shows poor resolution in delineating the *Garcinia* species, and that can be explained by the low genetic divergence within this marker (Supplementary Fig. S3).

Compound	LOD (mg/ml)	LOQ (mg/ml)	Relative standard deviation	Linear equation
(−)-Hydroxycitric acid in *Garcinia* fruit extracts	0.06	0.45	1.3%	$y = 1.0143 \times + 100.92\,R^2 = 0.9374$
(−)-Hydroxycitric acid lactone in *Garcinia* fruit extracts	0.04	0.71	2.1%	$y = 2.8848 \times + 163.45\,R^2 = 0.9974$

Table 2. Method validation data for quantification of (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone.
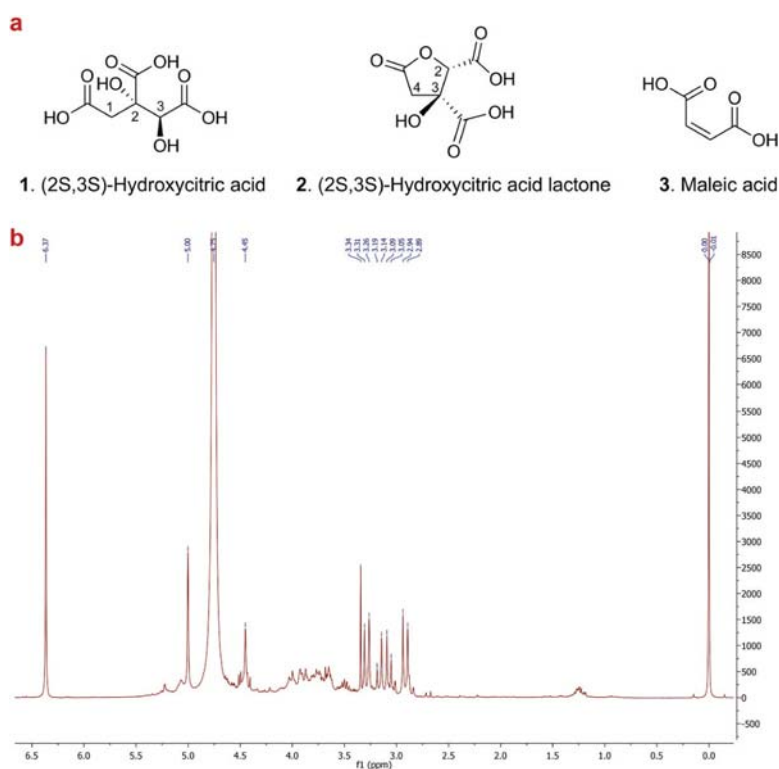
Figure 4. (**a**) Chemical structures of (−)-hydroxycitric acid (**1**), (−)-hydroxycitric acid lactone (**2**) and maleic acid (**3**). (**b**) ^{1}H NMR spectrum of *Garcinia gummi-gutta* (voucher no. HAS429) extract.

In order to authenticate the raw drugs traded as *Kodampuli* and *Kokum*, the marker nrITS was utilized due to its high amplification success rate from raw drugs (100%), whereas we were unable to amplify the chloroplast markers from the DNA isolated from the raw drug samples. Similarly, it was observed that the PCR amplification success rate in reference samples was 100% for nrITS compared to *psbA-trnH* (79%) and *rbcL* (93%) (Table 1). Analyses of nrITS sequences of *Kodampuli* and *Kokum* along with BRM barcode library revealed that there was no adulteration in the species (Fig. 3), and also that the morphological characters of fruits aided in the authenticity verification of collected raw drug samples from the market (Fig. 2). This was also apparently due to the absence of taxonomic complexities in fruit morphological characters of *Kodampuli* and *Kokum*[44].

A prerequisite for DNA-based molecular analysis of herbal products is the isolation of good quality DNA[23]. In this study, the yield of isolated DNA from the *Garcinia* food supplements ranged from 0.05 to 1 ng/ml. Plant DNA can also be removed or degraded during the manufacturing process of herbal products: extensive heat treatment, irradiation, ultraviolet light exposure, filtration, extractive distillation or supercritical fluid extraction all affect DNA yield and integrity[23].

NMR quantification method in *Garcinia* fruits. ^{1}H NMR was used to quantify the (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone content in raw herbal drugs of *Garcinia* species. The chemical formulas for (−)-hydroxycitric acid with the (2 S,3 S) configuration and its lactone form are shown in Fig. 4a. The (−)-hydroxycitric acid content was directly measured from areas of characteristic signals in the proton spectra of the water extracts (Fig. 4b). Limit of detection (LOD) and limit of quantification (LOQ) is the lowest concentration of an analyte in a sample which can be detected and quantified, respectively, for which the selected signals exhibit the minimal required S/N ratio. Table 2 shows LOD values of 0.06 and 0.04 mg/ml, and LOQ values of

Voucher of dried fruits	(−)-Hydroxycitric acid content, % (SD)	(−)-Hydroxycitric acid lactone content, % (SD)	Total (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone content, % (SD)
HAS370	8.5 (2.9)	14.8 (1.4)	23.3 (3.7)
HAS404	8.2 (0.4)	14.4 (0.4)	22.6 (0.8)
HAS391	10.3 (0.6)	19.1 (1.2)	29.4 (1.6)
HAS468	7.6 (0.4)	11.7 (1.0)	19.4 (0.9)
HAS378	6.3 (0.3)	7.8 (0.3)	14.1 (0.4)
HAS288	7.5 (0.2)	11.9 (0.9)	19.5 (1.1)
HAS470	8.8 (0.6)	14.9 (1.2)	23.6 (1.7)
HAS395	7.9 (0.1)	12.5 (0.2)	20.3 (0.1)
HAS388	8.1 (1.1)	14.1 (1.9)	22.2 (3.0)
HAS379	6.7 (0.2)	10.0 (0.5)	16.8 (0.4)
HAS443	8.1 (0.6)	11.3 (0.9)	19.4 (1.4)
HAS422	8.9 (0.4)	17.0 (0.7)	25.9 (1.0)
HAS414	7.8 (0.4)	12.5 (0.6)	20.3 (1.0)
HAS389	8.2 (0.1)	14.4 (1.5)	22.6 (2.5)
HAS409	7.6 (0.4)	13.1 (0.4)	20.7 (0.6)
HAS399	8.9 (0.1)	14.1 (0.2)	23.0 (0.2)
HAS429	8.9 (0.3)	14.5 (0.2)	23.4 (0.5)
HAS203	3.9 (1.3)	7.4 (0.3)	11.3 (1.3)
HAS469*	7.6 (0.3)	11.7 (0.4)	19.3 (0.6)
HAS457*	3.7 (1.3)	11.3 (1.0)	15.0 (2.3)
HAS473*	5.0 (0.8)	7.3 (0.7)	12.3 (1.3)
HAS365*	2.7 (0.4)	5.4 (0.2)	8.2 (0.5)
HAS369*	4.8 (0.1)	8.1 (0.1)	12.8 (0.1)
HAS396*	1.7 (0.2)	3.5 (0.4)	5.2 (0.6)

Table 3. (−)-Hydroxycitric acid, and (−)-hydroxycitric acid lactone content (%) of *Garcinia gummi-gutta* (*Kodampuli*) and *Garcinia indica* (*Kokum*) fruit rinds using ^{1}H NMR for quantification (**Garcinia indica* samples).

0.13 and 0.20 mg/ml for (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone respectively. Supplementary Fig. S4 shows NMR spectra of *Garcinia* fruit extracts in different concentrations with the resulting changes in signal intensities for (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone.

The ^{1}H NMR spectrum of the water extract of *G. gummi-gutta* HAS429 depicted in Fig. 4b clearly shows the presence of characteristic signals from (−)-hydroxycitric acid (**1**). The ^{1}H NMR signals for H-1 appear as two closely spaced doublets at δ 3.07 ppm (d, $J = 16.6$ Hz) and 3.16 ppm (d, $J = 16.6$ Hz), while H-3 appears as a singlet at δ 4.45 ppm. In the same spectrum the H-4 protons in (−)-hydroxycitric acid lactone (**2**) give rise to two doublets at δ 2.92 ppm (d, $J = 18.0$ Hz) and 3.29 ppm (d, $J = 18.0$ Hz), H-2 gives a singlet at δ 5.00 ppm. The two vicinal protons of the internal standard maleic acid (**3**) give rise to the singlet at δ 6.37 ppm. However, a slight variation in the characteristic signals of (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone within triplicates of each sample and between samples was observed (relative standard deviation < 0.03). These variations in chemical shifts values are possibly due to minor pH differences or interactions with other molecules in the extracts.

Though no prior sample clean-up after extraction was necessary in order to quantify the (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone content in the *Garcinia* fruit extracts, it was observed that the extracts are rich in sugar compounds (Fig. 4b & Supplementary Fig. S5). Table 3 shows the percentage of (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone (mass fraction) in the dried fruits of *Kodampuli* and *Kokum* raw drugs. The content of (−)-hydroxycitric acid in the dried *Kodampuli* fruits varied from 3.9% to 10.3% with an average of 7.9% and the content of (−)-hydroxycitric acid lactone varied from 7.4% to 19.1% with an average of 13.1%. On the other hand, the content of (−)-hydroxycitric acid in the dried *Kokum* fruits varied from 1.7% to 7.6% with an average of 4.3% and the content of (−)-hydroxycitric acid lactone varied from 3.5% to 11.7% with an average of 7.8%. Since the equilibrium state of (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone in the *Garcinia* fruits is not known, the total content of the acid and the lactone was calculated. The total average content of (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone in *Kodampuli* was 21.0% while the total content in *Kokum* was 12.1%. As far as we know, this is the first time that ^{1}H NMR has been used for the quantification of (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone. Gas chromatography[45], high performance liquid chromatography[46], and capillary zone electrophoresis[47] have previously been used to quantify the hydroxycitric acid and hydroxycitric acid lactone content in *Garcinia* fruits and food supplements. HPLC with UV detection at 210–220 nm is commonly used today and has replaced the more laborious GC methods[12,48]. A challenge with HPLC is to obtain a good resolution between the organic acids and their lactones in *Garcinia*. There are examples showing no discrimination between (−)hydroxycitric acid and the lactone or methods that suffer from poor resolution[46,49,50]. Another challenge is the low selectivity at 210 nm, therefore sample preparation may be needed to remove co−eluting interfering substances[46,49,50]. Compared to these methods, quantitative NMR (qNMR)

Herbal products code no.	Labeled content per capsule/tablet		(−)-Hydroxycitric acid content (mg/capsule) determined by qNMR
	Garcinia ingredients	mg HCA$	
1	G. cambogia extract (60% HCA), 500 mg	300	36 ± 2.9
2	G. cambogia extract (50% HCA), 500 mg	250	149 ± 0.7
3	G. indica extract, 350 mg		122 ± 9.2
4	G. cambogia, 100 mg		59 ± 1.4
5	G. cambogia extract (60% HCA), 525 mg	315	29 ± 1.9
6	G. cambogia extract (65% HCA), 250 mg G. cambodia powder, fruit rind and leaf (1% HCA), 350 mg	166	144 ± 3.7#
7	G. cambogia extract, 300 mg		69 ± 1.8*
8	G. indica powder, 400 mg		184 ± 7.3
9	G. cambogia extract, 350 mg		150 ± 4.3
10	G. cambogia fruit (60% HCA), 500 mg	300	289 ± 8.6

Table 4. (−)-Hydroxycitric acid, and (−)-hydroxycitric acid lactone content in *Garcinia* food supplements used in the study. $Calculated based on labeled content of HCA; #Contained 41 ± 0.6 mg (−)-hydroxycitric acid lactone, the other samples contained only detectable amounts or no lactone; *Samples were analyzed only twice.

currently employed has the advantage of being rapid (less than 8 minutes needed for 128 scans) with no need for additional cleanup of extracts or derivatization. NMR makes it possible to detect of all kind of organic molecules in the same sample, as long as they are soluble in the NMR solvent, and is also highly reproducible with little instrument-instrument variation; in addition it is non-invasive and non-destructive[51].

In previous studies, using an HPLC method, a (−)-hydroxycitric acid content of *G. gummi-gutta* of 16–18% was reported, and the leaves and rinds of *G. indica* were found to contain 4.1–4.6% and 10.3–12.7%, respectively, whereas only trace amounts of (−)-hydroxycitric acid lactone was reported[46,50]. For *G. cowa*, the (−)-hydroxycitric acid content was found to be 1.7, 2.3 and 12.7%, respectively in the fresh leaves, fruits and dried rinds[48]. These overlaps between the total content of (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone in *Garcinia* species suggest that these organic acids should not be used as marker compound to distinguish between *Garcinia* species.

Seasonal and geographic variations in chemical composition have been reported for a number of medicinal plants[52,53], and also chemical variability along the value chains, harvesting pressure, harvest time, collection of immature plant parts etc., have been reported to influence the composition and concentration of plant constituents[54–56]. The variation in the concentration of (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone in *Kodampuli* and *Kokum* raw drugs can be explained since the raw drugs were obtained from different geographic locations of South India (Fig. 1), and concentration of organic acids can also vary based on the harvest time, as has been shown in other fruits[57,58]. The raw drugs stocked in the herbal markets are normally obtained from a single source or from collectors of a specific region. Enquiries with the traders indicated that supplies are made usually by wholesale agents who in turn obtain raw drugs from sub-agents and collectors, and it is likely that the collectors get the *Kodampuli* and *Kokum* from across the Western Ghats of India. This could explain the variation in the concentration of (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone in the studied *Garcinia* fruits.

Validation of *Garcinia* food supplements. *G. cambogia* (syn. *G. gummi-gutta*) or unspecified *Garcinia* extracts rich in (−)-hydroxycitric acid are examples of food supplements freely available in pharmacies, health food stores, and via the internet without any regulations and quality control. In order to authenticate the validity of *Garcinia* products, ten *Garcinia* food supplements were randomly purchased via e-commerce and pharmacies (Supplementary Table S4). One of the major challenges with the authentication of *Garcinia* food supplements is the lack of processing information. The majority of the supplements were not labeled with the solvent used for extraction, and thus the composition of the extracts was difficult to predict, and it is not obvious if they contain water-soluble consituents such as hydroxycitric acid or lipophilic compounds like garcinol and camboginol. The content of (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone in the *Garcinia* supplements is shown in Table 4, and compared with the labeled contents. Product 1 and 5, labeled to contain 500 mg and 525 of extract with 60% HCA, were found to contain only 36 ± 2.9 mg (5.5%) and 29 ± 1.9 mg (4.6%) (−)-hydroxycitric acid, respectively. The other eight products contained more reasonable amounts of (−)-hydroxycitric acid compared to their labeled ingredients (59–289 mg (12.8–50.5%) per capsule or tablet). In comparison the analyzed *G. gummi-gutta* fruit rind water extracts were found to contain 32% of (−)-hydroxycitric acid and its lactone on average, and the average content found for *G. indica* water extracts was 19%. A previous study utilizing capillary zone electrophoresis concluded that one sample out of five did not contain *G. atroviridis* Griff extract as claimed on the label[47]. On the contrary, a study utilizing HPLC revealed no adulteration among *G. gummi-gutta* and *G. indica* commercial products[59] and another study using HPLC revealed that *G. gummi-gutta* commercial products contained 51–55% of (−)-hydroxycitric acid with small quantities of tartaric, citric, and malic acids[49]. From quantitative NMR analysis of water extracts obtained from the supplements, it was found that five products contained both (−)-hydroxycitric acid and (−)-hydroxycitric acid lactone. Among these, only one product contained quantifiable amounts of (−)-hydroxycitric acid lactone, whereas (−)-hydroxycitric acid lactone was missing or only present in trace amounts in the remaining products (Table 4 & Supplementary Fig. S6). The absence of (−)-hydroxycitric acid lactone in the supplements with *Garcinia* extracts might be due to the extraction method employed[60].

^{1}H NMR spectra of *G. indica* and G. *gummi-gutta* (syn. *G. cambogia*) fruit rind methanol extracts showed characteristic signals from polyisoprenylated xanthones (garcinol or camboginol). Signals from aromatic protons at δ 6.7, 7.0 and 7.2 ppm and unsaturated tertiary methyls groups at δ 1.5–1.7 ppm were observed[61,62]. These signals were also observed in product no. 6, labeled to contain both *G. cambogia* extract (250 mg) and powder of fruit rinds and leaves (350 mg), while methanol extracts of the other supplements did not give rise to signals from garcinol or camboginol. These signals were not present in spectra of the fruit rind or herbal supplement water extracts. Thus, garcinol or camboginol, which are likely not present in the *Garcinia* commercial extracts, should not be used as authentication compounds for *Garcinia* supplements.

Conclusions

In this study, we used DNA barcoding and ^{1}H NMR spectroscopy to authenticate *Garcinia* fruits and food supplements. ^{1}H NMR is useful for qualitative and quantitative analysis of constituents in medicinal plants and herbal products[24]. However, NMR has, so far, not received a wider application in the official food supplements testing and medicine control laboratories than other chromatographic methods, mostly because the NMR technique was judged to be complicated and instruments too expensive[25]. However, several improvements in instrumentation during the last decade have made NMR available for routine applications[25]. In the future, a metabolomic approach using NMR would be useful for large-scale analysis of *Garcinia* food supplements and this study has shown that qNMR is a fast and sensitive enough method for the quantification of (−)-hydroxycitric acid content. Several studies have advocated the use of DNA barcoding in herbal product authentication and pharmacovigilance[19,23,29,63] due to its cost effectiveness and ability to identify plant species. In this study, DNA barcoding was used to demonstrate that there was no adulteration or substitution of *Garcinia* species in major herbal markets of south India. These results show the usefulness of DNA barcoding and ^{1}H NMR spectroscopy for use in complementing traditional methods of quality control for consumer safety, and demonstrates that DNA barcoding can be used as a screening method for the identification of *Garcinia* species in herbal trade, and NMR for detection and quantification of hydroxycitric acid and (−)-hydroxycitric acid lactone in *Garcinia* fruits and food supplements.

Our ^{1}H NMR results suggest that in order to increase consumer confidence by advocating and promoting a higher standard of quality in herbal products, there is an urgent need to study food supplements derived from traditional medicinal plants.

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Author Contributions

G.S.S., H.W., H.D.B., R.U.S., G.R. conceived the experiment. G.S.S. carried out molecular and phytochemical lab work. M.T. and H.W. developed phytochemical method, and M.T., G.S.S., K.E.M. were involved in phytochemical lab work. G.S.S., J.U.S., S.V.G., R.V. were involved in plant collection and interviewing the farmers. G.S.S. wrote the manuscript, and all authors have read and approved the final version of the manuscript.

Additional Information

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Authentication of *Garcinia* fruits and food supplements using DNA barcoding and NMR spectroscopy

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Supplementary Information

Supplementary Table S1. Details of biological reference material samples collected from different parts of South India used in the analysis.

Supplementary Table S2. List of trade/vernacular names used during the collection of *Garcinia* raw drugs samples from raw drug markets of South India.

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Supplementary Fig. S1. Maximum Likelihood tree (RAxML) of *Garcinia* species using nrITS.

Supplementary Fig. S2. Maximum Likelihood tree (RAxML) of *Garcinia* species using *psbA-trnH*.

Supplementary Fig. S3. Maximum Likelihood tree (RAxML) of *Garcinia* species using *rbcL*.

Supplementary Fig. S4. ^{1}H NMR spectrum of (-)-hydroxycitric acid, and (-)-hydroxycitric acid lactone in different concentration of *Garcinia* fruits.

Supplementary Fig. S5. ^{1}H NMR spectrum of *Garcinia* fruits extract.

Supplementary Fig. S6. ^{1}H NMR spectrum of analyzed *Garcinia* food supplements.

Supplementary Table S1. Details of biological reference material samples collected from different parts of South India used in the analysis.

Sl. no.	Species name	Voucher no.	Collection site	GenBank Accession numbers		
				ITS	psbA-trnH	rbcL
1	*Garcinia gummi-gutta* (L.) Roxb.	ATREE119	Ramagondanahalli (FRLHT), Bangalore	KP318329	KP318368	KP318400
		ATREE120	Vaddi ghat, Uttara kannada	KP318330	KP318369	KP318431
		ATREE121		KP318331	KP318370	KP318432
		ATREE122	Sirsi, Karnataka	KP318332	KP318371	KP318401
		ATREE123		KP318333	-	-
2	*Garcinia xanthochymus* Hook.f. ex T.Anderson	ATREE144	Ramagondanahalli (FRLHT), Bangalore	KP318354	KP318387	KP318428
		ATREE145	Chikka Bommasandra (GKVK, Bangalore)	KP318355	KP318388	KP318429
		ATREE146	Sirsi, Karnataka	KP318356	KP318389	KP318433
		ATREE147	Nilkund, Uttara kannada	KP318357	KP318390	KP318430
		ATREE148	Sirsi, Karnataka	KP318358	-	-
3	*Garcinia indica* (Thouars) Choisy	ATREE124	Salkani, Uttara kannada	KP318334	KP318372	KP318402
		ATREE125	Ramagondanahalli (FRLHT), Bangalore	KP318335	KP318373	KP318403
		ATREE126	Chikka Bommasandra (GKVK, Bangalore)	KP318336	KP318374	KP318404
		ATREE127	Sirsi, Karnataka	KP318337	-	KP318405
		ATREE128		KP318338	-	-
4	*Garcinia morella* (Gaertn.) Desr.	ATREE139		KP318349	KP318385	KP318415
		ATREE140		KP318350	KP318386	KP318416
		ATREE141	Sirsi, Karnataka	KP318351	-	KP318417
		ATREE142		KP318352	-	KP318418
		ATREE143		KP318353	-	KP318419
5	*Garcinia mangostana* L.	ATREE137	Sirsi, Karnataka	KP318347	KP318383	KP318413
		ATREE138		KP318348	KP318384	KP318414
6	*Garcinia talbotii* Raizada ex Santapau	ATREE150	Vaddi ghat, Uttara kannada	KP318360	KP318392	KP318434
		ATREE151		KP318361	KP318393	KP318425
		ATREE152	Sirsi, Karnataka	KP318362	KP318394	KP318426

No.	Species	Location	Voucher			
7	*Garcinia livingstonei* T.Anderson	Sirsi, Karnataka	ATREE153	KP318363	KP318395	KP318427
			ATREE154	KP318364	KP318396	KP318410
			ATREE155	KP318365	KP318397	KP318411
			ATREE156	KP318366	KP318398	KP318435
			ATREE157	KP318367	-	KP318412
8	*Garcinia pedunculata* Roxb. ex Buch.-Ham.	Jorhat, Assam	ATREE133	KP318343	KP318379	KP318420
			ATREE134	KP318344	KP318380	KP318421
			ATREE135	KP318345	KP318381	KP318422
			ATREE136	KP318346	KP318382	KP318423
9	*Garcinia lanceifolia* Roxb.	Jorhat, Assam	ATREE129	KP318339	KP318375	KP318406
			ATREE130	KP318340	KP318376	KP318407
			ATREE131	KP318341	KP318377	KP318408
			ATREE132	KP318342	KP318378	KP318409
10	*Garcinia spicata* Hook.f.	Ramagondanahalli (FRLHT), Bangalore	ATREE149	KP318359 EU128390.1*	KP318391	KP318424
		Sirsi, Karnataka	ATREE159	EU128389.1*	KP318399	HQ332063.1*
11	*Garcinia cowa* Roxb. ex Choisy	Sirsi, Karnataka	ATREE158	AF367213.1* AB110799.1*	-	KJ510948 *

*Sequences downloaded from NCBI

Supplementary Table S2. List of trade/vernacular names used during the collection of *Garcinia* raw drugs samples from raw drug markets of South India.

Sl. no.	Species name	Trade name /Vernacular name
1	*Garcinia gummi-gutta* (L.) Roxb.	Kodampuli/ kodukkappuli
2	*Garcinia xanthochymus* Hook.f. ex T.Anderson	Deavkai/ malaippuli
3	*Garcinia indica* (Thouars) Choisy	Kukam/ kokam
4	*Garcinia morella* (Gaertn.) Desr.	Kadukaai puli/ punarpuli
5	*Garcinia mangostana* L.	Shulampuli/ mangosteen
6	*Garcinia talbotii* Raizada ex Santapau	Tavir
7	*Garcinia livingstonei* T.Anderson	-
8	*Garcinia pedunculata* Roxb. ex Buch.-Ham.	Nerinnampuli
9	*Garcinia lanceifolia* Roxb.	-
10	*Garcinia spicata* Hook.f.	Kaadu jaarige
11	*Garcinia cowa* Roxb. ex Choisy	Dvipaja/ paravata

Supplementary Table S3. Details of raw drug samples collected from different parts of south India used in the analysis.

Sl. No	Location of Shops/State	Kodampuli	Kokum
1.	Salem/Tamil Nadu	HAS443	-
2.	Vellore/Tamil Nadu	HAS429	-
3.	Panruti/Tamil Nadu	HAS414	-
4.	Villupuram/Tamil Nadu	HAS409	-
5.	Cuddalore/Tamil Nadu	HAS404	-
6.	Pondicherry/Tamil Nadu	HAS399	-
7.	Trichy/Tamil Nadu	HAS379	-
8.	Coimbatore/Tamil Nadu	HAS378	-
9.	Tambaram/Tamil Nadu	HAS203	-
10.	Pattukkottai/Tamil Nadu	HAS288	-
11.	Davangere/Karnataka	HAS365	-
12.	Sirsi/Karnataka	HAS370	HAS369
13.	Bangalore/Karnataka	HAS395	HAS396
14.	Virajpet/Karnataka	HAS470	HAS469
15.	Kannur/Kerala	HAS388	-
16.	Thalassery/Kerala	HAS389	-
17.	Thiruvanathapuram/Kerala	HAS391	-
18.	Wayanad/Kerala	HAS422	-
19.	Kaviyoor/Kerala	HAS468	-
20.	Kolhapur/Maharashtra	-	HAS473
21.	Mumbai/Maharashtra	-	HAS457

Supplementary Table S4. Details of *Garcinia* herbal products used in the study.

Herbal products code no.	Species on label	Scientific names of the plant ingredients as indicated in the product	Product type	Country of origin	Country of acquisition	Vendor type	Product classification as indicated in the product	Remarks from label	Weight of capsules/tablets used in the study
1	1	*Garcinia cambogia*	Capsules	USA	India	Pharmacy	Dietary supplement	Each capsule contains *Garcinia cambogia* extracts 500 mg, containing 60% HCA. Potassium 50 mg, calcium 50 mg	1.4481 g (2 capsules)
2	1	*Garcinia cambogia*	Capsules	USA	India	Pharmacy	Dietary supplement	Each capsule contains *Garcinia cambogia* extracts 500 mg, containing 50% HCA. Calcium 160 mg	1.4677 g (2 capsules)
3	1	*Garcinia indica*	Capsules	India	India	Pharmacy	Not stated	Each capsule contains 350 mg of *Garcinia indica* extract	1.3220 g (3 capsules)
4	4	*Commiphora mukul, Garcinia gummi-gutta, Allium sativum Plumbago zeylanica*	Capsules	India	India	Pharmacy	Not stated	*Garcinia gummi-gutta* 100 mg	1.3940 g (3 capsules)
5	1	*Garcinia cambogia*	Capsules	USA	USA	Internet	Dietary supplement	*Garcinia cambogia* extract 1050 mg, standardized to 60% HCA Potassium 160 mg	1.2608 g (2 capsules)
6	1	*Garcinia cambogia* Desr.	Tablets	India	USA	Internet	Herbal Supplement	*Garcinia cambogia* extract 250 mg, standardized to 65% HCA. Garcinia powder 350 mg (fruit rind and leaf), 1% HCA	1.2243 g (2 tablets)
7	5	*Garcinia cambogia Gymnema sylvestre Terminalia chebula Trigonella foecum-graeceum Balsamodendron mukul*	Capsules	Indua	Romania	Pharmacy	Not stated	Extracts: *Garcinia cambogia* (300 mg), *Gymnema sylvestre* (10 mg), *Terminalia sylvestre* (10 mg), *Trigonella foecum-graeceum* (10 mg) Powder: *Balsamodendron mukul* (70 mg)	1.3445 g (3 capsules)
8	2	*Garcinia indica Commiphora mukul,*	Tablets	India	India	Pharmacy	Not stated	Each tablet contains 400 mg powders of *Garcinia indica,*	1.6856 g (3 tablets)

		Emblica officinalis, Terminalia bellirica, Terminalia chebula						50 mg of Shuddha Guggul (*Commiphora mukul*), 90 mg of Triphala Ghan (Three myrobalons)
9	1	*Garcinia indica*	Tablets	India	Sweden	Internet	Not stated	Each capsule contains 350 mg of *Garcinia indica* extract	1.2419 g (2 tablets)
10	1	*Garcinia cambogia*	Capsules	USA	Norway	Internet	Herbal Supplement	*Garcinia cambogia* fruit 500 mg, standardized to 60% HCA	1.1432 g (2 capsules)

Supplementary Table S5. Details of the barcode primers used in the study.

DNA barcode regions	Primer name	Sequence (5'-3')	Reference
nrDNA-*ITS*	ITS1	TCCGTAGGTGAACCTGCGG	[1]
	ITS4	TCCTCCGCTTATTGATATGC	
psbA–trnH	psbA	GTTATGCATGAACGTAATGCTC	[2]
	trnH	CGCGCATGGTGGATTCACAAATC	
rbcL	F2N	CCAAGTTGAGAGAGATAAATTGAACAAG	[3]
	1460R	TCCTTTTAGTAAAAGATTGGGCCGAG	

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**Supplementary Table S6.** ^{1}H NMR shifts of (2S, 3S)-Hydroxycitric acid observed in the water extract of *G. gummi-gutta* HAS429 depicted in Figure 4b.

Number	δ_H (multiplicity, J in Hz)
1a	3.07 (d, J = 16.6 Hz)
1b	3.16 (d, J = 16.6 Hz)
3	4.45 (s)

Supplementary Table S7. ^{1}H NMR shifts of (2S, 3S)-Hydroxycitric acid lactone observed in the water extract of *G. gummi-gutta* HAS429 depicted in Figure 4b.

Number	δ_H (multiplicity, J in Hz)
2	5.00 (s)
4a	2.92 (d, J = 18.0 Hz)
4b	3.29 (d, J = 18.0 Hz)

Supplementary Fig. S1. Maximum Likelihood tree (RAxML) of *Garcinia* species using nrITS.
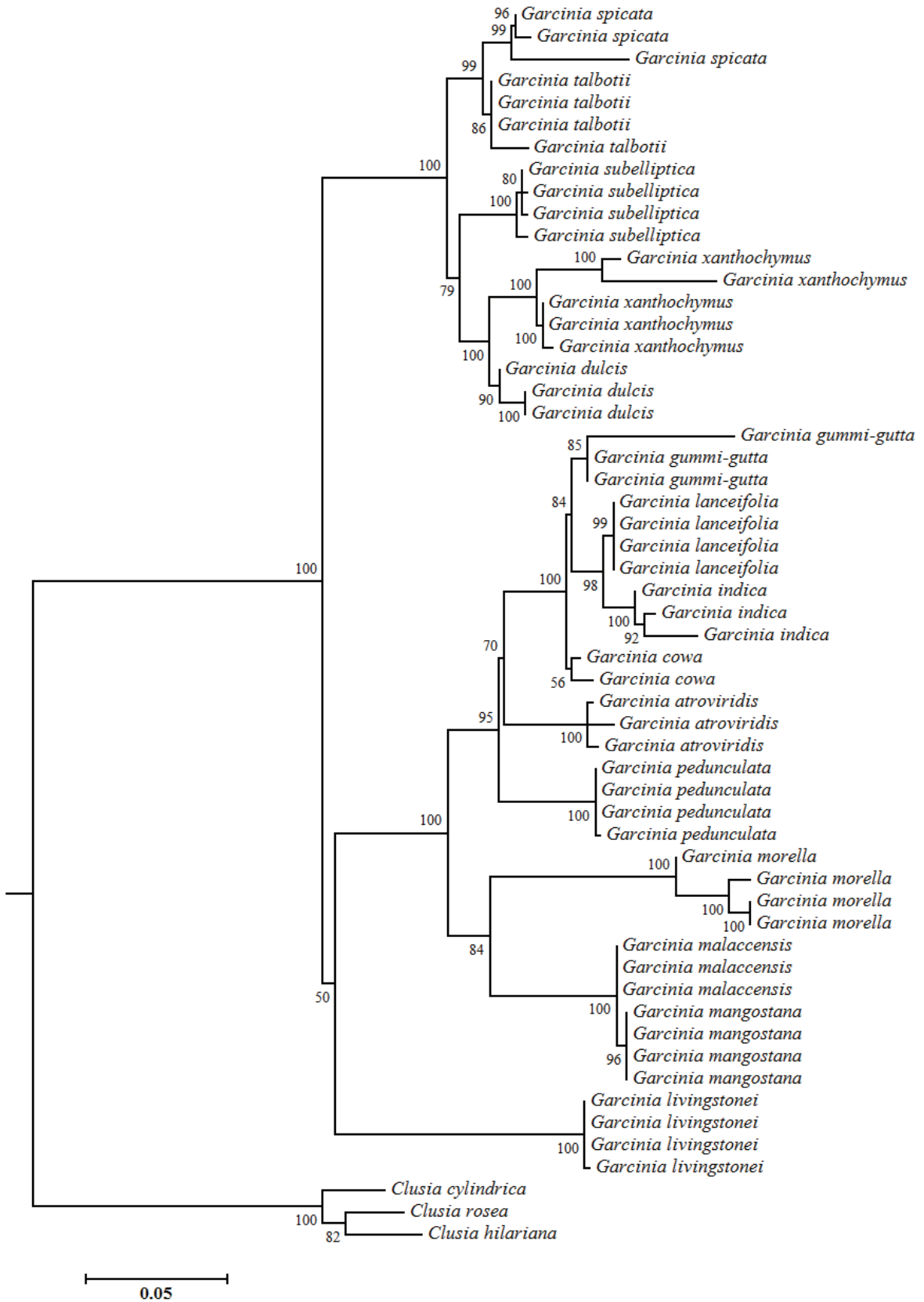
Supplementary Fig. S2. Maximum Likelihood tree (RAxML) of *Garcinia* species using *psbA-trnH*.
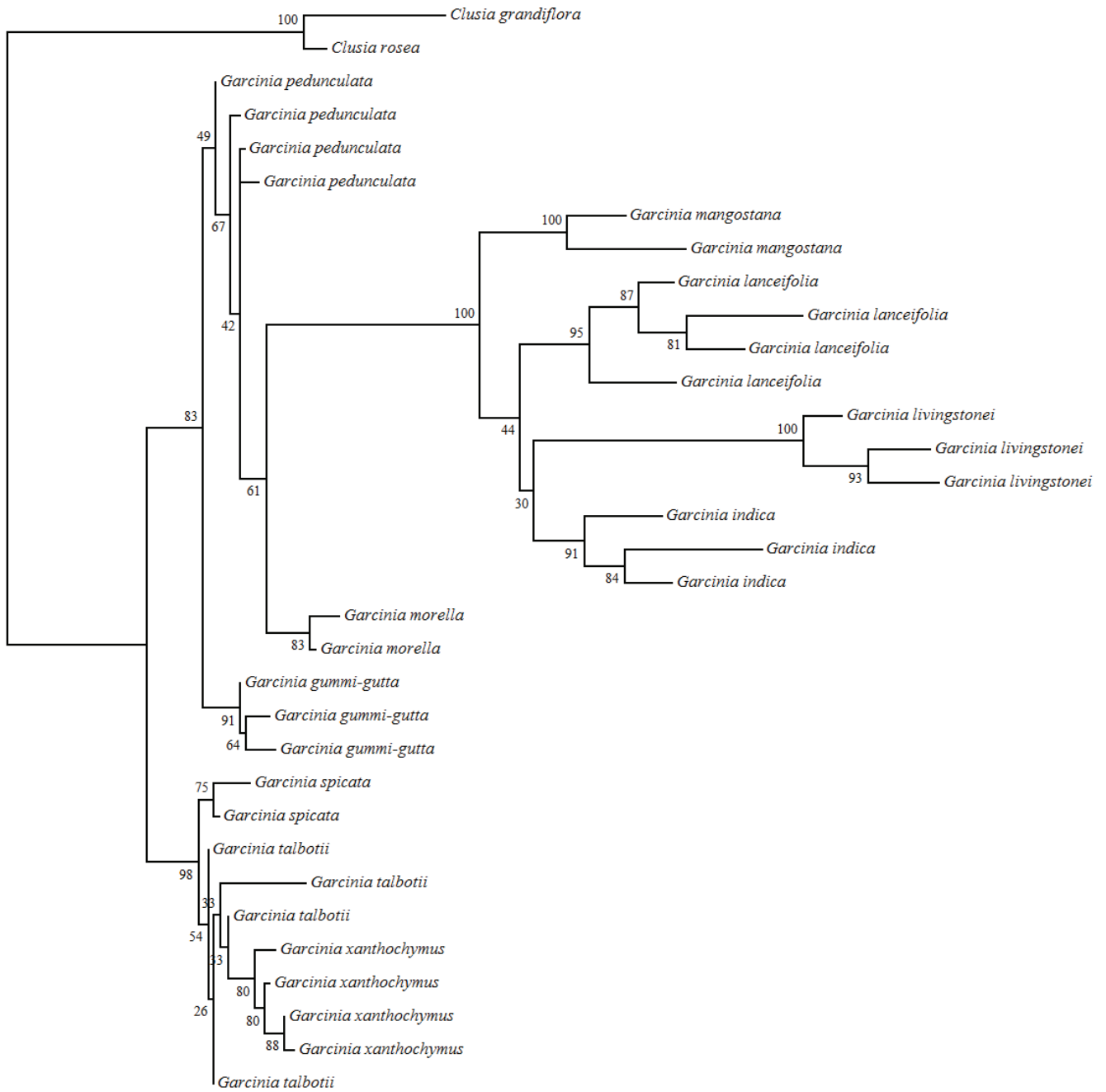
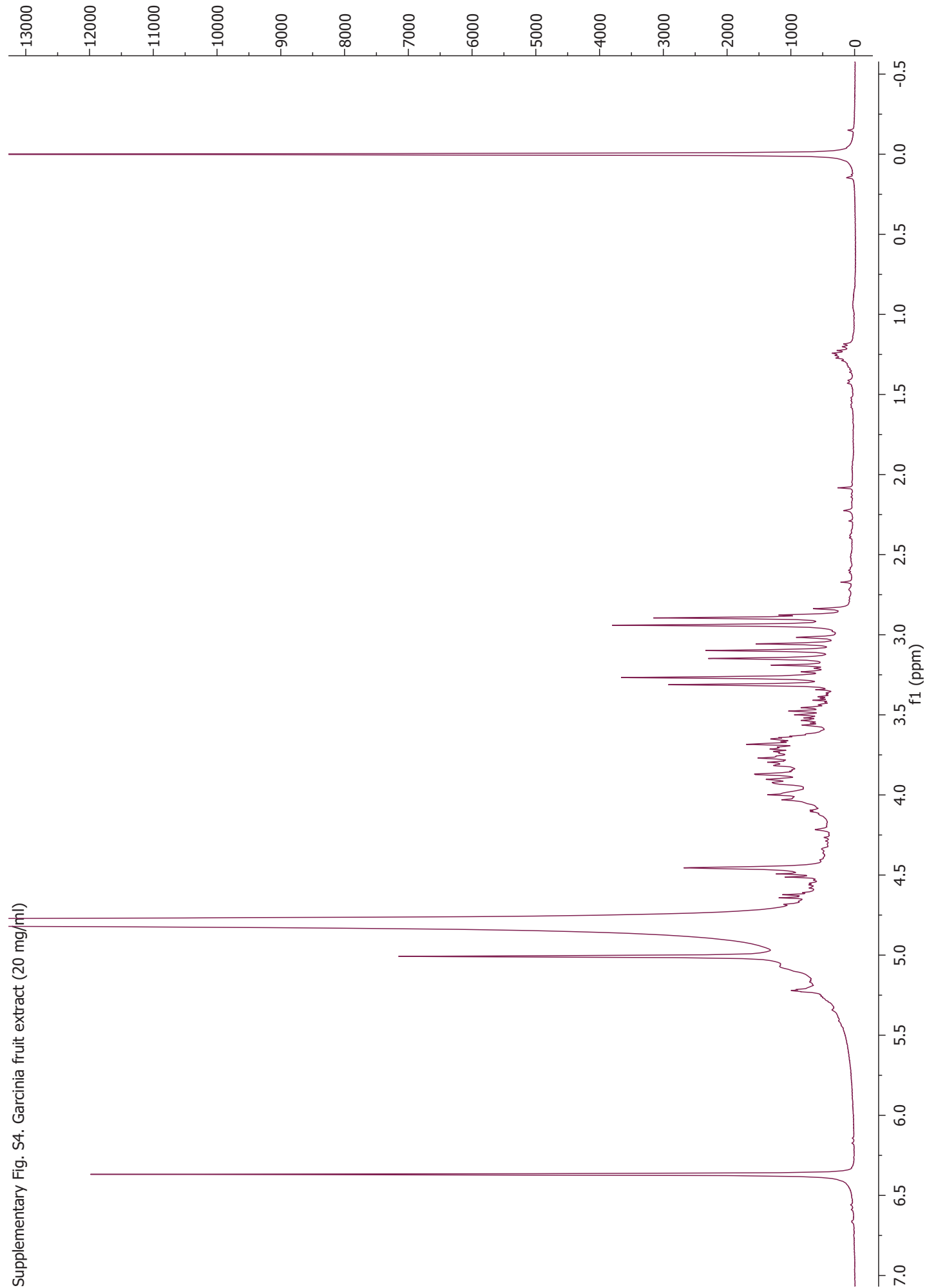**Supplementary Fig. S3.** Maximum Likelihood tree (RAxML) of *Garcinia* species using *rbcL*.
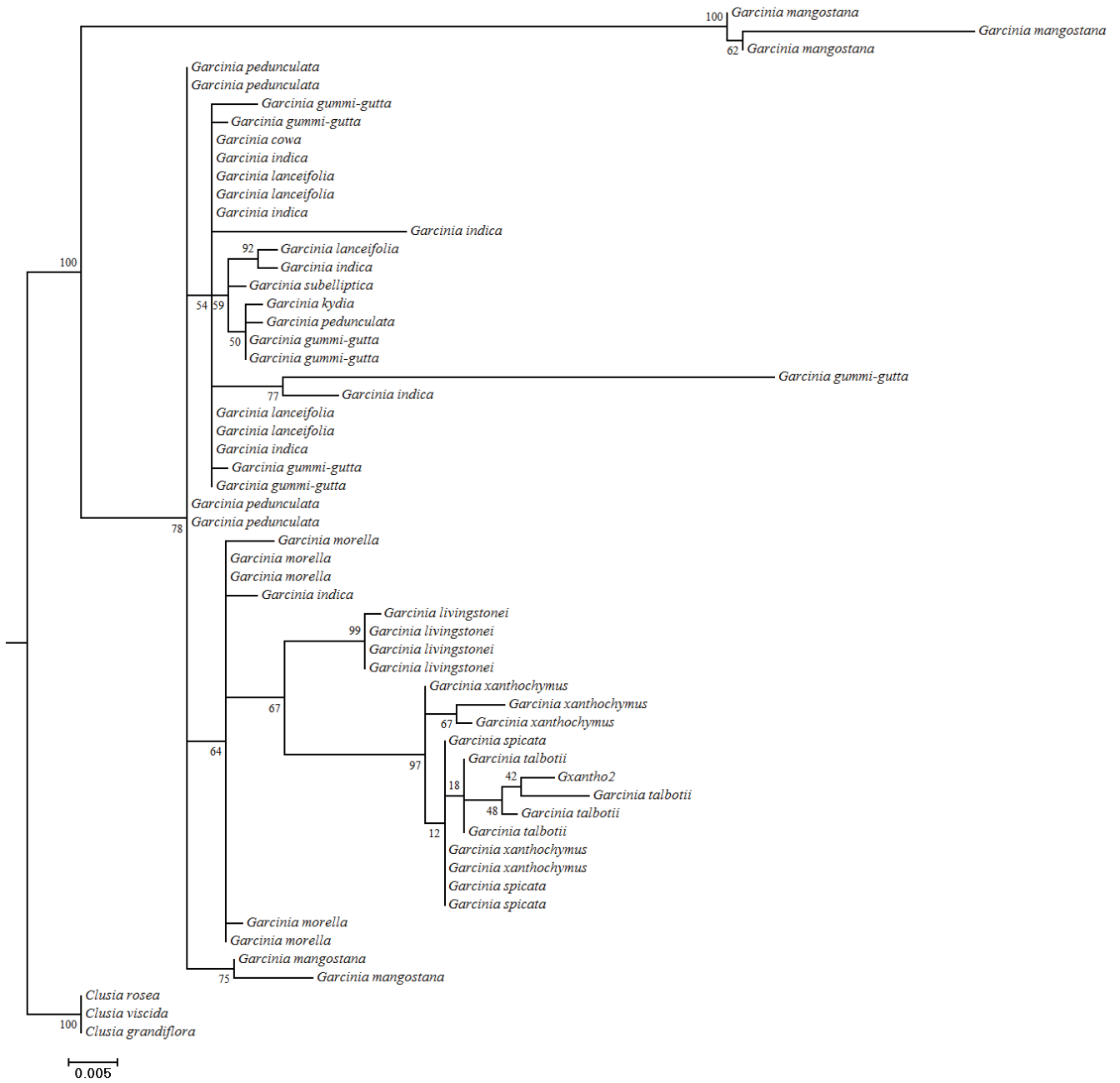
Supplementary Fig. S4. ^{1}H NMR spectrum of (‐)-hydroxycitric acid, and (‐)-hydroxycitric acid lactone in different concentration of *Garcinia* fruits
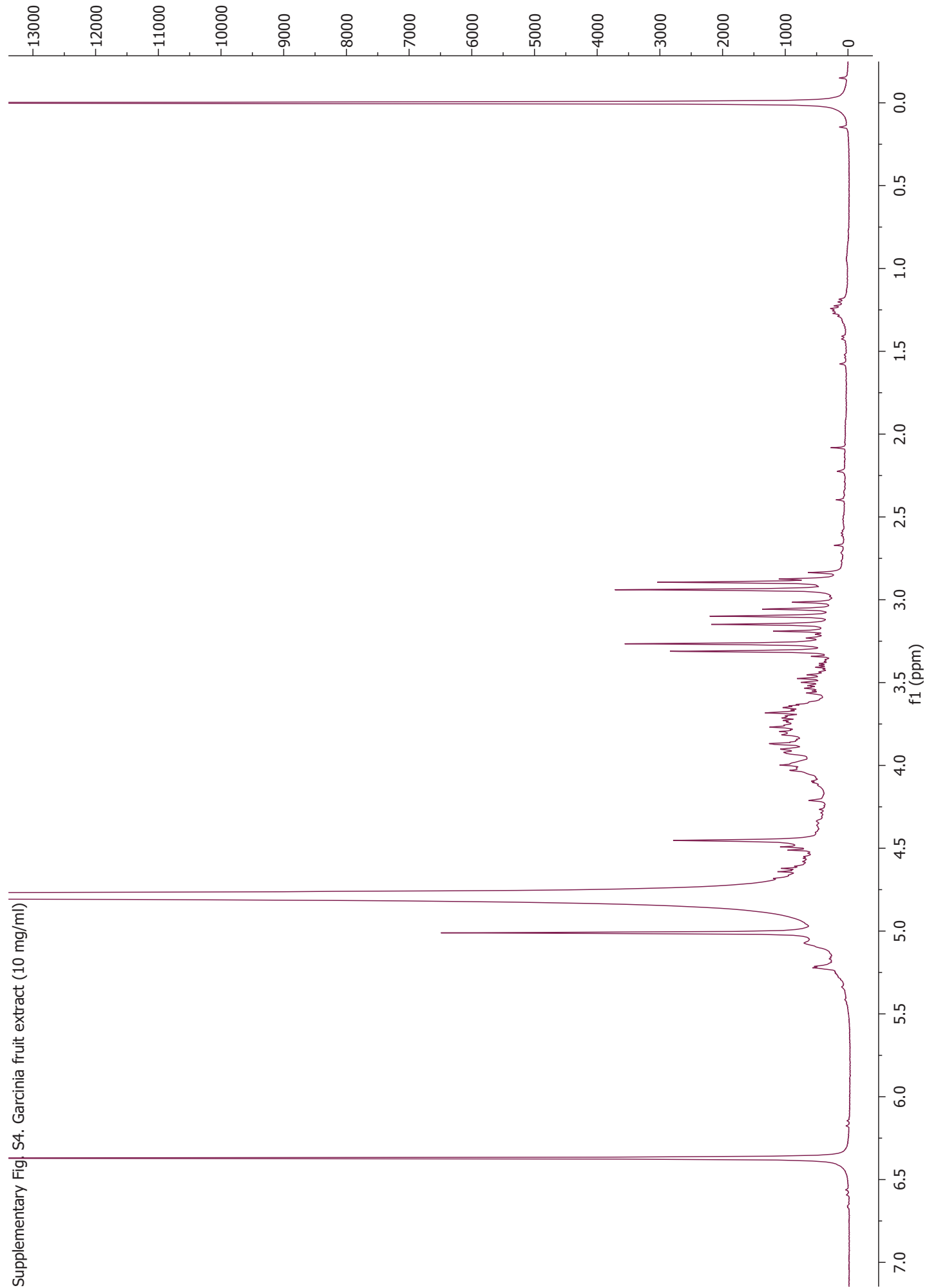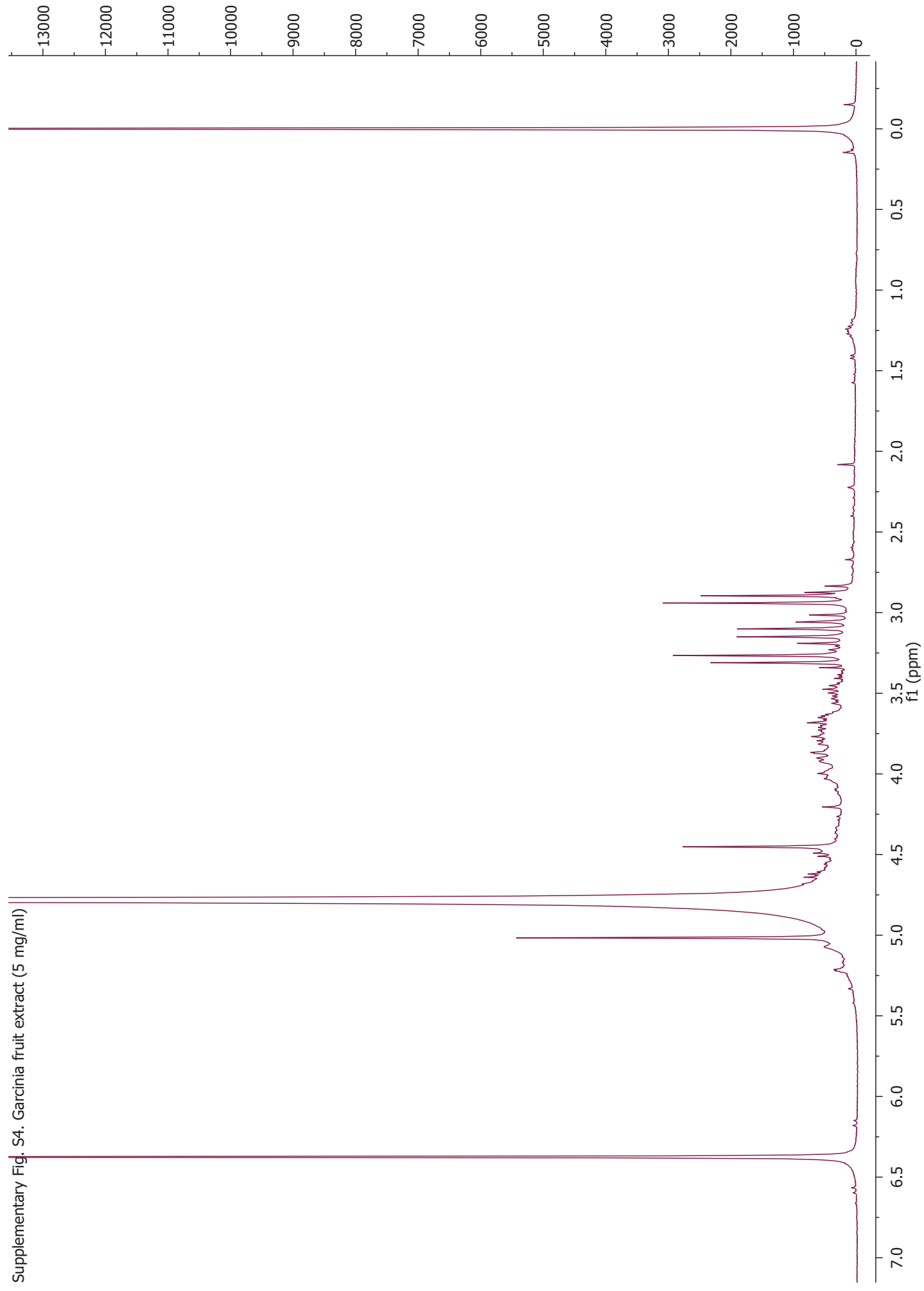
Supplementary Fig. S4. Garcinia fruit extract (40 mg/ml)
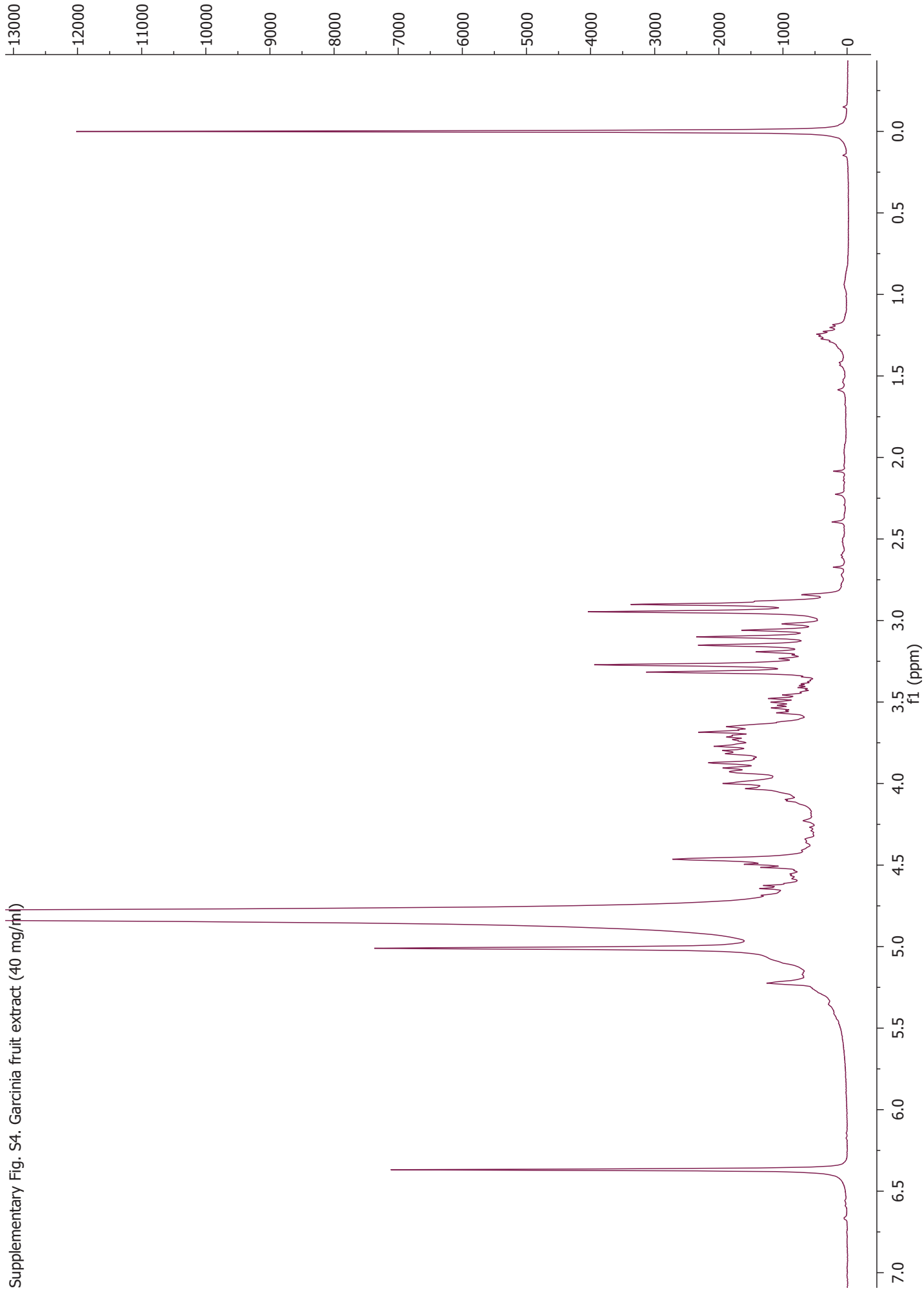
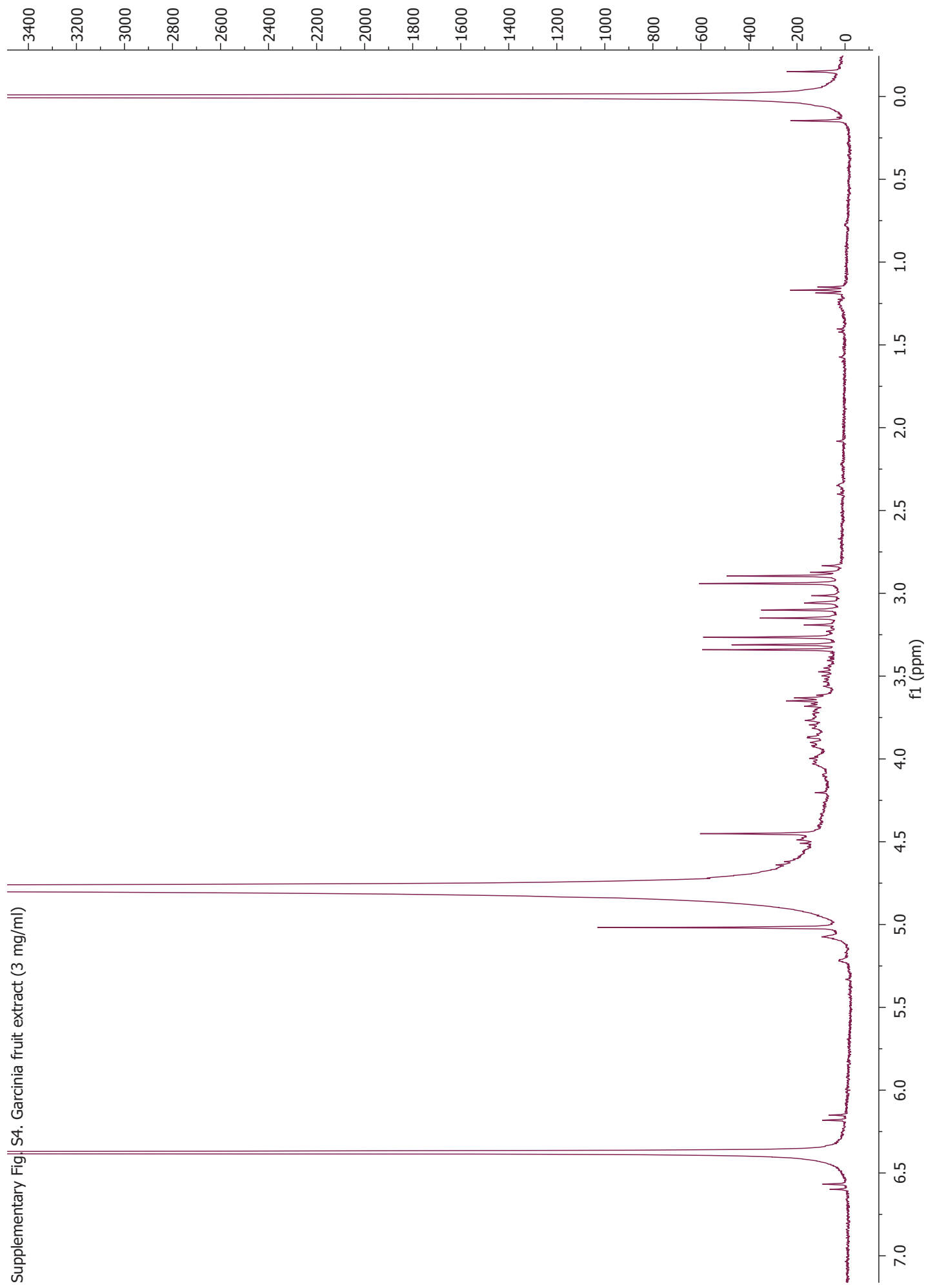Supplementary Fig. S4. Garcinia fruit extract (20 mg/ml)

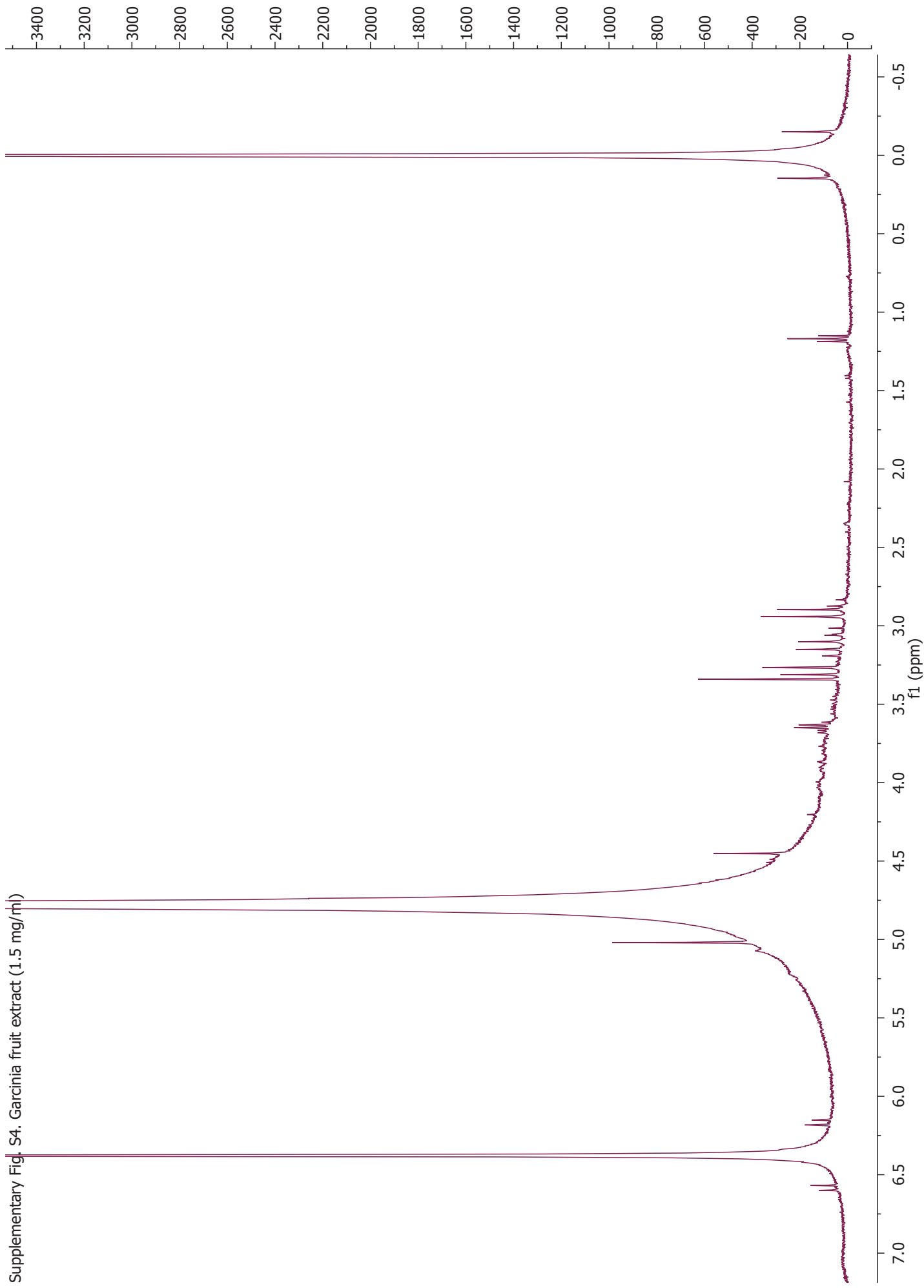Supplementary Fig. S4. Garcinia fruit extract (10 mg/ml)

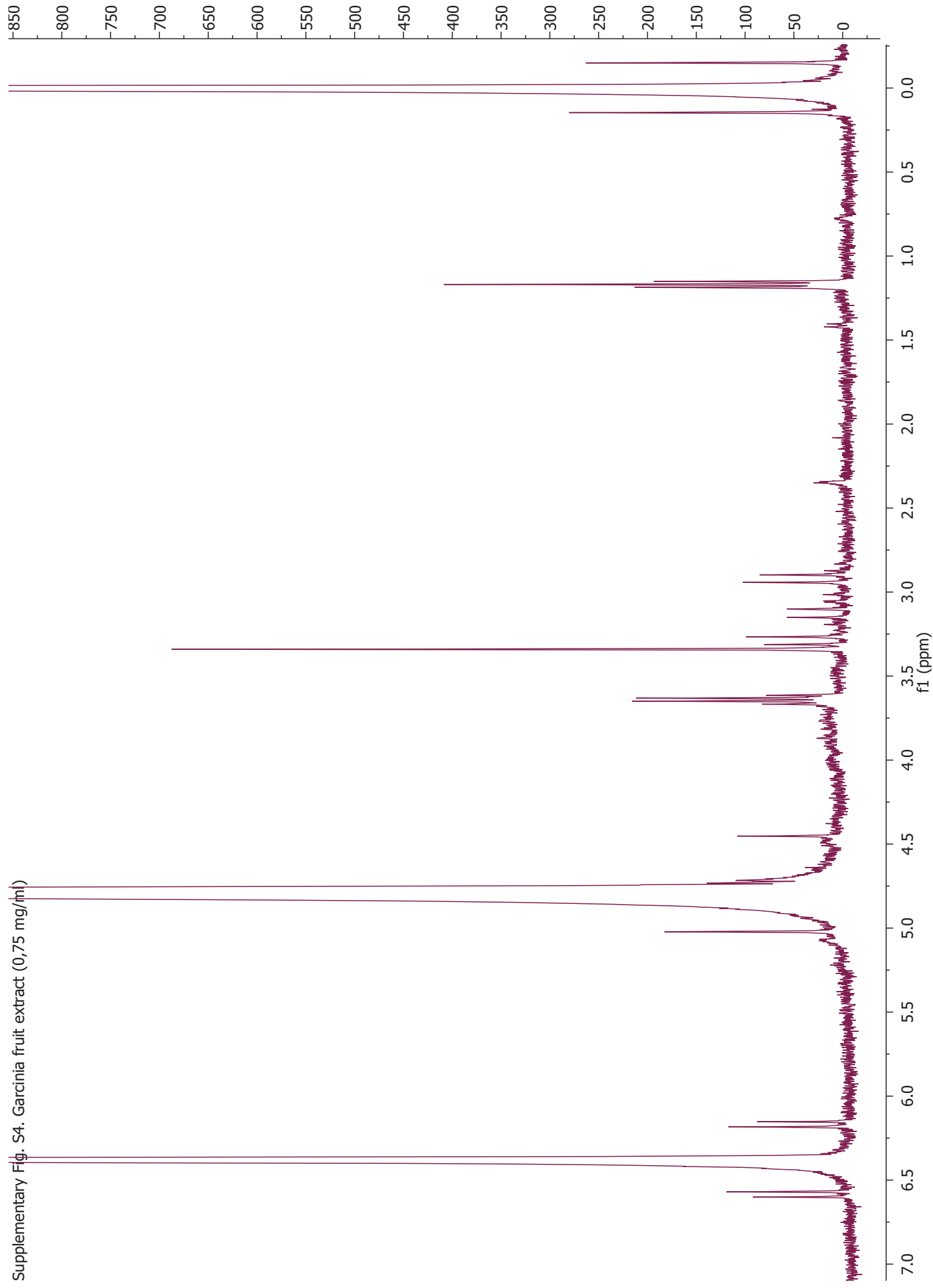Supplementary Fig. S4. Garcinia fruit extract (5 mg/ml)

Supplementary Fig. S4. Garcinia fruit extract (3 mg/ml)

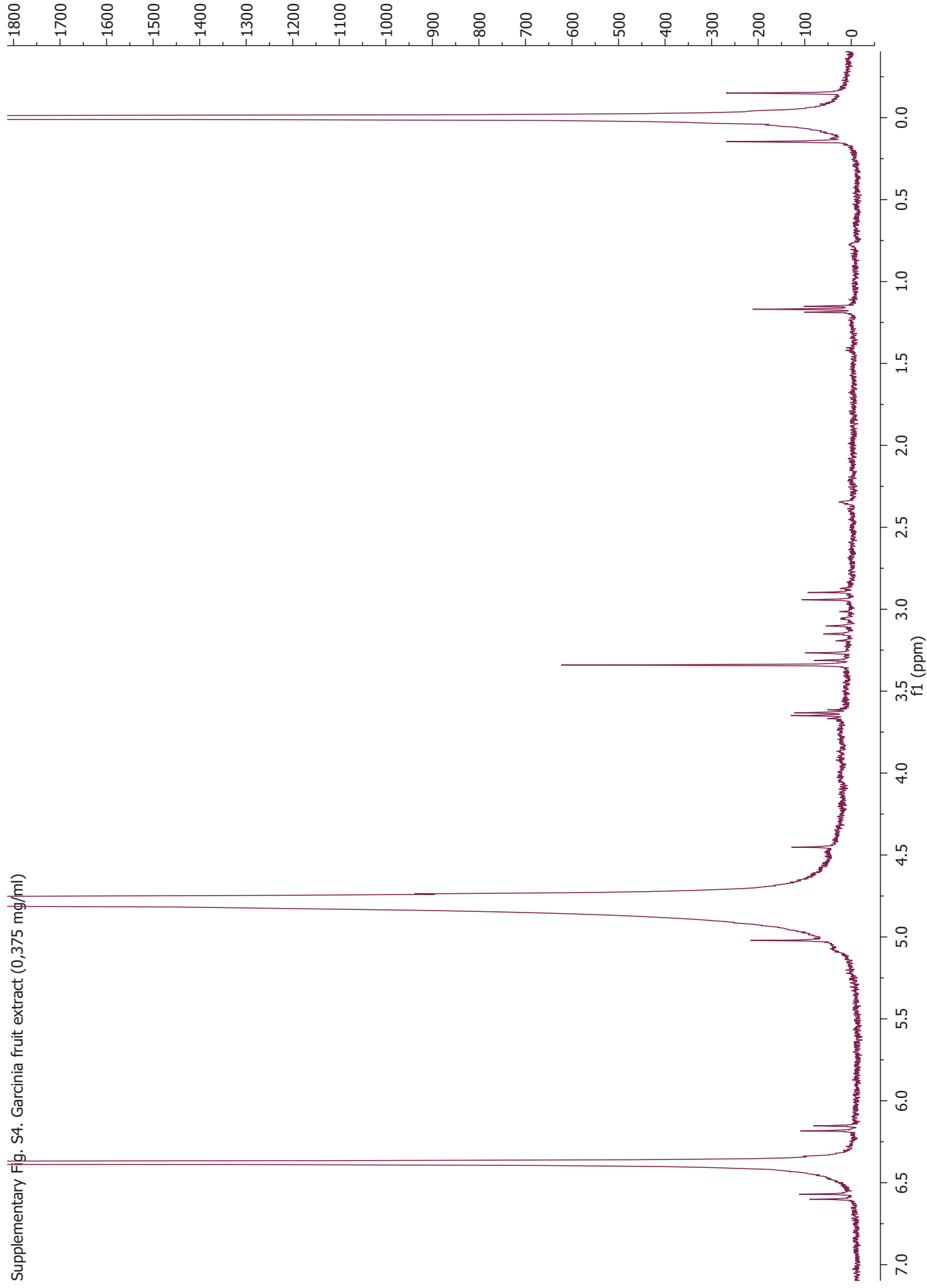Supplementary Fig. S4. Garcinia fruit extract (1.5 mg/ml)

Supplementary Fig. S4. Garcinia fruit extract (0,75 mg/ml)

Supplementary Fig. S4. Garcinia fruit extract (0.375 mg/ml)

Supplementary Fig. S4. Garcinia fruit extract (0,1875 mg/ml)
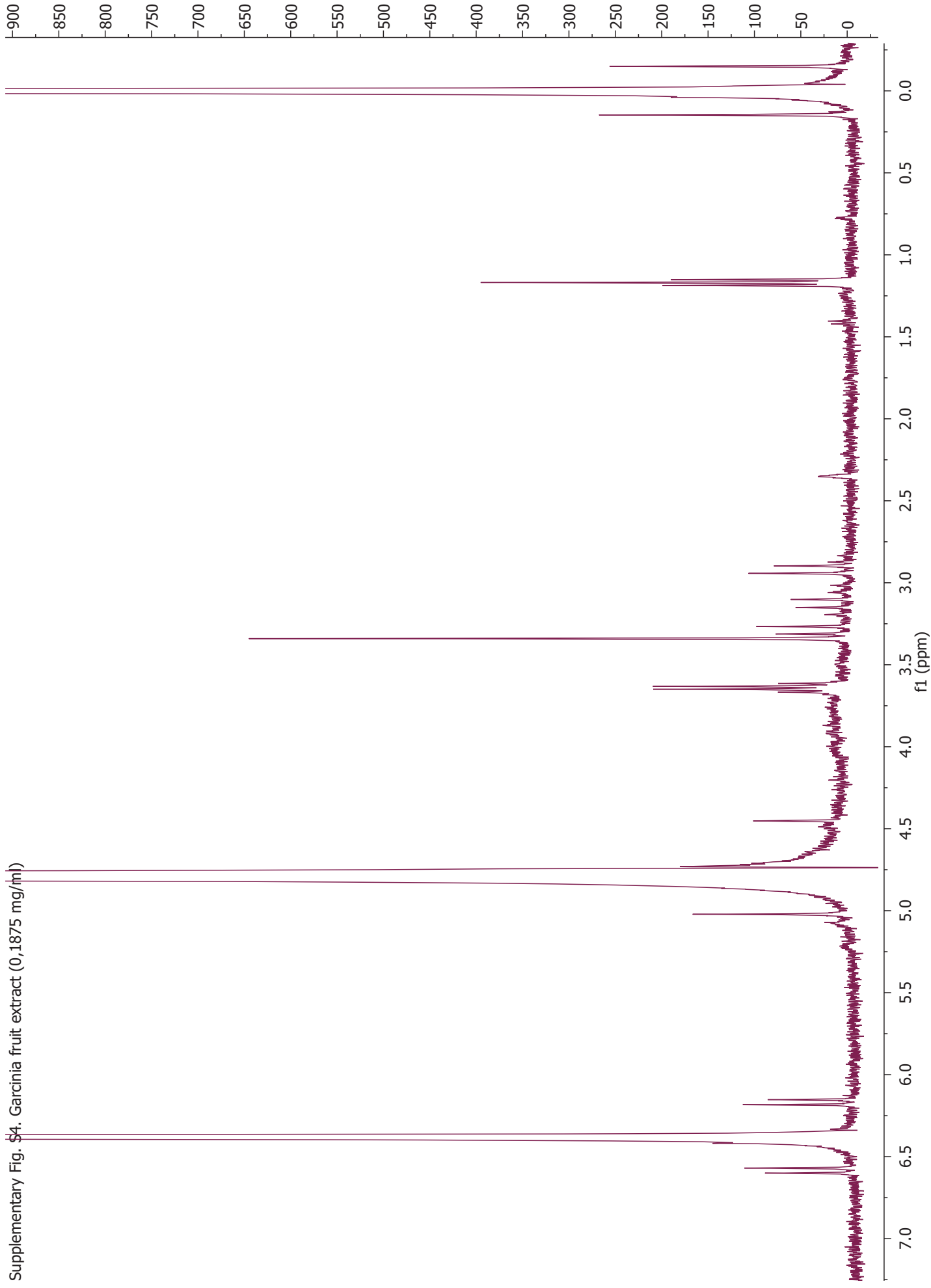
Supplementary Fig. S5. ¹H NMR spectrum of *Garcinia* fruits extract

Supplementary Fig. S5. Garcinia fruit (HAS388)
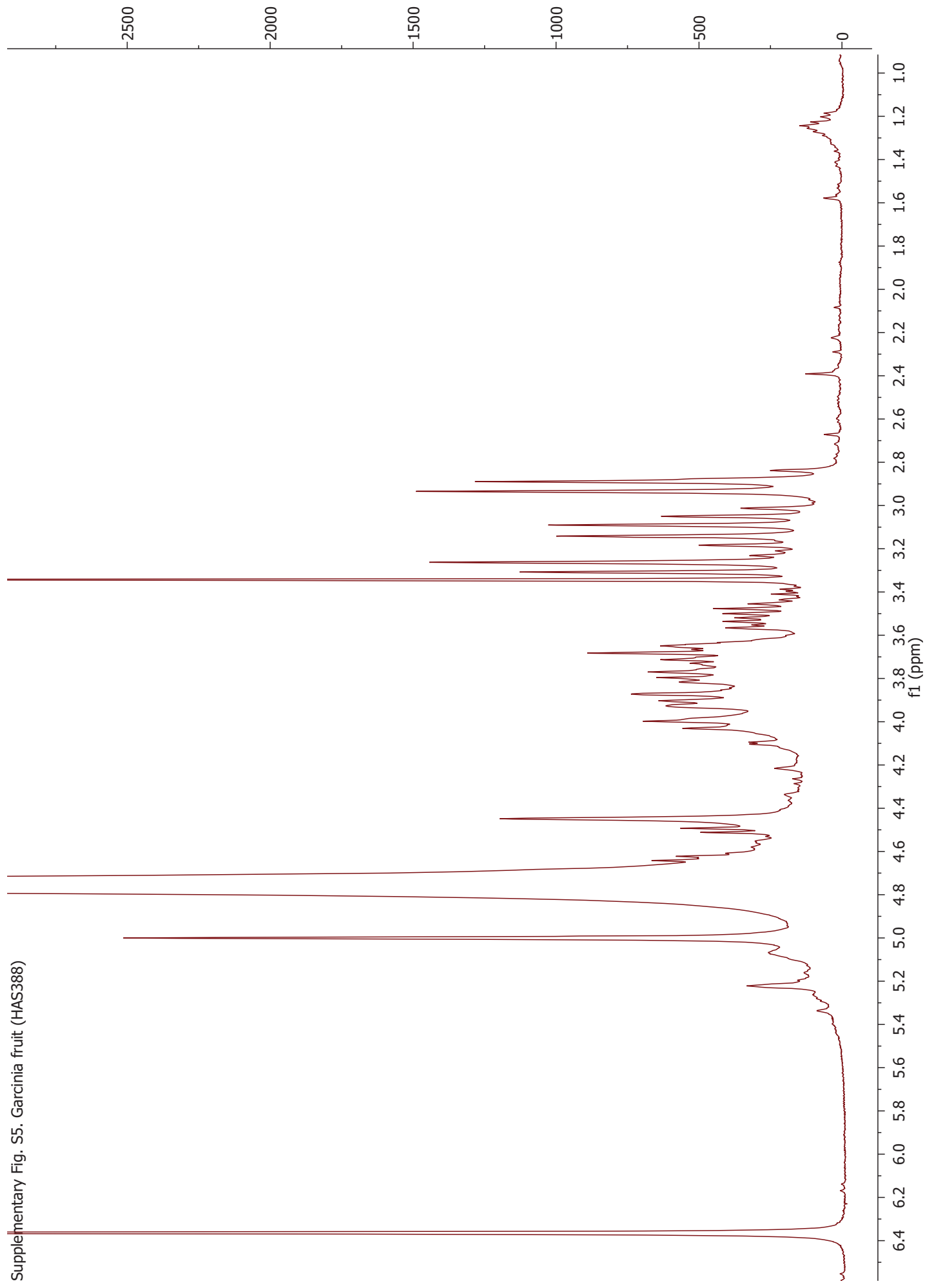
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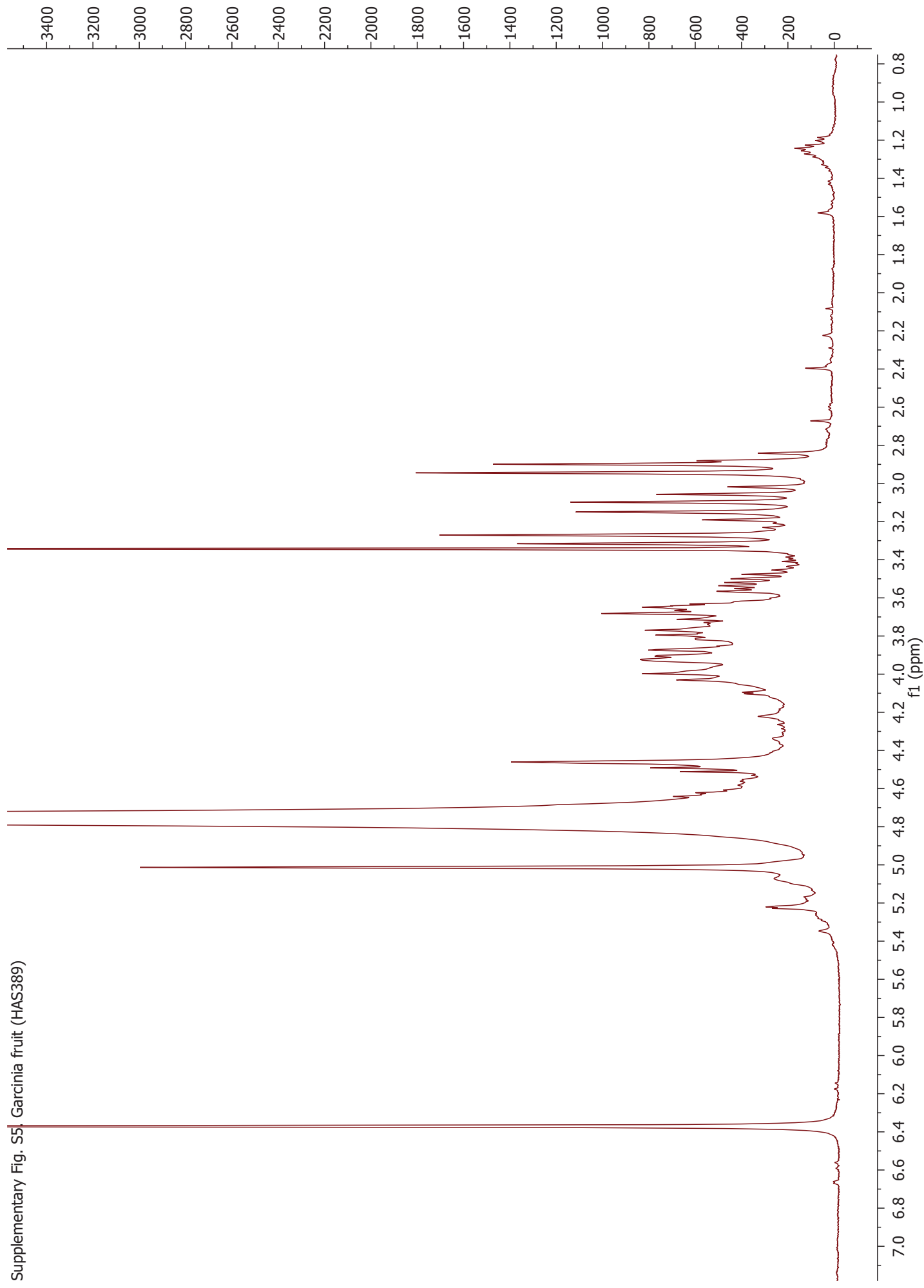
Supplementary Fig. S5. Garcinia fruit (HAS473)
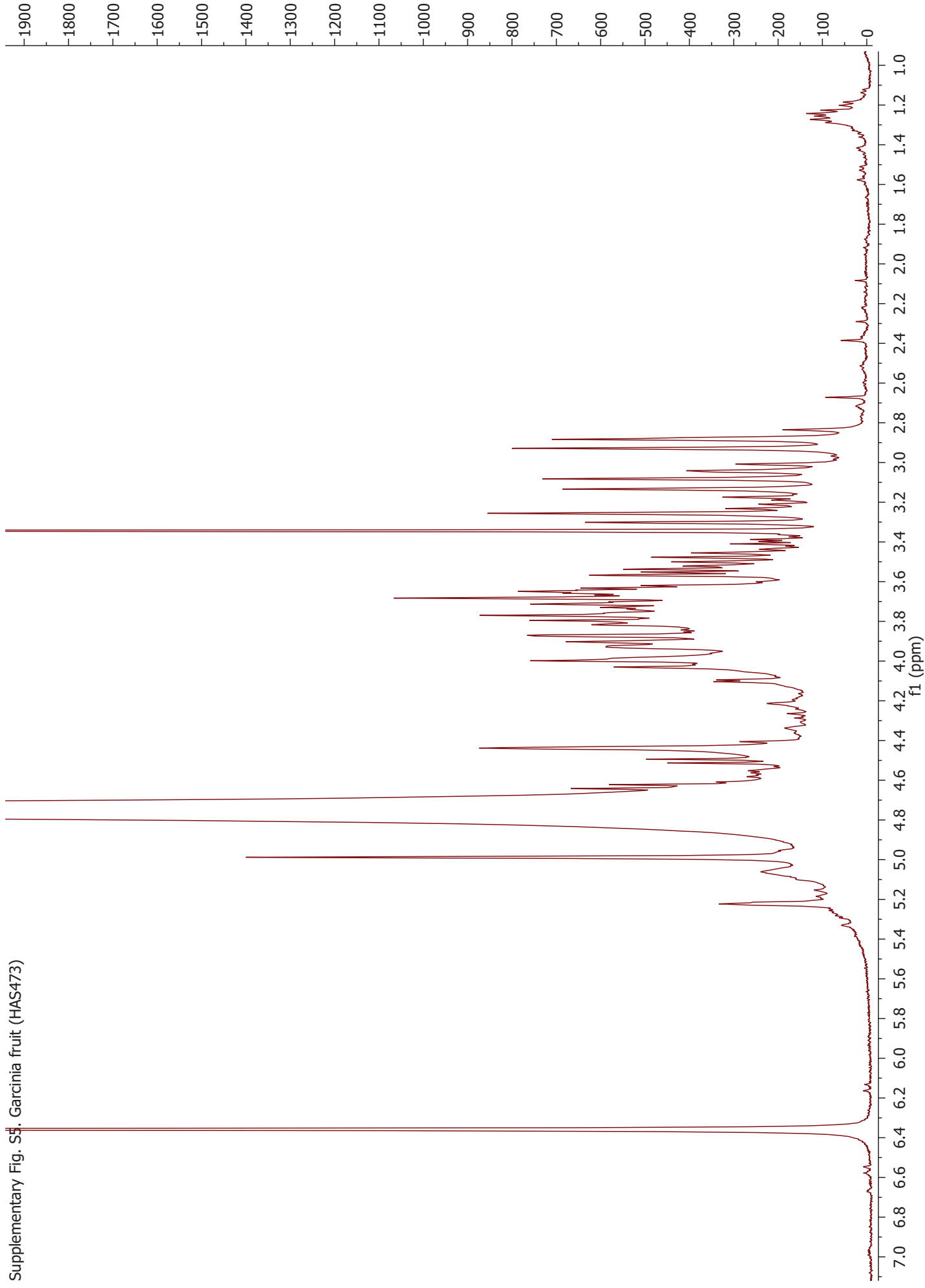
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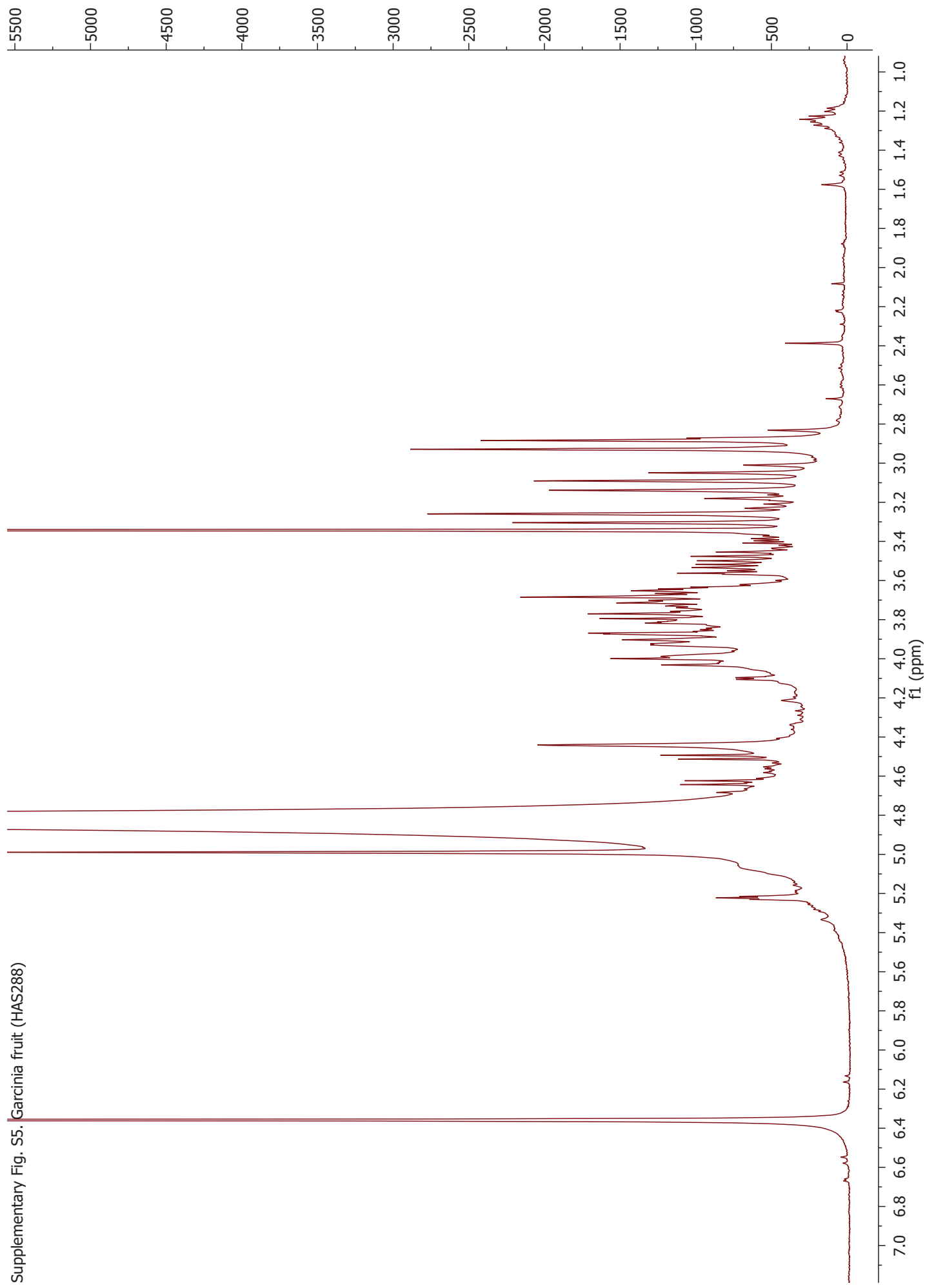
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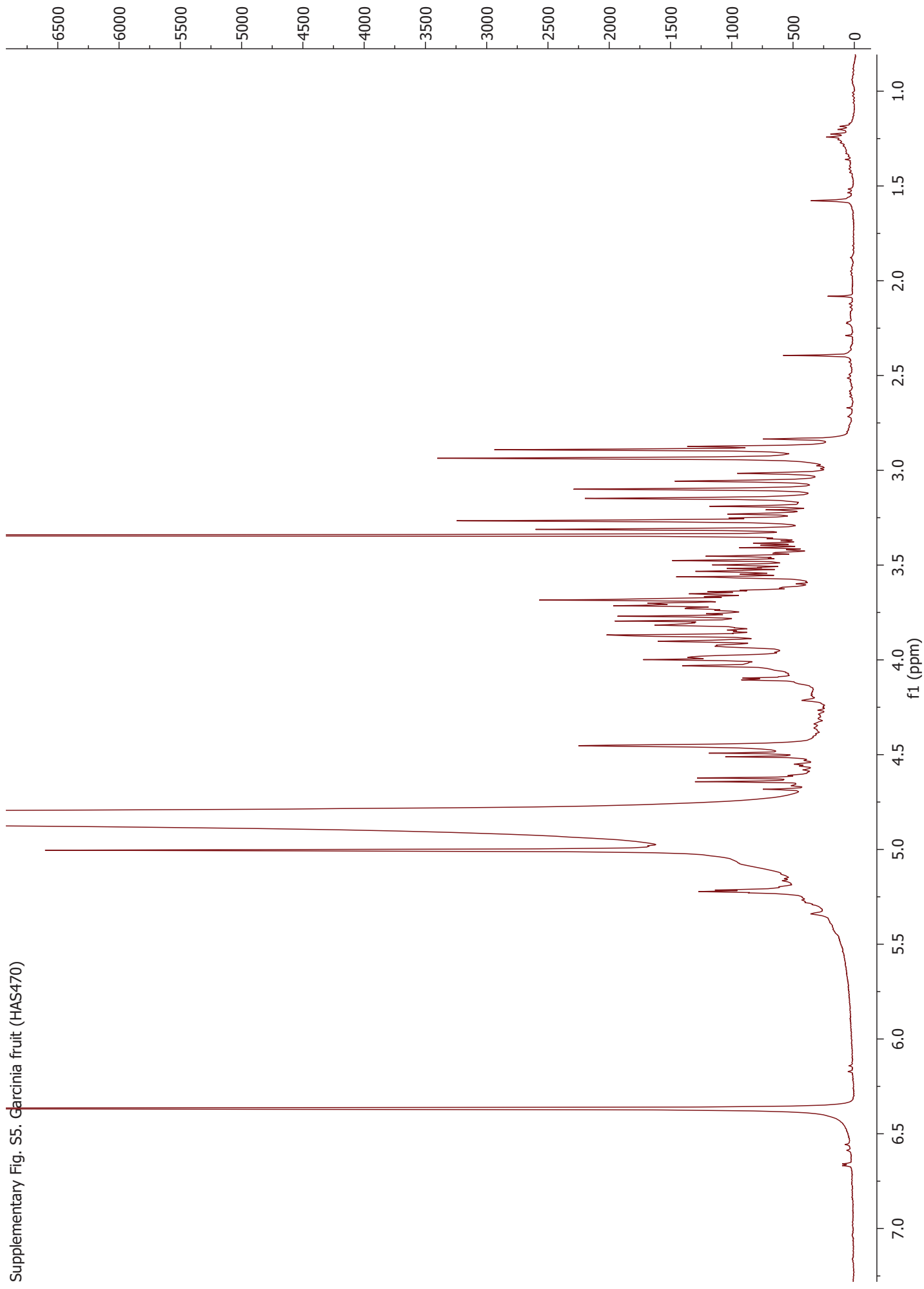
Supplementary Fig. S5. Garcinia fruit (HAS203)
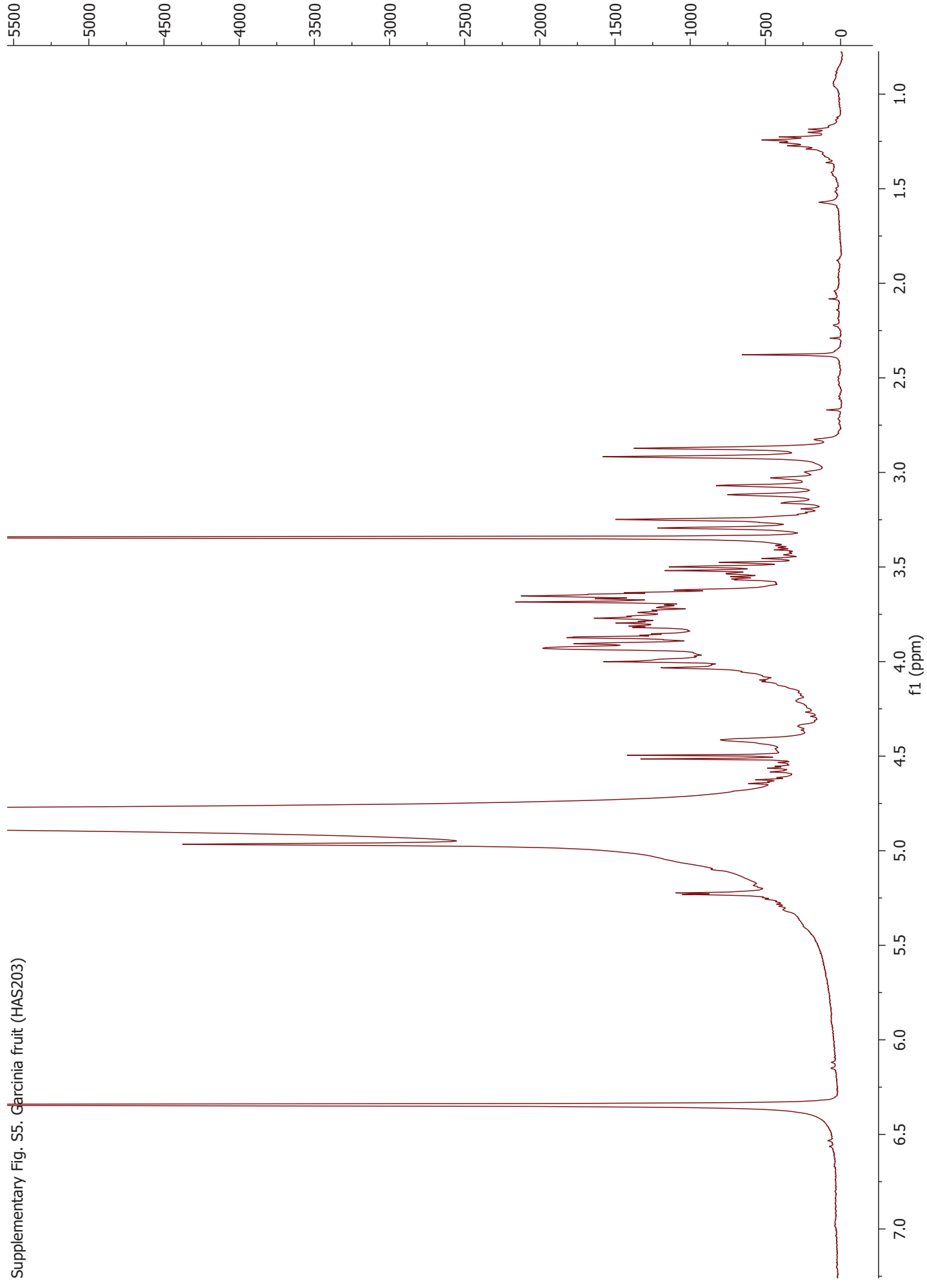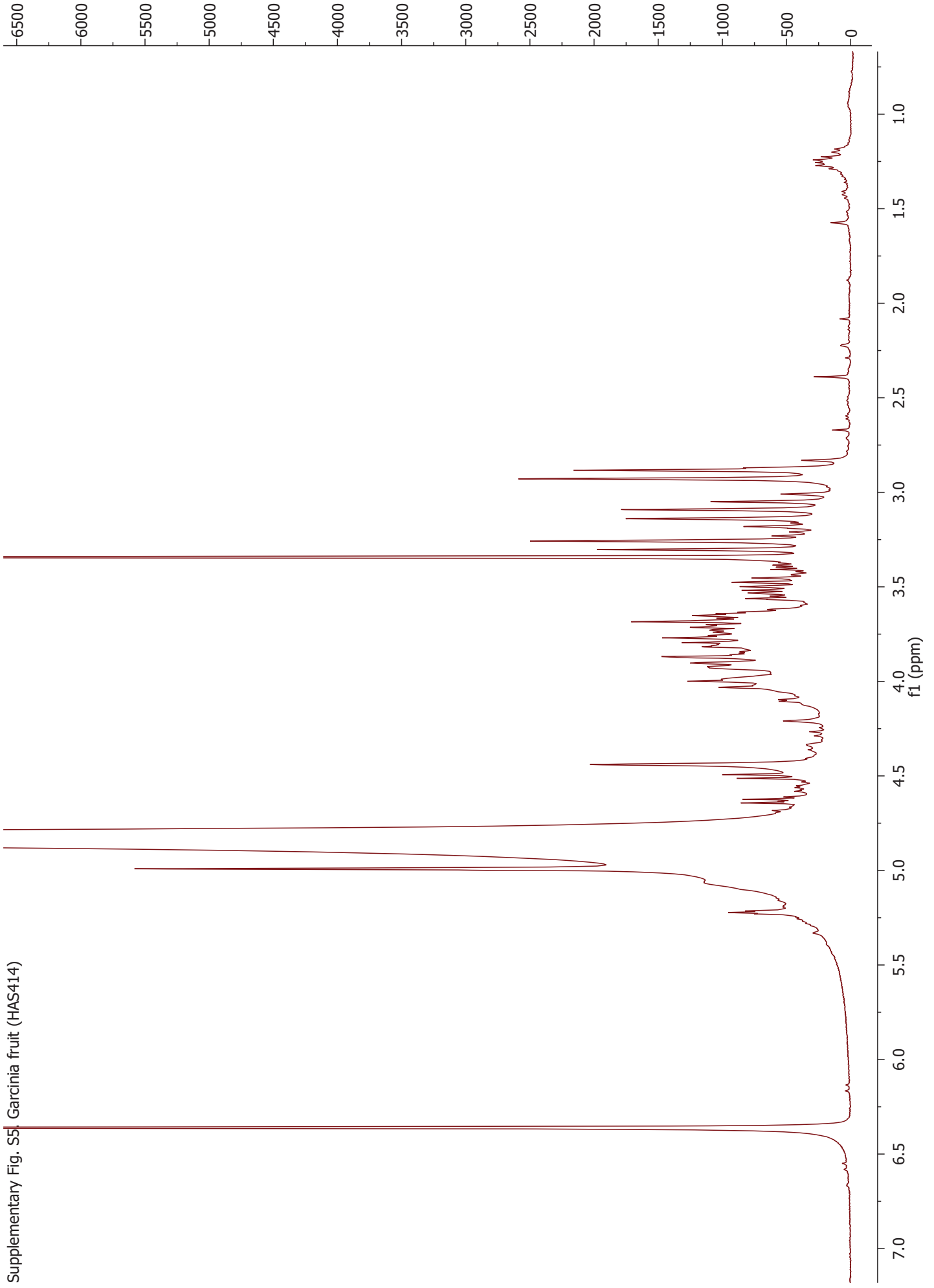
Supplementary Fig. S5. Garcinia fruit (HAS414)

Supplementary Fig. S5. Garcinia fruit (HAS399)
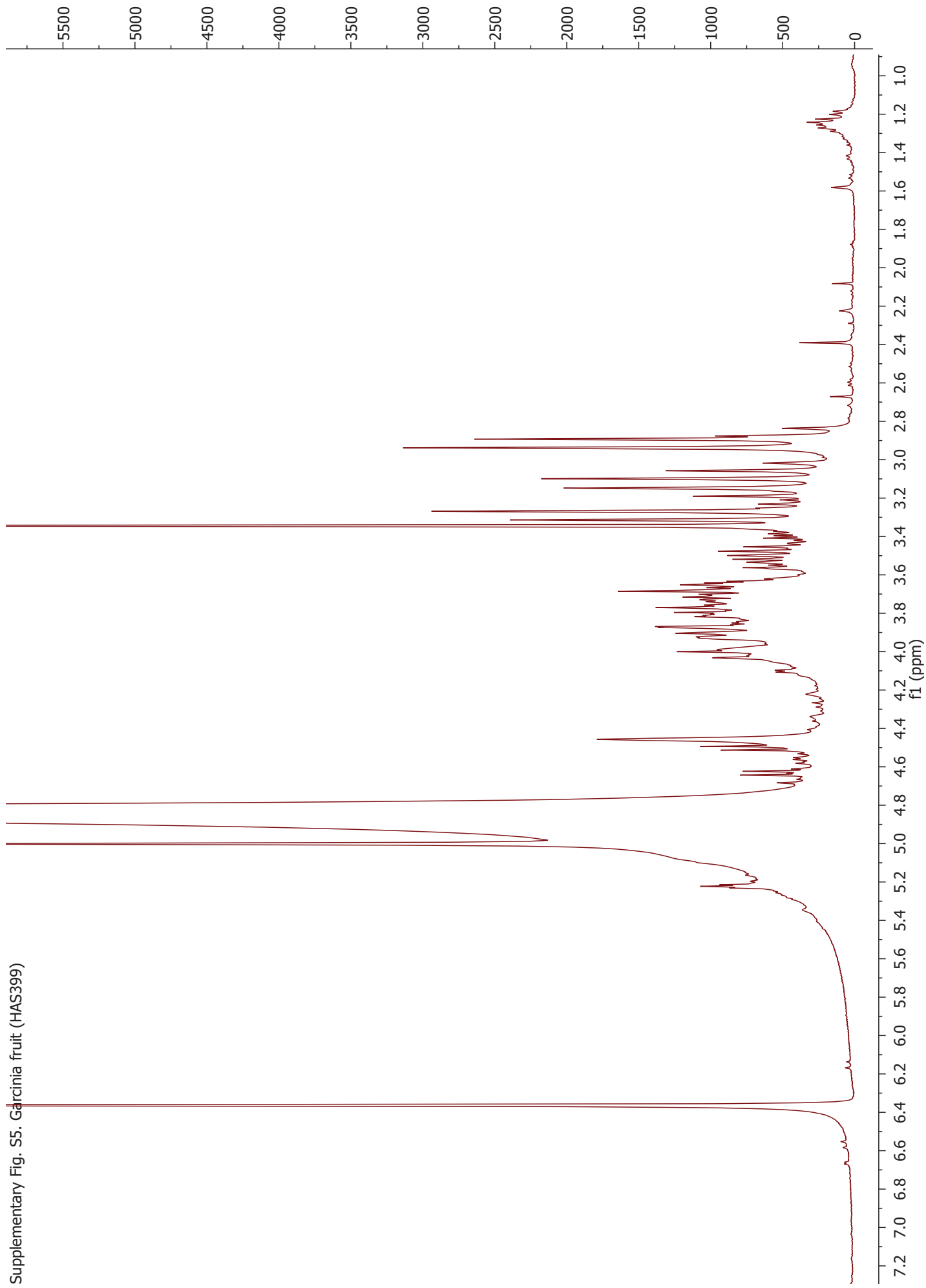
Supplementary Fig. S5. Garcinia fruit (HAS365)
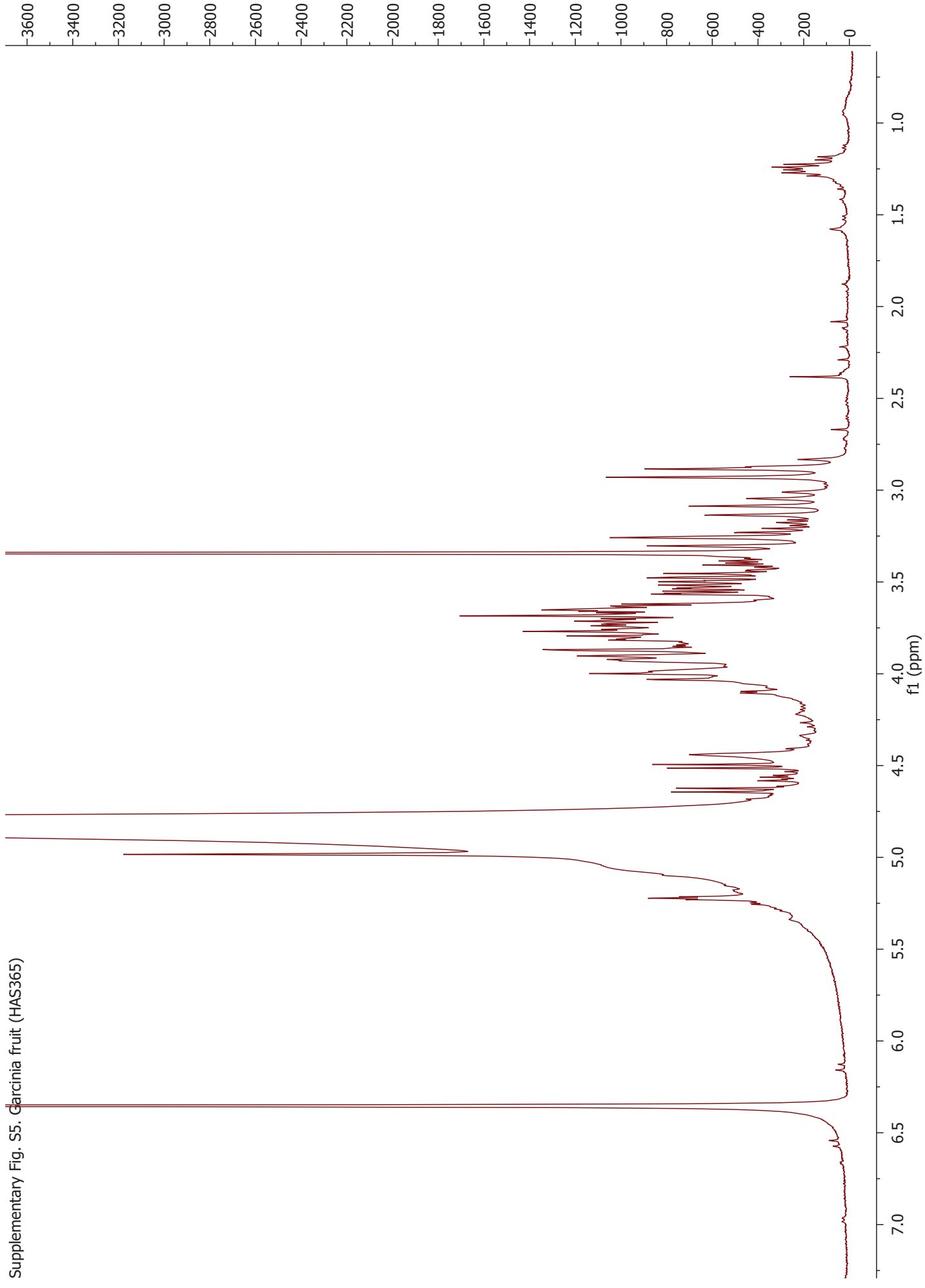
Supplementary Fig. S5. Garcinia fruit (HAS291)
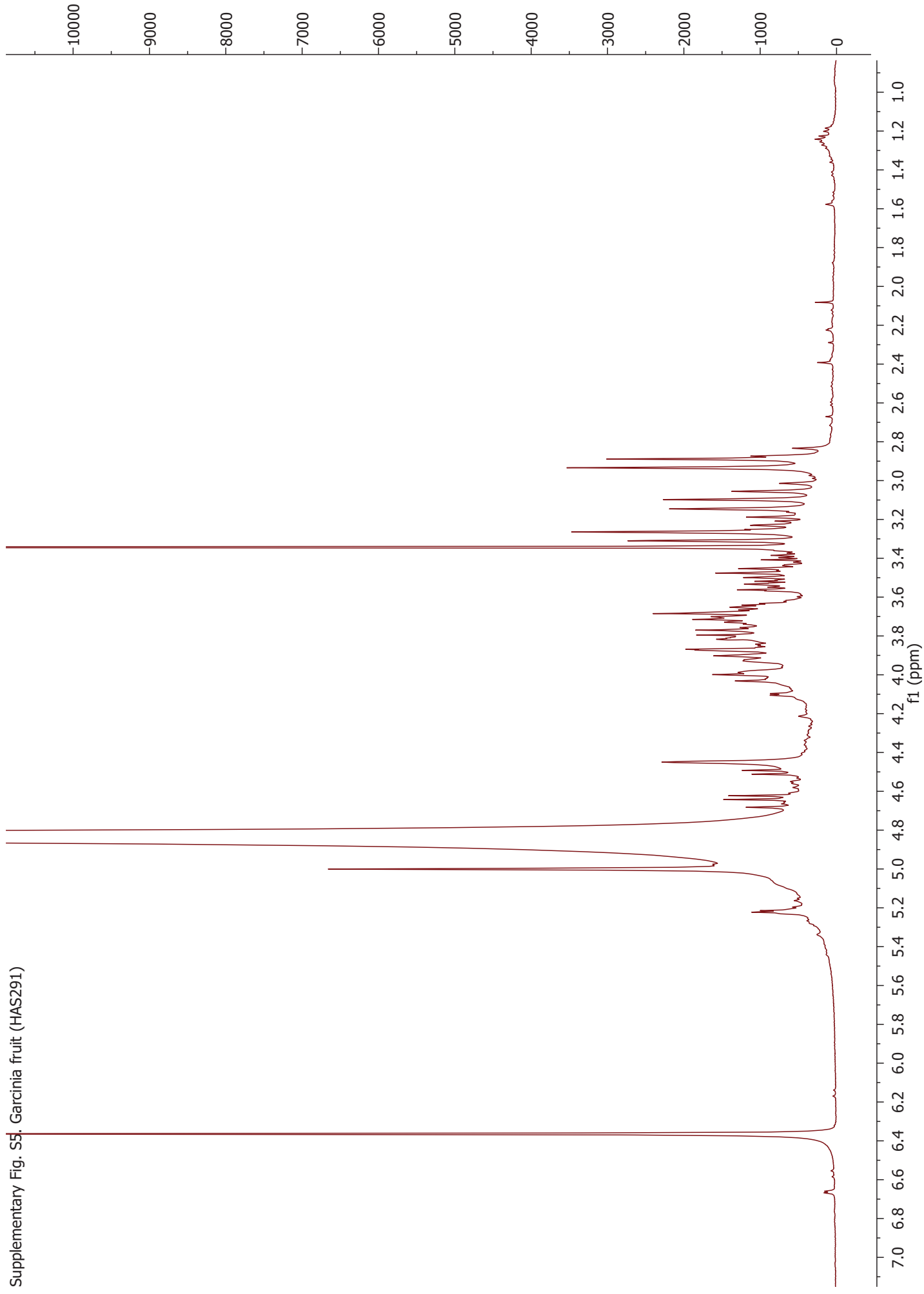
Supplementary Fig. S6. ^{1}H NMR spectrum of analyzed *Garcinia* food supplements.

Supplementary Fig. S6. Garcinia food supplement-1
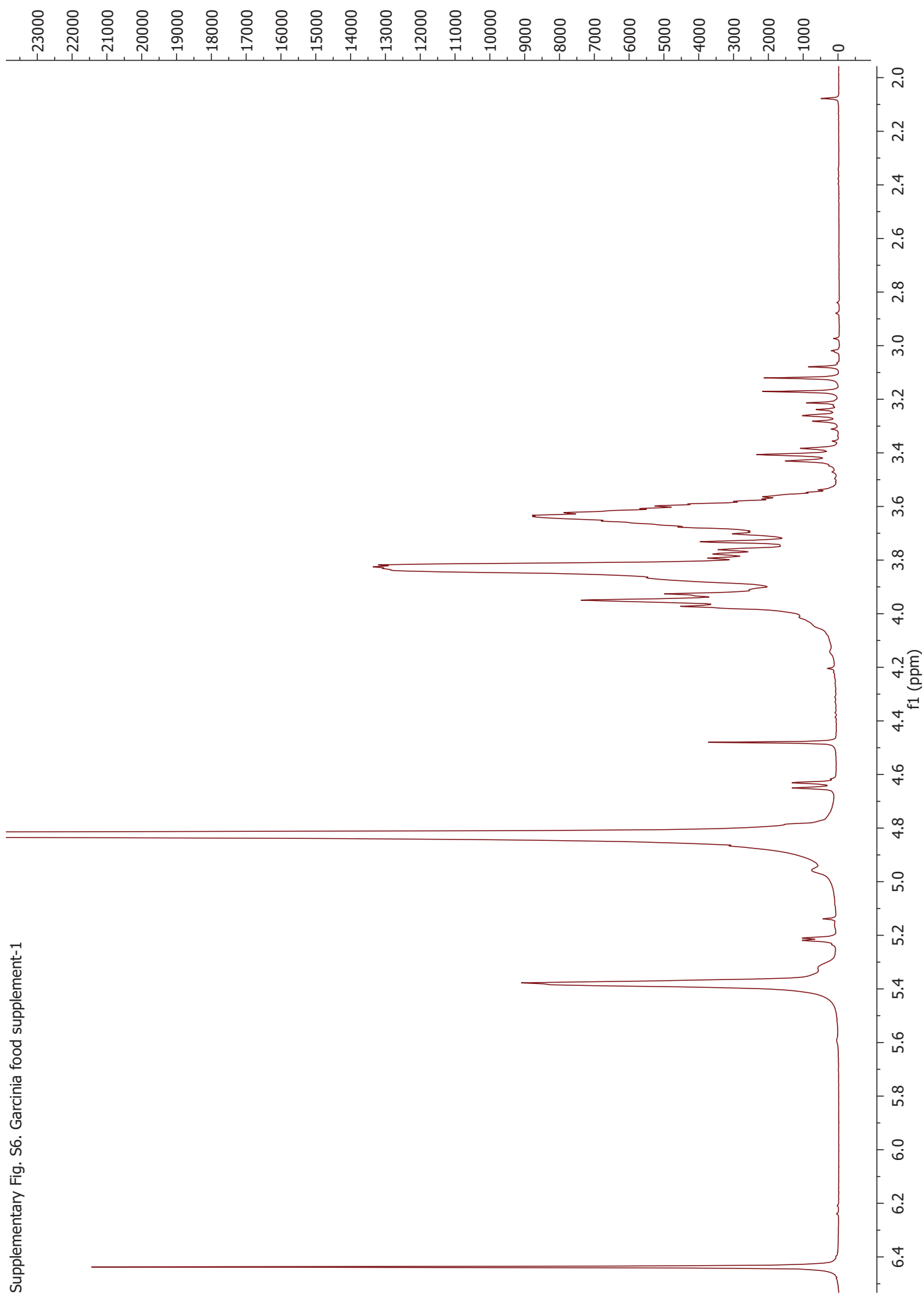
Supplementary Fig. S6. Garcinia food supplement-2
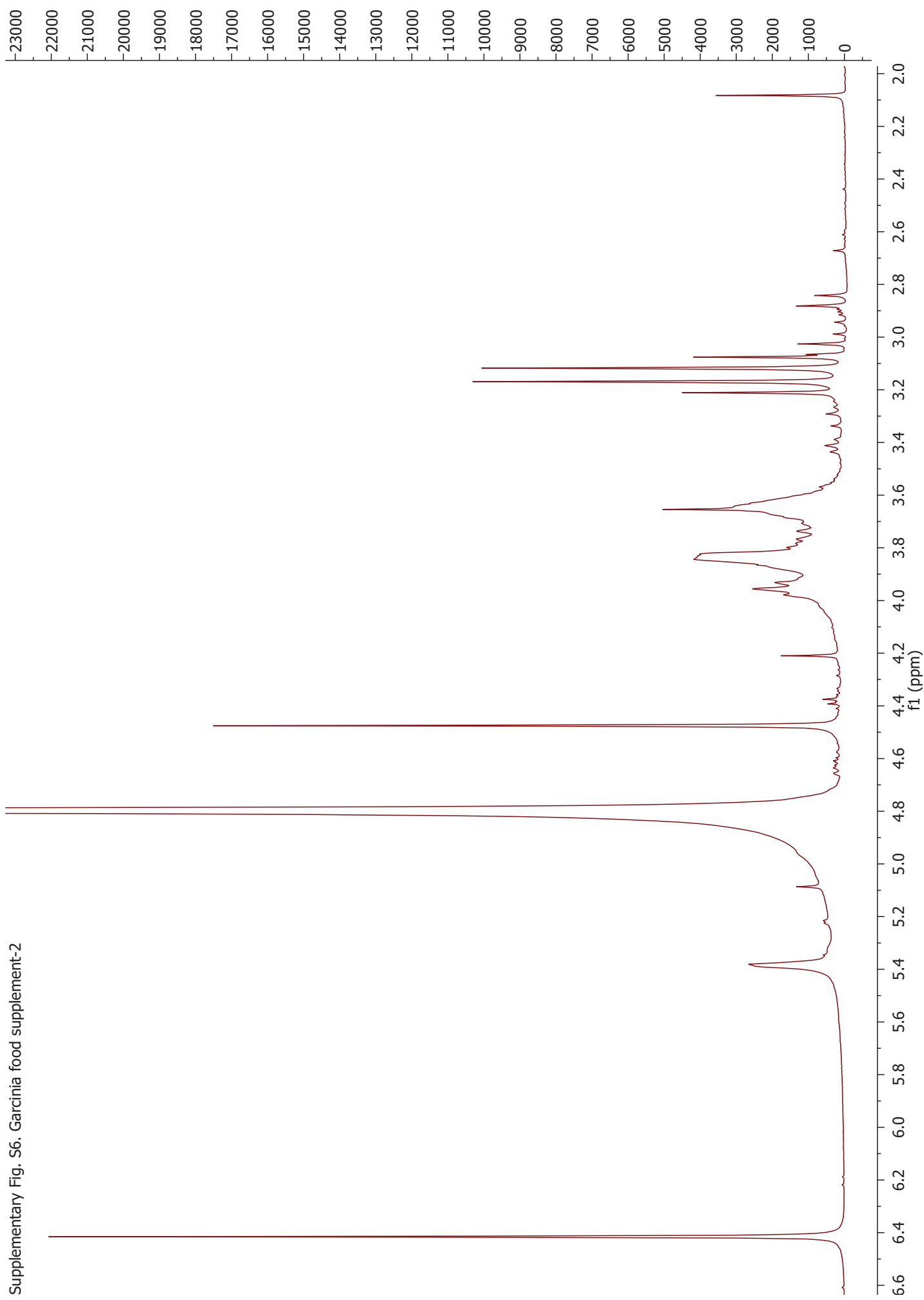
Supplementary Fig. S6. Garcinia food supplement-3

Supplementary Fig. S6. Garcinia food supplement-4

Supplementary Fig. S6. Garcinia food supplement-5

Supplementary Fig. S6. Garcinia food supplement-6
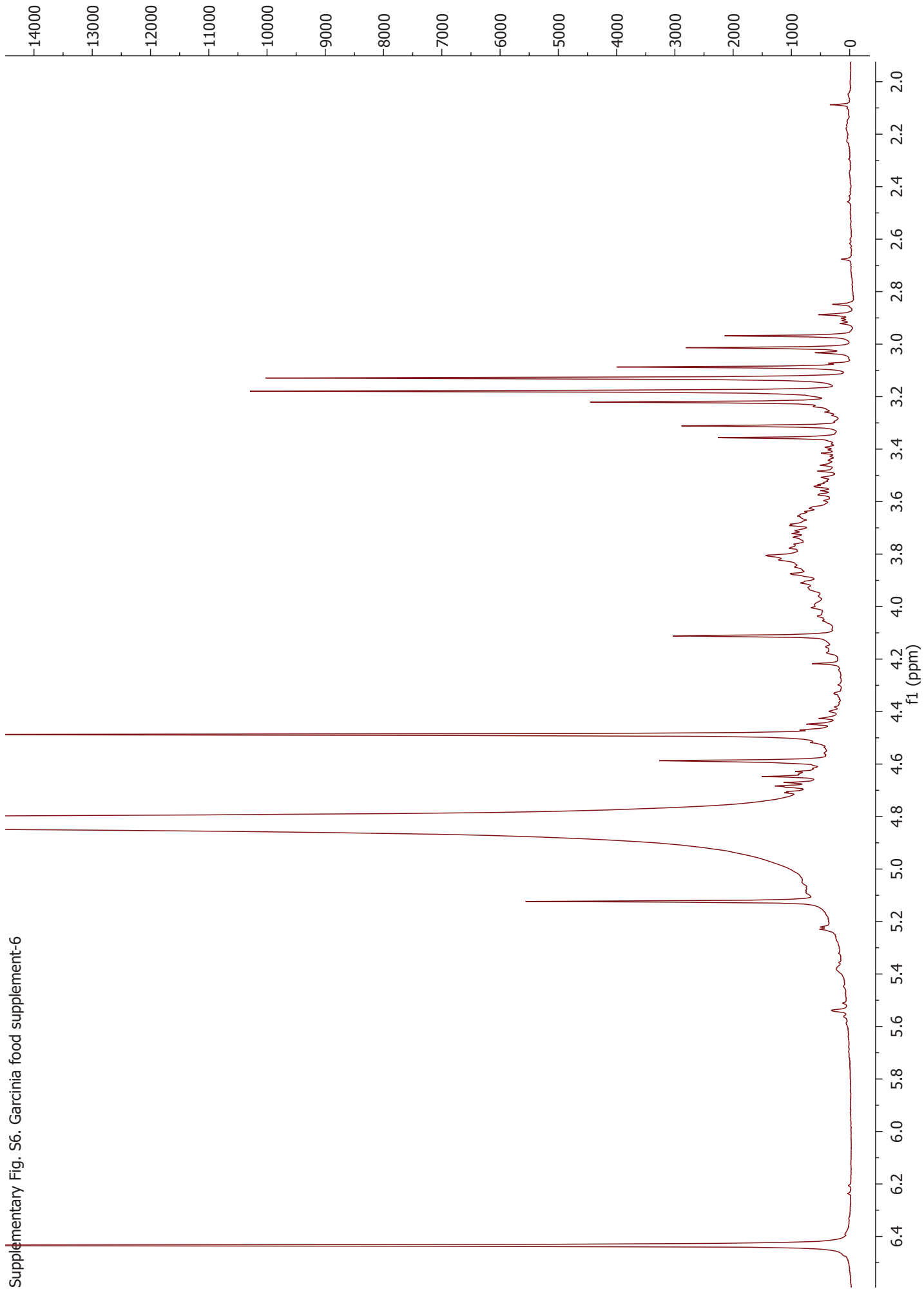
Supplementary Fig. S6. Garcinia food supplement-7
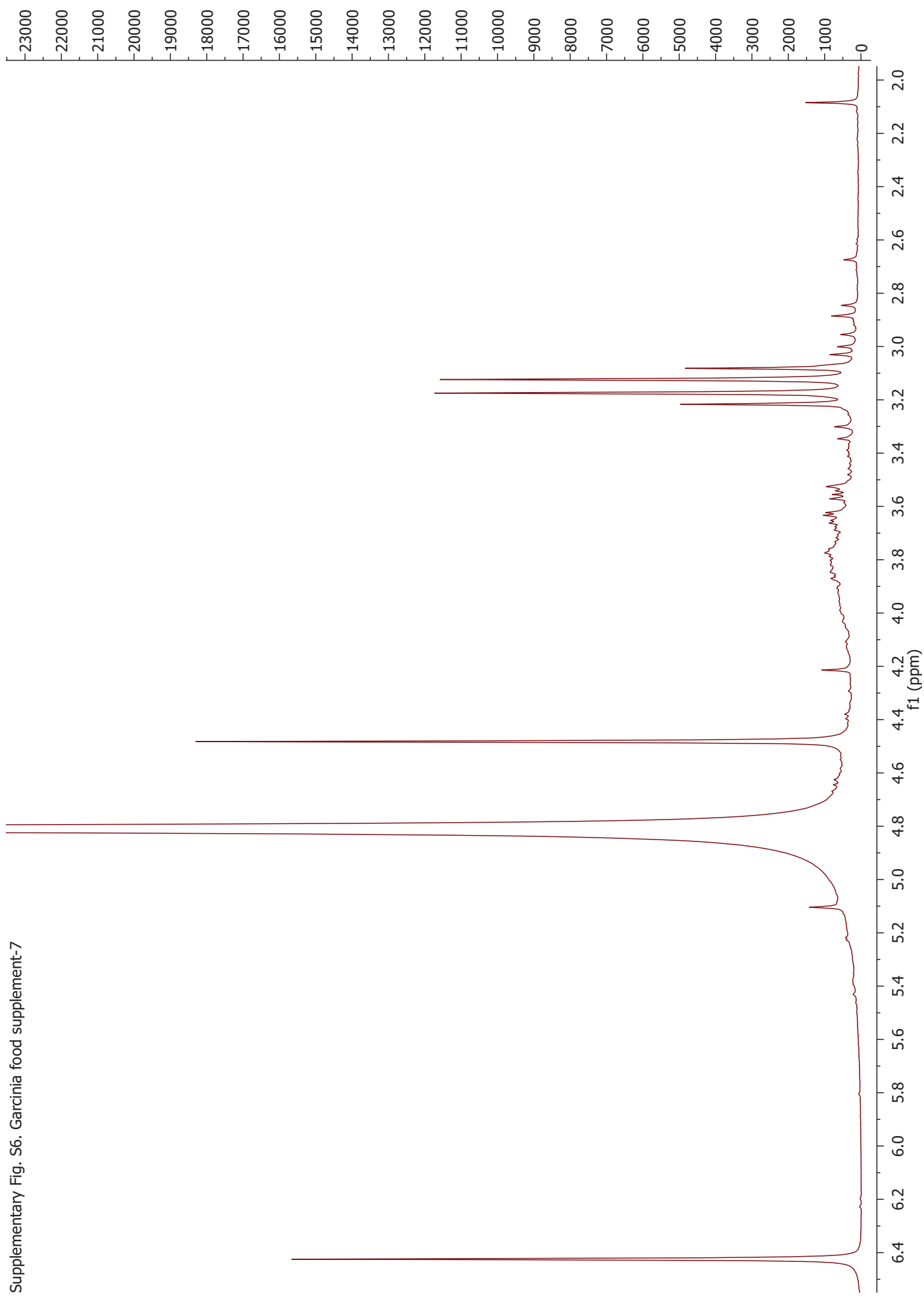
Supplementary Fig. S6. Garcinia food supplement-8
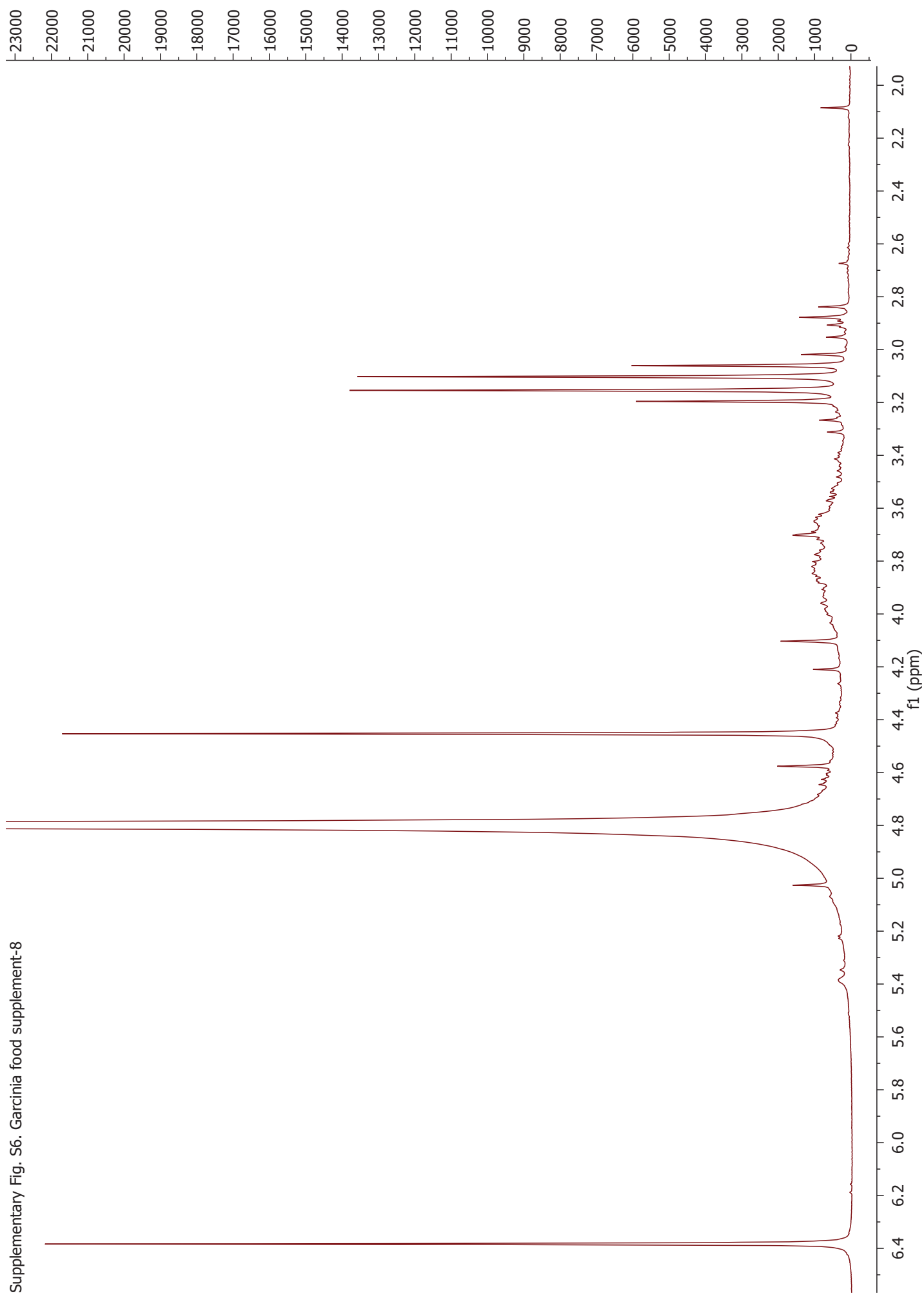
Supplementary Fig. S6. Garcinia food supplement-9
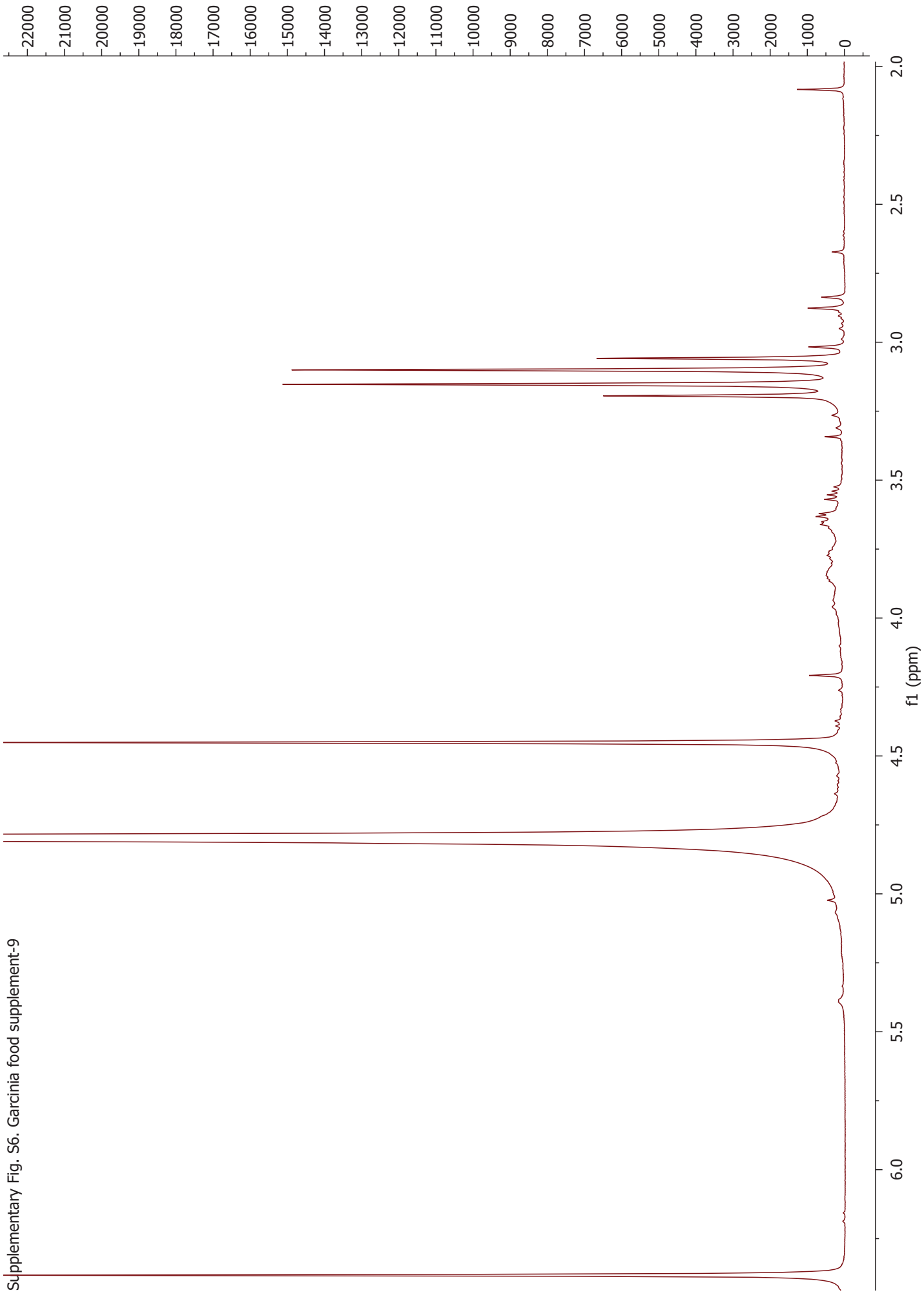
Supplementary Fig. S6. Garcinia food supplement-10
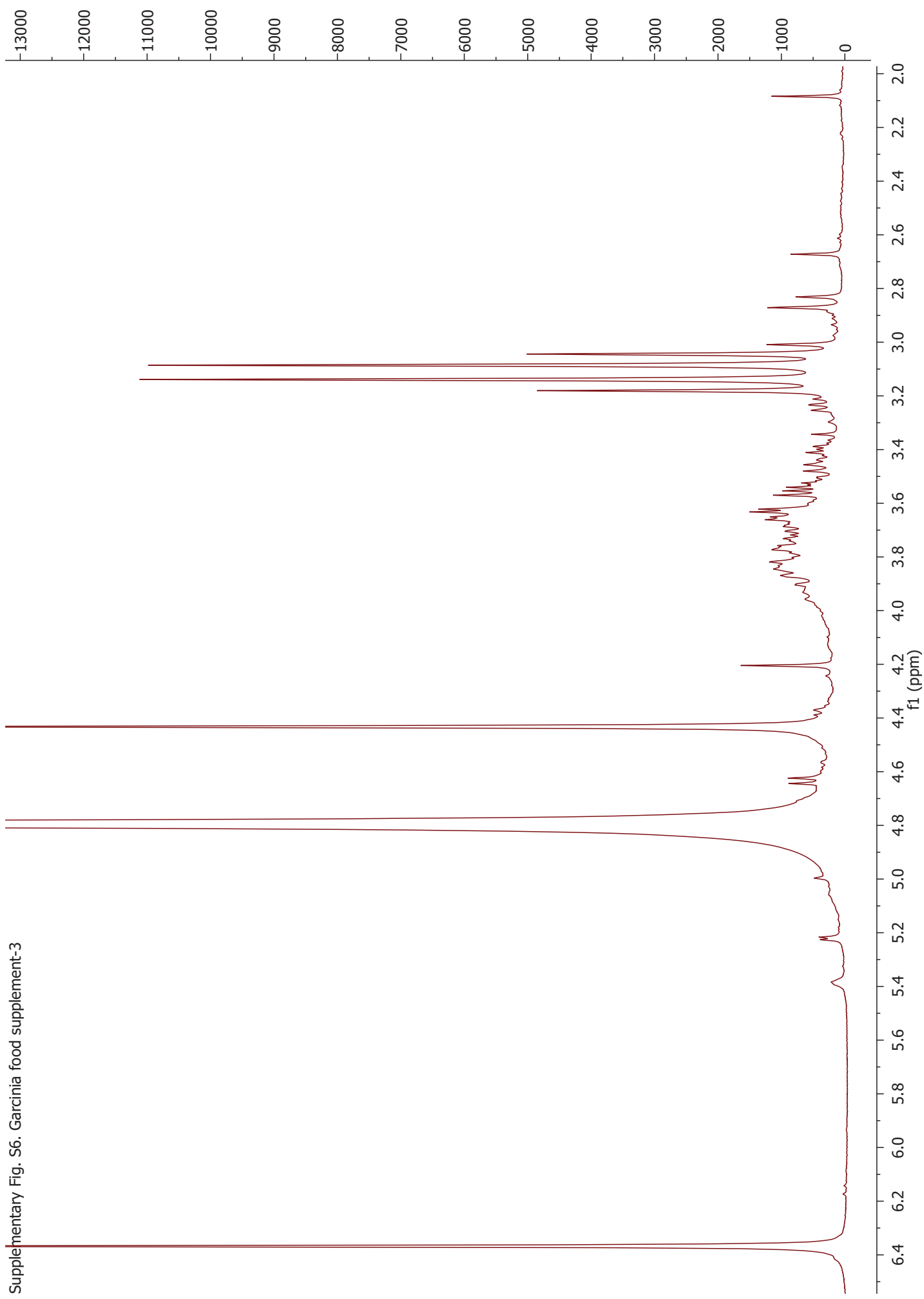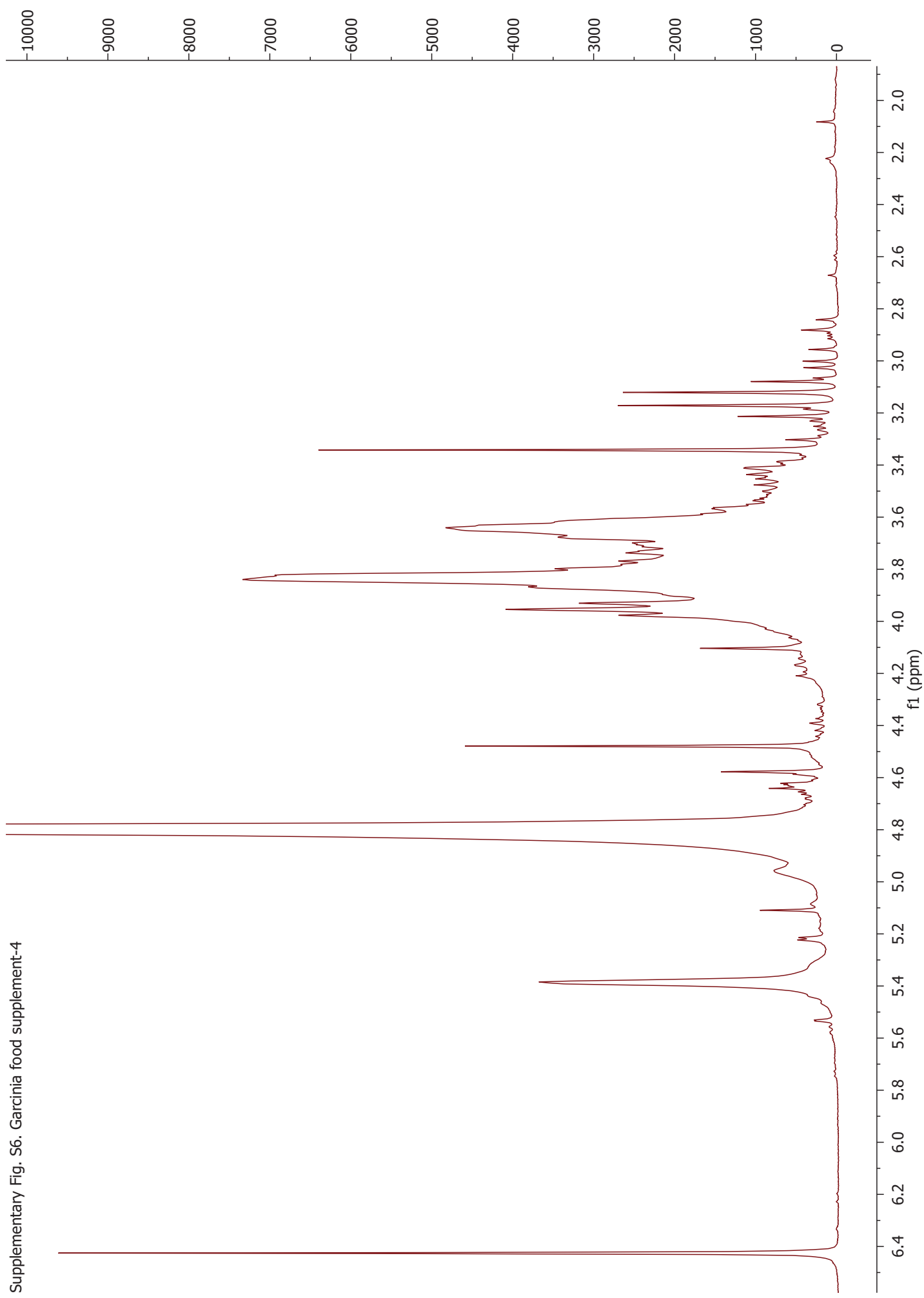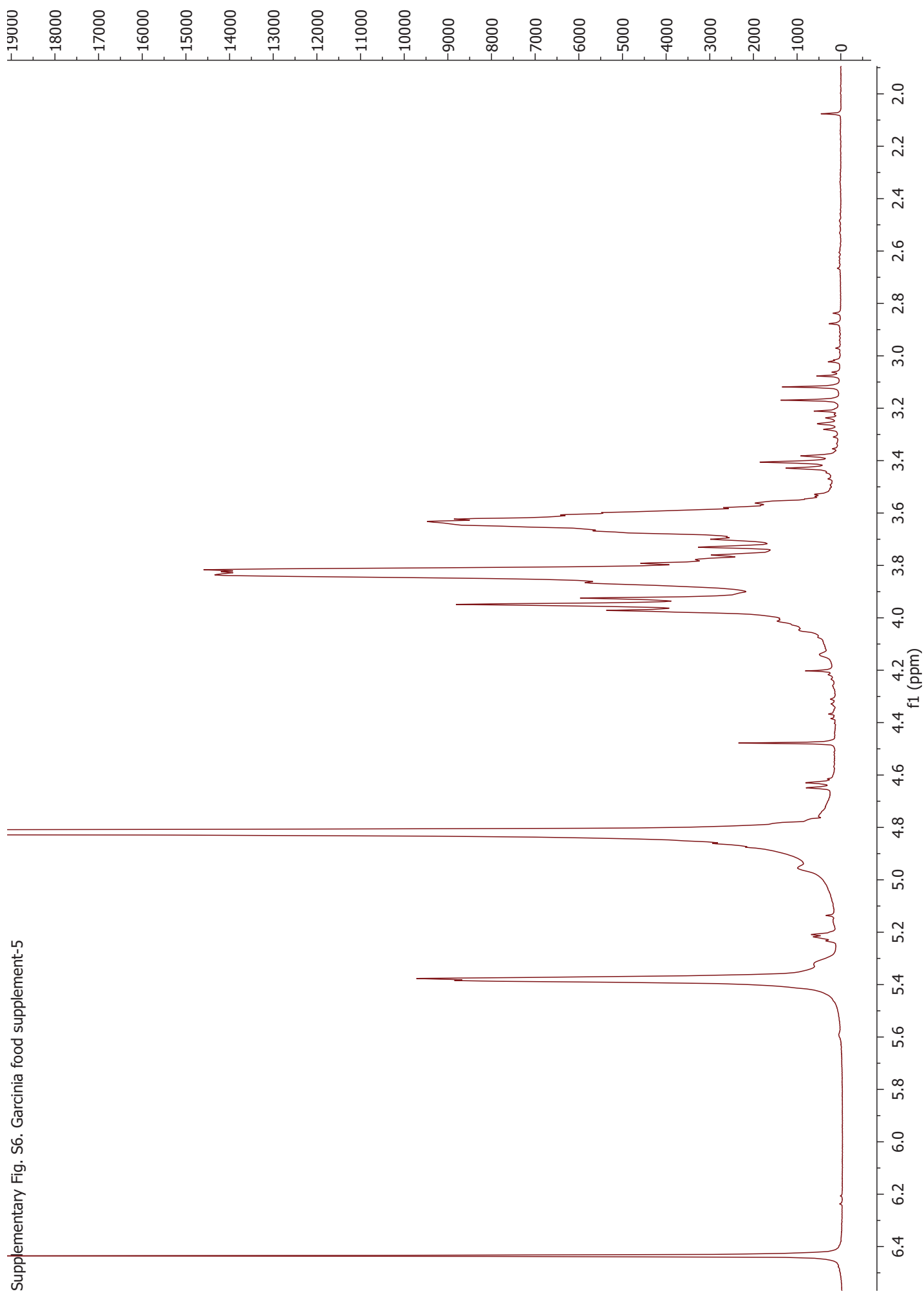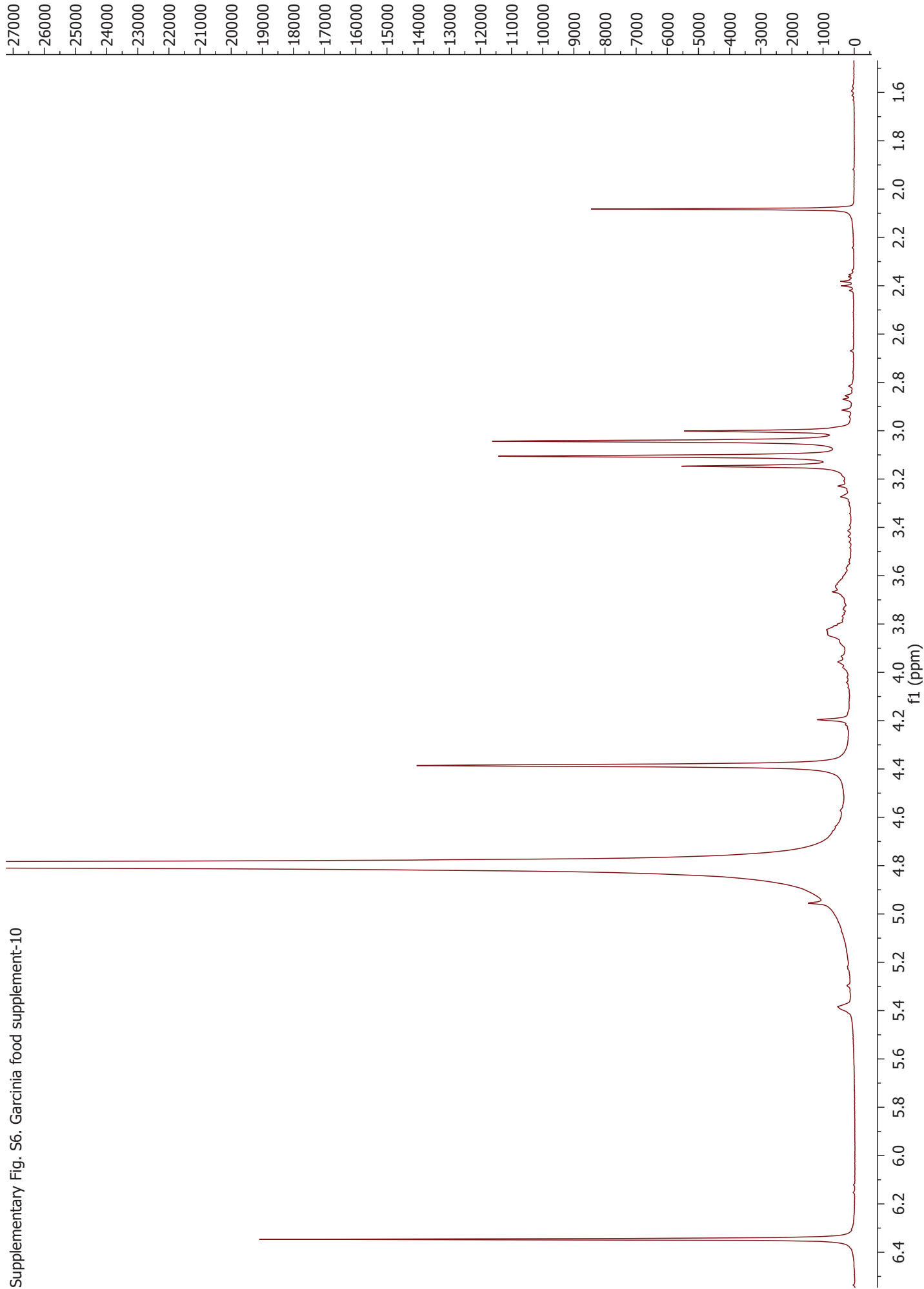
IV

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DNA Metabarcoding Authentication of Ayurvedic Herbal Products on the European Market Raises Concerns of Quality and Fidelity

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Ayurveda is one of the oldest systems of medicine in the world, but the growing commercial interest in Ayurveda based products has increased the incentive for adulteration and substitution within this herbal market. Fraudulent practices such as the use of undeclared fillers and use of other species of inferior quality is driven both by the increased as well as insufficient supply capacity of especially wild plant species. Developing novel strategies to exhaustively assess and monitor both the quality of raw materials and final marketed herbal products is a challenge in herbal pharmacovigilance. Seventy-nine Ayurvedic herbal products sold as tablets, capsules, powders, and extracts were randomly purchased via e-commerce and pharmacies across Europe, and DNA metabarcoding was used to assess the ability of this method to authenticate these products. Our analysis reveals that only two out of 12 single ingredient products contained only one species as labeled, eight out of 27 multiple ingredient products contained none of the species listed on the label, and the remaining 19 products contained 1 to 5 of the species listed on the label along with many other species not specified on the label. The fidelity for single ingredient products was 67%, the overall ingredient fidelity for multi ingredient products was 21%, and for all products 24%. The low level of fidelity raises concerns about the reliability of the products, and detection of threatened species raises further concerns about illegal plant trade. The study highlights the necessity for quality control of the marketed herbal products and shows that DNA metabarcoding is an effective analytical approach to authenticate complex multi ingredient herbal products. However, effort needs to be done to standardize the protocols for DNA metabarcoding before this approach can be implemented as routine analytical approaches for plant identification, and approved for use in regulated procedures.

Keywords: Ayurvedic herbal products, botanical authentication, DNA barcoding, herbal medicines, pharmacovigilance, quality control

INTRODUCTION

Ayurveda, or Ayurvedic medicine, is one of the oldest systems of traditional medicine (TM), with origins in India more than 3,000 years ago. Nowadays Ayurveda is popular and used worldwide in complementary and alternative healthcare and medical practices (CAM) (World Health Organization [WHO], 2013). Ayurvedic formulations are obtained using an average of 80% botanicals, 12% animals, and 8% minerals, and are used as raw materials and preparations such as extracts (Joshi et al., 2017). About 7,000 plant species are used for medicinal purposes in India, from which, about 1,200 species have been reported to be actively traded (Goraya and Ved, 2017). The total commercial demand for herbal material in India, in 2014 and 2015, was estimated to be in excess of 512,000 tons, with a market value of 1 billion USD (Goraya and Ved, 2017). India has more than 8,000 licensed manufacturing units for medicinal products and the increasing level of consumption of herbal products exceed the supply capacity for some plant species (Goraya and Ved, 2017). In order to ensure a level of uniformity of the therapeutic formula and the ingredients used, the Ayurvedic formulary and Ayurvedic Pharmacopeia of India was published by the Government of India as a legally binding document describing the quality, purity, and strength of selected drugs that are manufactured, distributed and sold by the licensed manufacturers in India (Joshi et al., 2017).

As many other TMs, Ayurvedic herbal medicines, require quality assurances for their wider usage and acceptability in CAM practicing countries (World Health Organization [WHO], 2013). The growing demand for Ayurveda encourages an industry for mass production of herbal products, leading to the use of large quantities of plant raw material, mainly harvested from the wild flora (Valiathan, 2006; Goraya and Ved, 2017; Joshi et al., 2017). Many of the Indian medicinal plant species are in short supply due to the lack of cultivation and several wild species are not available in sufficient quantities for commercial exploitation (Goraya and Ved, 2017). The intensive use of herbal products increases the incentive for adulteration and substitution in the medicinal plant trade (Newmaster et al., 2013). This awareness of content irregularities calls attention to the quality of the traded mass produced herbal products with direct impact on their efficacy and safety (Leonti and Casu, 2013). One of the pharmacognostic parameters to assure quality, safety and efficacy of a herbal medicine is the utilization of correctly identified medicinal plants used as raw material (Evans, 2009). Several new strategies and appropriate standard methods have been proposed to exhaustively assess and monitor both the quality of raw materials and marketed herbal products (Barnes, 2003; De Boer et al., 2015). Standard methods routinely used to assess herbal material, preparations and products rely on morphological characters, microscopy, and chemical fingerprinting [i.e., thin–layer chromatography', high–performance liquid chromatography (HPLC), and gas chromatography (GC)] (De Boer et al., 2015; Parveen et al., 2016). These methods are quick and cost-effective techniques for primary qualitative analysis of raw material and derived herbal products. Alternatively, the use of more advanced methods for identification and quantification of chemical marker compounds

is becoming popular [i.e., liquid chromatography (LC)–mass spectrometry (MS), GC-MS, and LC-nuclear magnetic resonance (NMR)], but requires valuable instrumentation (Jiang et al., 2010; Wang et al., 2017; Zhang et al., 2017; Raclariu et al., 2018a).

Various important issues influence the quality of Ayurvedic herbal products and they need to be carefully taken into consideration when determining the analytical method of choice for quality control. The herbal products are usually complex mixtures of plant material and/or extracts and excipients, and results of manifold processing steps. To apply only standard analytical methods may pose serious challenges to the accuracy of herbal product quality control. Furthermore, adulteration by the deliberate use or admixture of substitutes and undeclared plant fillers, fraudulent adulteration by using fillers of botanical origin or plant materials of inferior quality (Zhang et al., 2012), the addition of pharmaceuticals or other synthetic substances in order to reach an expected effect or a certain level of marker compounds (Calahan et al., 2016; Rocha et al., 2016) raises concerns about the quality and safety of the herbal products. Multiple plant species as source for botanical drug as allowed in different pharmacopeias, as well as the accidental substitutions, all raise concerns ranging from simple misleading labeling to potential serious adverse drug reactions (Ernst, 1998; Heubl, 2010; Gilbert, 2011) or poisoning due to toxic contaminants (Chan, 2003).

All the standard analytical approaches, including sensory and chemical inspection may have a good resolution in quality control by detecting the quality and quantity of specific lead or phytochemical marker compounds. However, they are generally not applicable in identifying target plant species within a complex herbal product, and show low ability to detect non-targeted plant ingredients in herbal products (De Boer et al., 2015). To overcome this limitation, DNA-based approaches have been proposed as useful analytical tools for the quality control of herbs and herbal products (Parveen et al., 2016). DNA barcoding is a cost-effective, species-level identification based upon the use of short and standardized gene regions, known as 'barcodes' (Hebert et al., 2003). Several reviews have corroborated the diverse applicability of DNA barcoding in the field of medicinal plant research (Techen et al., 2014; De Boer et al., 2015). Initially used as an identification tool, DNA barcoding is now applied in the industrial quality assurance context to authenticate a wide range of herbal products (De Boer et al., 2015; Parveen et al., 2016; Sgamma et al., 2017).

The combination of High-Throughput Sequencing (HTS) and DNA barcoding, known as DNA metabarcoding, enables simultaneous high-throughput multi-taxa identification by using the extracellular and/or total DNA extracted from complex samples containing DNA of different origins (Taberlet et al., 2012). Several studies have utilized this approach in identifying and authenticating medicinal plants and derived herbal products. For example, *Echinacea* species, *Hypericum perforatum*, and *Veronica officinalis* were detected in 89, 68 and 15%, respectively, of the investigated herbal products (Raclariu et al., 2017a,b, 2018b). Similarly, Ivanova et al. (2016) found that 15 tested herbal supplements contained non-listed, non-filler plant DNA, and Cheng et al. (2014) showed that the quality of 27 tested herbal

preparations was highly affected by the presence of contaminants. Coghlan et al. (2012) revealed the species composition of 15 highly processed traditional Chinese medicines using DNA metabarcoding, and showed that the products contained species included on CITES appendices I and II. A number of studies in India have surveyed herbal raw drug markets and tested the authenticity of the herbal drugs using DNA barcoding. These studies reported that 24% of raw drug samples of *Phyllanthus amarus* Schumach. & Thonn. were substituted with other phenotypically similar *Phyllanthus* species (Srirama et al., 2010). Similar substitution were reported for other species, such as *Sida cordifolia* L. (76%) (Vassou et al., 2015), *Cinnamomum verum* J.Presl (70%) (Swetha et al., 2014), *Myristica fragrans* Houtt. (60%) (Swetha et al., 2017), *Senna auriculata* (L.) Roxb. (50%) (Seethapathy et al., 2015), *Senna tora* (L.) Roxb. (37%) (Seethapathy et al., 2015) and *Senna alexandrina* Mill. (8%) (Seethapathy et al., 2015). Furthermore, Vassou et al. (2016) reported that 21% of raw drugs in Indian herbal markets were unauthentic. Shanmughanandhan et al. (2016) found that 60% of 93 herbal products sold in the form of capsules and plant powders in local stores in India were adulterated. Studies that combined spectroscopic methods, such as NMR, with DNA barcoding or microscopy to authenticate herbal products, reported 80% adulteration in *Saraca asoca* (Urumarudappa et al., 2016), 80% in *Berberis aristata* (Srivastava and Rawat, 2013) and 22% in *Piper nigrum* (Parvathy et al., 2014). All these studies utilizing DNA barcoding and metabarcoding have highlighted the concerns over the quality and good labeling practices of herbal products (Coghlan et al., 2012; Ivanova et al., 2016; Raclariu et al., 2017a,b; Veldman et al., 2017).

The aim of this study was threefold. First, we aimed to test the composition and fidelity of Ayurvedic products marketed in Europe using DNA metabarcoding. Secondly, we aimed to analyze the presence of any red listed species listed on the product label and used as ingredients using DNA metabarcoding. Our final aim was to evaluate the ability of DNA metabarcoding to identify the presence of authentic species, any substitution and adulteration and/or presence of other off labeled plant species.

MATERIALS AND METHODS

Sample Collection

Seventy-nine Ayurvedic herbal products sold as tablets ($n = 30$), capsules ($n = 30$), powders ($n = 16$), and extracts ($n = 3$) were purchased via e-commerce ($n = 53$) and pharmacies ($n = 26$), from Norway ($n = 21$), Romania ($n = 26$), and Sweden ($n = 32$). Based on the label information, 26 were single plant ingredient products, 39 contained between two to ten plant ingredients, and 14 products contained between eleven to 27 plant ingredients (**Supplementary Table S1**). The products contained a total of 159 plant species belonging to 132 genera and 60 families (**Supplementary Table S2**). It was also confirmed that nrITS sequences of all the 159 plant species labeled in the analyzed herbal products were available within the NCBI/GenBank database (**Supplementary Table S2**). The accepted binomial

names and authors of the plants species used as ingredients were validated using The Plant List (2013). The Ayurvedic herbal products were imported into Norway for scientific analyses under Norwegian Medicines Agency license no. 16/04551–2. An overview of the products, including label information, but not the producer/importer name, lot number, expiration date or any other information that could lead to the identification of that specific product, can be found in **Supplementary Table S1**.

DNA Extraction, Amplicon Generation, and High Throughput Sequencing

The 79 Ayurvedic herbal products were processed depending on their pharmaceutical formulation, in addition to an extraction blank per DNA extraction round. A small amount of each herbal product, about 200 mg, was homogenized using 3–5 zirconium grinding beads in a Mini-Beadbeater-1 (Biospec Products Inc., Bartlesville, Oklahoma, United States). The total DNA from each product was extracted from homogenized contents using CTAB extraction (Doyle and Doyle, 1987). The final elution volume was 100 μl. Extracted DNA was quantified using a Qubit 2.0 Fluorometer and Qubit dsDNA HS Assay Kit (Invitrogen, Carlsbad, California, United States). All amplicon libraries, defined as PCR amplified products from a study sample, were prepared in three replicates. For each replicate two nuclear ribosomal target sequences were amplified, the internal transcribed spacers nrITS1 and nrITS2, respectively. The fusion primers included the annealing motif from the Sun et al. (1994) plant-specific primer pairs 17SE and 5.8I1, and 5.8I2 and 26SE. The forward primers included the Ion Torrent A adapter, a 10 bp multiplex identifier tag following the IonXpress setup for Ion Torrent (Thermo Fisher Scientific, Carlsbad, California, United States). The reverse primer included the truncated P1 (trP1) tags in addition to the annealing motif. Expected amplicon sizes were 300–350 bp.

Polymerase chain reactions were carried out using DNA extracted from the herbal products in final reaction volumes of 25 μl including 0.5 μl of template DNA solution (ranging from 0.5 to 2 ng/μl), 1X Q5 reaction buffer (New England Biolabs Inc., United Kingdom), 0.6 μM of each primer (Biolegio B.V., Netherlands), 200 nM dNTPs, 5 U Q5 High-Fidelity DNA Polymerase (New England Biolabs Inc., United Kingdom) and 1X Q5 High GC enhancer. The PCR cycling protocol consisted of initial denaturation at 98°C for 30 s, followed by 35 cycles of denaturation at 98°C for 10 s, annealing at 56°C for nrITS1 or 71°C for nrITS2 for 30 s, and elongation at 72°C for 30 s, followed by a final elongation step at 72°C for 2 min. Three PCR negative controls of the extraction blanks were included per amplification to control for external and cross sample contamination. After PCR, the amplicons were purified using Illustra Exostar (GE Healthcare, Chicago, Illinois, United States) in accordance with the manufacturer protocols. The molarity of each amplicon library was measured using a qPCR based assay (CFX96 Touch Real-Time PCR Detection System, Bio-Rad, Hercules, California, United States). The equimolar amounts of each amplicon library were merged

and sequenced using an Ion Torrent Personal Genomic Machine (Thermo Fisher Scientific) as described by Raclariu et al. (2017a).

Bioinformatics Analysis

The sequencing read data were analyzed and demultiplexed into FASTQ files, per sample, using Torrent Suite version 5.0.4 (LT), and each of the replicates was analyzed individually. FASTQ read files were processed using the HTS-barcode-checker pipeline available as a Galaxy pipeline at the Naturalis Biodiversity Center[1] (Lammers et al., 2014). Using the HTS pipeline, nrITS1 and nrITS2 primer sequences were used to demultiplex the sequencing reads per sample and to filter out reads that did not match any of the primers. PRINSEQ was used to determine filtering and trimming values based on read lengths and Phred read quality. All reads with a mean Phred quality score of less than 26 were filtered out, as well as reads with a length of less than 200 bp. The remaining reads were trimmed to a maximum length of 380 bp. CD-HIT-EST was used to cluster reads into molecular operational taxonomic units (MOTUs) defined by a sequence similarity of >99% and a minimum number of ten reads. The consensus sequences of non-singleton MOTUs were queried using BLAST against a reference nucleotide sequence database, with a maximum e-value of 0.05, a minimum hit length of 100 bp and sequence identity of >97%. The number of reads per MOTU, as well as the BLAST results per MOTU, were compiled using custom scripts from the HTS Barcode Checker pipeline (Lammers et al., 2014). The reference sequence database consisted of a local copy of the NCBI/GenBank nucleotide database that is refreshed monthly. These parameters were applied to each of the replicates. A species was considered and validated as being present within the product only if this was detected in at least 2 out of the 3 replicates.

Presence and Abundance of Species Across Samples

To assess species diversity within each sample, and to obtain insights into the dominant species within the Ayurvedic herbal products, the read abundances were normalized by dividing the number of reads for a MOTU by the total number of reads per sample. As a result, the read counts are transformed into a proportion of reads found per species within each sample (**Supplementary Tables S3, S4**). Furthermore, MOTUs detected in at least two out of the three replicates, for each sample, were categorized into expected-detected (MOTUs corresponding to species listed on the product label versus species detected in the analysis), expected-not detected (MOTUs corresponding to species listed on the product label but not detected in the analysis), and not expected-detected (MOTUs corresponding to species non-listed on the product label but detected in the analysis) (**Supplementary Table S5**). The total occurrences of MOTUs per category of expected and detected were evaluated (**Supplementary Table S5**), and a matrix of

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[1]http://145.136.240.164:8080/

correlation was generated using ClustVis (Metsalu and Vilo, 2015).

RESULTS

Fidelity of Ayurvedic Products

The genomic DNA extracts were highly variable in quantity and quality. Total DNA concentration for each of the 79 herbal products is provided in **Supplementary Table S6**. **Table 1** shows the average DNA yield for each of the investigated herbal product types. The result shows that three samples labeled as containing only standardized extracts yielded an average of 0.5 ng/μl DNA, whereas tablets, capsules and powders yielded an average of 5.8, 9.6, and 44.7 ng/μl DNA, respectively. Out of 79 products used in the study, 10 tablets were also labeled to contain extracts in addition to crude plant material (#6, #12, #13, #14, #17, #18, #20, #21, #38, and #74). PCR amplification for nrITS1 and nrITS2 regions were performed for all 79 samples, and amplicons were generated for all replicates for nrITS1 and nrITS2 (for samples and concentrations see **Supplementary Table S6**). The extraction blanks yielded no molecular operational taxonomic units (MOTUs) with nrITS1 and nrITS2 primers.

The sequencing success rate was 44% for ITS1 and 41% for ITS2 (**Supplementary Table S6**). Thirty-five products out of 79 (44%) yielded no MOTUs in any of the replicates either for nrITS1 or nrITS2 that fulfilled our quality criteria, and they were excluded from the results and the further discussion (#11, #20–22, #28, #29, #33, #35, #37–39, #41–51, #53, #54, #56, #57, #59, #62, 64, #65, #67, #71, #72, #76, and #78). These products consisted of 13 tablets, 11 capsules, and 11 powders (**Supplementary Table S6**). The products that yielded MOTUs were represented by 17 tablets, 19 capsules, 5 powders, and 3 extracts (**Supplementary Table S7**).

A total of 188 different plant species belonging to 154 genera and 65 families were identified from the retained MOTUs using BLAST. The separate analyses resulted in 131 plant species (110 genus and 53 families) for nrITS1, and 101 plant species (84 genus and 39 families) for nrITS2. The number of species detected per sample ranged from one to 42. After applying our quality selection criteria, where a species was considered and validated as being present within the product only if it was detected in at least 2 out of the 3 replicates, five additional products (#4, #15, #24, #25, and #26 includes 2 tablets and 3 extracts) that failed to yield the same MOTU in any of the replicates were discarded. The remaining 39 products resulted in a total of 97 plant species belonging to 40 families (62 species for nrITS1, and 60 species for nrITS2). The species detected for all the replicates for both ITS1 and ITS2, were merged for each sample for further analyses (**Figure 1** and **Supplementary Tables S3, S7**).

Figure 2 illustrates the fidelity of herbal products between various product forms, country, and method of acquisition. In ten out of twelve single ingredient products that were labeled as containing only one species, we detected multiple species (exceptions #5 and #52), from which six contained the species labeled on the product together with other species, whereas four products did not contained the species listed on the product

TABLE 1 | Genomic DNA yield and amplicon concentrations per herbal product type.

Product type	No. of herbal products	Average genomic DNA concentration (ng/µl) (SD)	Average amplicon concentration quantified by qPCR (ng/µl)						No. of products yielding DNA sequences	No. of products analyzed post filtering
			nrITS1			nrITS2				
			Replicate 1 (SD)	Replicate 2 (SD)	Replicate 3 (SD)	Replicate 1 (SD)	Replicate 2 (SD)	Replicate 3 (SD)		
Tablets	30	5.8 (6)	5.0 (7)	4.3 (4)	7.7 (15)	7.1 (10)	4.9 (4)	15.3 (18)	20	19
Capsules	30	9.6 (12)	6.2 (11)	6.0 (11)	5.1 (9)	5.8 (9)	4.2 (5)	8.3 (13)	16	15
Powders	16	44.7 (63)	19.2 (25)	3.4 (6)	2.4 (3)	9.4 (19)	4.0 (11)	8.3 (18)	5	5
Extracts	3	0.5 (0.5)	3.4 (0.4)	2.4 (2)	2.4 (2)	20.3 (13)	8.5 (4)	21 (19)	3	–
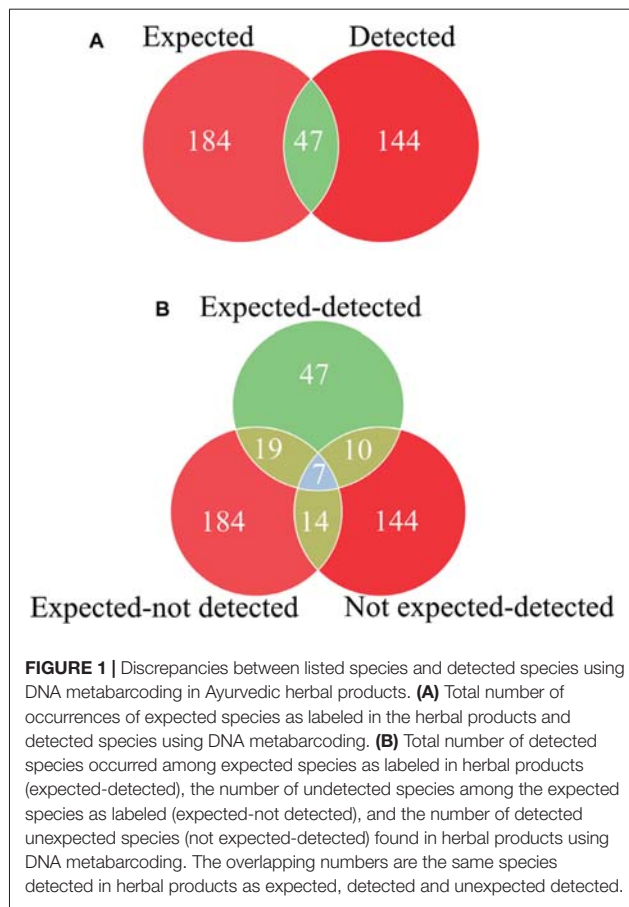
FIGURE 1 | Discrepancies between listed species and detected species using DNA metabarcoding in Ayurvedic herbal products. **(A)** Total number of occurrences of expected species as labeled in the herbal products and detected species using DNA metabarcoding. **(B)** Total number of detected species occurred among expected species as labeled in herbal products (expected-detected), the number of undetected species among the expected species as labeled (expected-not detected), and the number of detected unexpected species (not expected-detected) found in herbal products using DNA metabarcoding. The overlapping numbers are the same species detected in herbal products as expected, detected and unexpected detected.

label but contained several other non-listed species. Out of 27 successfully analyzed multiple ingredient products, 8 (29.6%) products contained none of the species listed on the label, and the remaining 19 products contained between one to five species listed on the label along with many other species not specified on the product label. The fidelity rate for single ingredient products was 67% (8 out of 12), and the overall ingredient fidelity (detected species from product label/total number of species on label) for multi ingredient products was 21% and for all products 24%. **Table 2** shows the top ten products with highest fidelity is also relatively high in the level of substitution, whereas **Table 3** shows the top ten products with highest adulteration and its fidelity.

Figure 3 depicts all 97 detected species based on the relative abundance of read numbers in 39 herbal products per type under the categories of expected-detected, expected-not detected, and not expected-detected.

Plant Ingredients in Herbal Products

A total of 159 plant species belonging to 132 genera and 60 families were specified on the labels of the 79 Ayurvedic herbal products used in this study. Assessing the source and availability of these plants, we found that 83 plants species are solely harvested from wild, and 31 of these are under various threat levels, including critically endangered and protected species, such

FIGURE 2 | Fidelity of herbal products **(A)** per product form; **(B)** per country; **(C)** per acquisition method. *n* = total number of herbal products.
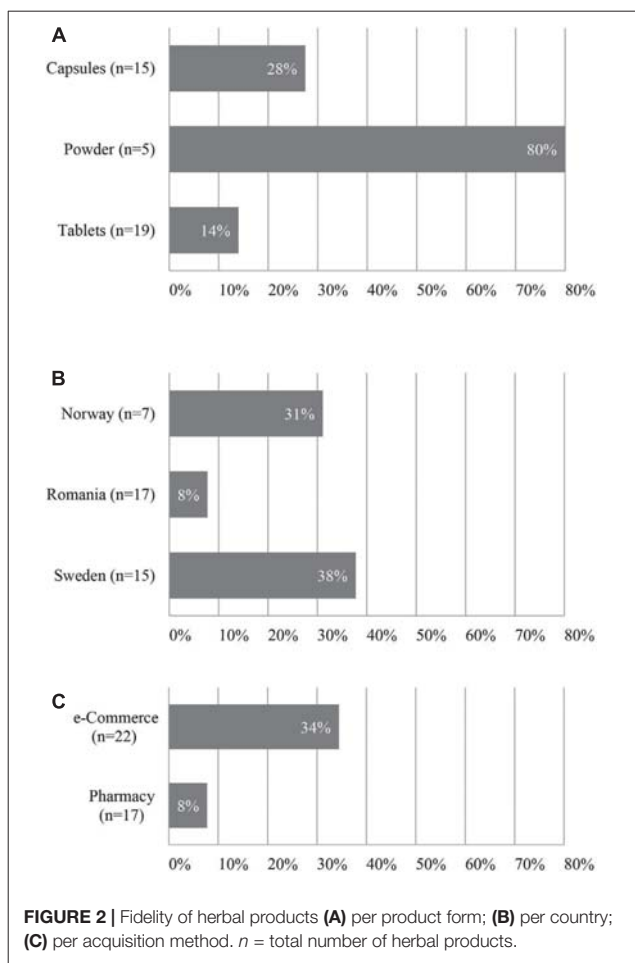
as *Pterocarpus marsupium* Roxb., *Pterocarpus santalinus* L.f., *Santalum album* L., and *Saraca asoca* (Roxb.) Willd. (**Figure 4** and **Supplementary Table S2**; Ved and Goraya, 2007; Envis Frlht, 2017; Goraya and Ved, 2017). The DNA metabarcoding analysis confirms the presence of four of these threatened species,

i.e., *Celastrus paniculatus, Glycyrrhiza glabra, Gymnema sylvestre,* and *Saraca asoca,* whereas the remaining threatened species were not detected despite being included as labeled ingredients (**Figure 3** and **Supplementary Table S7**). The following species were found in over 20% of the products: *Withania somnifera* (L.) Dunal (39%), *Tribulus terrestris* L. (27%), *Convolvulus prostratus* Forssk. (23%), *Coriandrum sativum* L. (23%), *Ipomoea parasitica* (Kunth) G. Don (23%), *Ocimum basilicum* L. (23%) and *Senna alexandrina* Mill. (23%) (**Figure 3** and **Supplementary Table S3**). Seventeen are present in more than 10% of samples are listed in the **Supplementary Table S3**.

DISCUSSION

The British Pharmacopeia is one the first to publish a specific methods section on DNA barcoding, and in the 2016 version it included a new methods appendix on "Deoxyribonucleic acid (DNA) based identification techniques for herbal drugs" to create a framework for compliance of DNA barcoding with regulatory requirements (British Pharmacopeia Commission, 2016; Sgamma et al., 2017). However, DNA barcoding and metabarcoding are not yet widespread validated methods for use in the regulatory context of quality control. Several studies advocate its usefulness for herbal product authentication and pharmacovigilance either as a standard method or as a complementary method (Ivanova et al., 2016; Raclariu et al., 2017a,b, 2018a; Sgamma et al., 2017). In this study, DNA metabarcoding was used as an analytical approach in Ayurveda herbal product authentication.

A number of studies have shown that the quality of the extraction substrate influences amplification and sequencing success (Ivanova et al., 2016; Raclariu et al., 2017a, 2018b). In addition the presence of DNA in the extraction substrates is influenced by degradation during the harvesting, drying, storage, and industrial processing of plant material (Novak et al., 2007). The success rate in generating raw sequence reads from the herbal products, and the number of products from which MOTUs could be identified per product after applying strict trimming and filtering quality criteria, reduced the number of

TABLE 2 | Top ten products with the highest fidelity and their level of adulteration.

Herbal product code	Product type	No. species on label	Detected by DNA metabarcoding	Fidelity (Expected-detected, absolute)	Fidelity (Expected-detected, relative)	Adulteration (Detected-Not expected, absolute)	Adulteration (Detected-Not expected, relative)
34	Tablets	8	15	5	63%	10	67%
31	Tablets	10	7	5	50%	2	29%
36	Tablets	13	7	4	31%	3	43%
73	Tablets	14	14	3	21%	11	79%
74	Tablets	9	5	3	33%	2	40%
66	Capsules	6	5	3	50%	2	40%
7	Capsules	6	5	2	33%	3	60%
75	Tablets	3	3	2	67%	1	33%
69	Capsules	1	13	1	100%	12	92%
3	Tablets	4	9	1	25%	8	89%

TABLE 3 | Top ten products with the highest adulteration and their fidelity.

Herbal product code	Product type	No species on label	Detected by DNA metabarcoding	Fidelity (Expected-detected, absolute)	Fidelity (Expected-detected, relative)	Adulteration (Detected-Not expected, absolute)	Adulteration (Detected-Not expected, relative)
69	Capsules	1	13	1	100%	12	92%
32	Tablets	6	12	0	0%	12	100%
73	Tablets	14	14	3	21%	11	79%
34	Tablets	8	15	5	63%	10	67%
3	Tablets	4	9	1	25%	8	89%
68	Capsules	1	8	0	0%	8	100%
40	Capsules	4	8	1	25%	7	88%
27	Capsules	1	8	1	100%	7	88%
6	Tablets	9	7	1	11%	6	86%
23	Tablets	4	7	1	25%	6	86%

samples yielding DNA metabarcoding results from 79 to 39 samples. In this study, 44% of products did not yield MOTUs in any of the replicates either for nrITS1 or nrITS2. Also, in the herbal products labeled to contain only extracts, no plant DNA was detected. The undetected MOTUs in these products could be related to the methodological framework of DNA metabarcoding such as DNA extraction protocol, suitability of primer pair sequences, amplification protocols in PCR for the library preparation, sequencing platform, filtering, quality thresholds, and chimera removal, and clustering thresholds (De Boer et al., 2017; Sgamma et al., 2017; Raclariu et al., 2018b). In addition, extraction of crude herbal drugs either in pre-processing or manufacturing can reduce the availability of plant DNA from those species, especially if material is extracted in boiling water or alcohol, and evaporated or dried at high temperatures.

Considerable incongruences were observed between the detected species and those listed on the label of the products. Similarly, Raclariu et al. (2017b) demonstrated the ability of DNA metabarcoding in detecting *Hypericum* species in complex herbal formulations, and revealed the incongruence between constituent species and those listed on the label in all products. Also, De Boer et al. (2017) performed DNA metabarcoding analyses on 55 commercial products based on orchids (*salep*) purchased in Iran, Turkey, Greece, and Germany, and concluded that there are significant differences in labeled and detected species. They also highlighted the applicability of DNA metabarcoding in targeted efforts for conservation of endangered orchid species. In our study, we detected a total of 97 species in 39 products that passed our quality criteria, and most of the identified species are likely ingredients of Ayurvedic herbal products. Detection of certain species is improbable given their distribution or unlikely use, and these include *Achillea millefolium* L., *Anchusa italica* Retz., *Calluna vulgaris* (L.) Hull, *Damrongia cyanantha* Triboun, *Fraxinus albicans* Buckley and *Trigastrotheca mulluginea* F.Muell. The identification of these plant species may be explained by *(i)* amplified PCR chimeras; *(ii)* false-positive BLAST identifications due to incomplete or error-prone reference databases; or *(iii)* presence of pollen from wind pollinating species, and this confirms previously raised concerns about the hypersensitivity of DNA metabarcoding (De Boer et al., 2017).

Out of 97 species detected in the DNA metabarcoding analysis, 40 species are sourced from wild, 38 species are cultivated, and 15 species are sourced from both wild and cultivation. Similarly, among the 89 species which were not detected in the analysis, 62 species are mainly sourced from wild, including endangered species such as *Embelia ribes* Burm.f., *Pterocarpus marsupium* Roxb., *Pterocarpus santalinus* L.f., *Pueraria tuberosa* (Willd.) DC., and *Santalum album* L. Understanding, the discrepancies between the species detected using DNA metabarcoding and those listed on the label of the products require careful consideration. In DNA metabarcoding analyses, the level of similarity clustering thresholds (>97, >99, and 100%) have an impact on the number and size of assigned MOTUs (Raclariu et al., 2017a). In this study, we used a 99% clustering threshold similar to previously published studies (Raclariu et al., 2017a; Veldman et al., 2017). Furthermore, to limit the impact of sequencing errors, which are known to affect the Ion Torrent sequencing platform (Salipante et al., 2014) and which could lead to the formation of false MOTUs, we used only the clusters that contained a minimum of 10 reads. In addition, by using three replicates for each sample and marker, we reduced further noise by accepting MOTUs only if present in more than one replicate. Furthermore, the strict filtering and trimming thresholds for base calling, length and quality, and strict clustering criteria for MOTUs formation, increase confidence of the results. As reported by previous studies (Ivanova et al., 2016; Raclariu et al., 2017b), the results related to the authentication of herbal products using DNA metabarcoding need to focus primarily on checking the presence of the labeled ingredients and contaminants. The presence of non-listed species may be explained by various factors, including but not limited to the deliberate adulteration and unintentional substitution that may occur from the early stage of the supply chain of medicinal plants (i.e., cultivation, transport, and storage), to the manufacturing
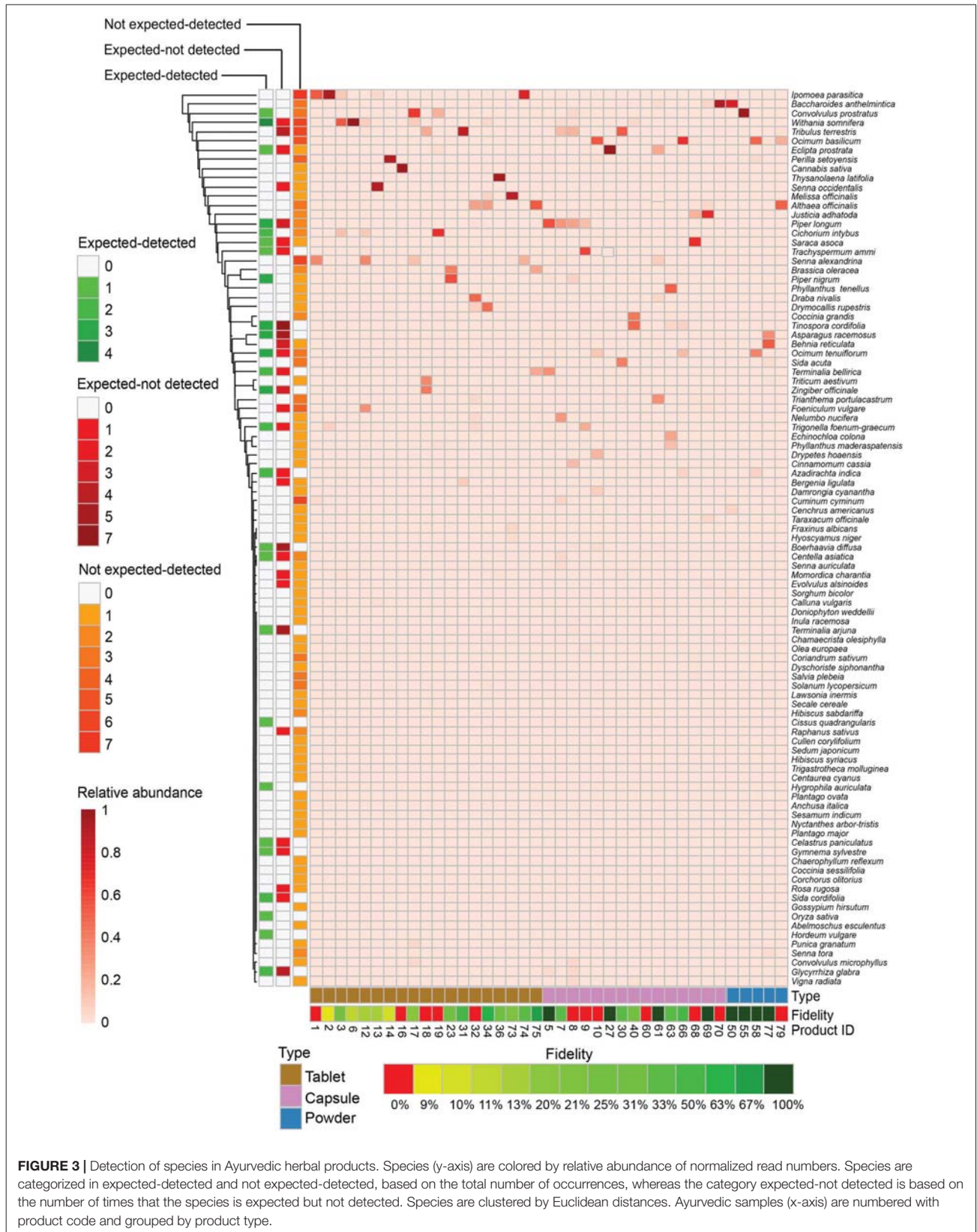
FIGURE 3 | Detection of species in Ayurvedic herbal products. Species (y-axis) are colored by relative abundance of normalized read numbers. Species are categorized in expected-detected and not expected-detected, based on the total number of occurrences, whereas the category expected-not detected is based on the number of times that the species is expected but not detected. Species are clustered by Euclidean distances. Ayurvedic samples (x-axis) are numbered with product code and grouped by product type.

FIGURE 4 | Source and conservation status of species in Ayurvedic products. **(A)** Source of plants labeled as ingredients in the herbal products studied. **(B)** Conservation status of plants labeled as ingredients in the herbal products studied. N = total number of species. *Wild/Cultivation denotes that the plants species are sourced both from wild and cultivation.
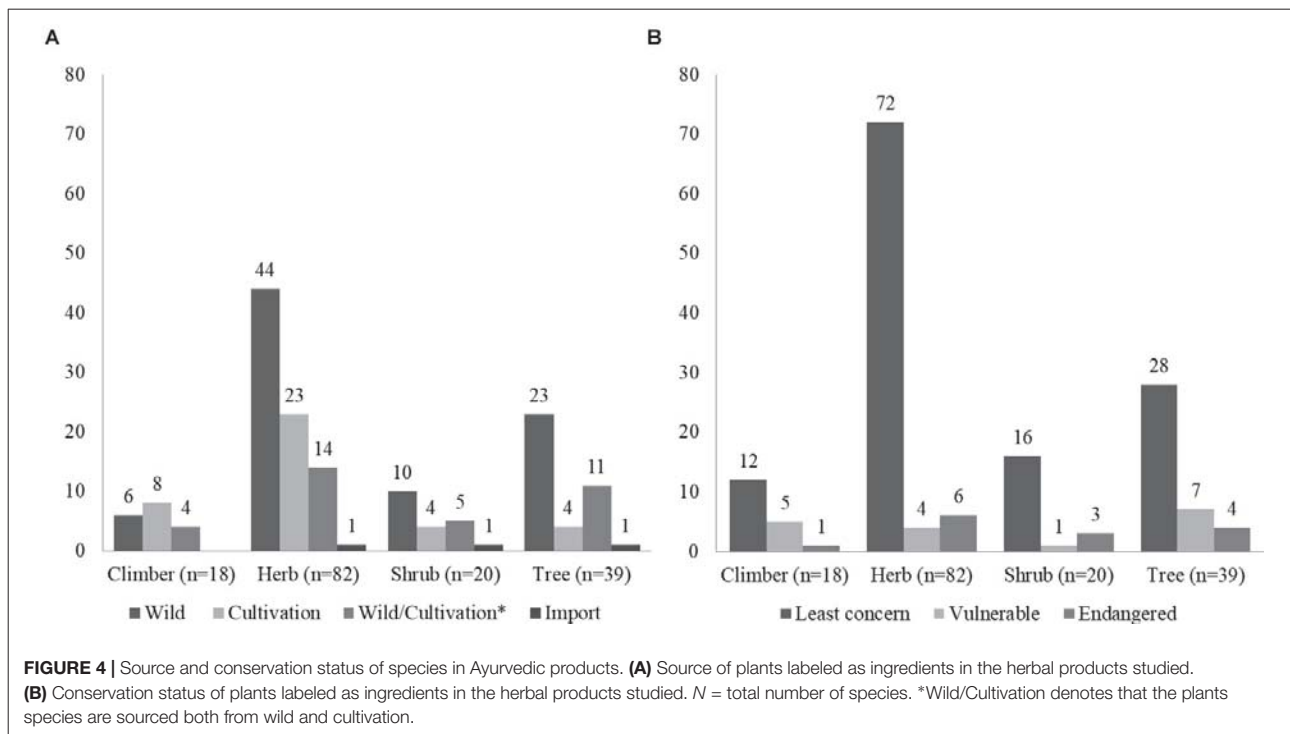
process and the commercialization of the final products. DNA metabarcoding is a highly sensitive method and even traces of DNA, e.g., contamination from grains of pollinating species or plant dust in the manufacturing process, can be detected and identified.

The advantage of DNA metabarcoding is its ability to simultaneously identify total species diversity within complex multi-ingredient and processed mixtures. Importantly, DNA metabarcoding data is used for qualitative evaluation only, to determine presence of taxa, and not for quantitative assessment of relative species abundance based on read numbers, as many variables considerably impact the obtained sequence read results (Staats et al., 2016). In the context of the quality control of herbal products, DNA metabarcoding does not provide any quantitative nor qualitative information of the active metabolites in the raw plant material or the resulting preparation, and this narrows its applicability only to identification and authentication procedures. Thus, if product safety control relies on threshold levels of specific marker compounds, absence of toxins, allergens and admixed pharmaceuticals, then other methods may be more relevant than DNA-based composition analysis. On the other hand, if product fidelity, species substitution or adulteration is suspected then the latter method outperforms in terms of resolution.

The results of this study reveal that there is a need for a better quality control of herbal products. A novel analytical approach should eventually use a combination of innovative high throughput methods that complement the standard ones recommended today.

CONCLUSION

Assessment of Ayurvedic herbal medicines using DNA metabarcoding provides insight into species diversity in these products and highlights a marked incongruence between species listed as ingredients on the product labels and those detected from DNA present in the samples. Detection of not-listed and not-expected species first and foremost suggests irregularities in the manufacturing process. The presence of foreign plant material could be due accidental reasons, such as contamination from insufficiently cleaned bags, containers, mills, conveyors, and other equipment, or co-occurrence of weeds in cultivation, pollen from wind pollinated species or seeds from wind-dispersed species. However, foreign plant material could also result from fraud, i.e., substitution, adulteration and/or admixture of other species. Interpretation of incongruences should focus on the detected species in the products, and less on the failure to detect species as there are many steps in manufacturing processes that could lead to degradation or loss of DNA beyond detectable limits, e.g., alcoholic extraction, decoction and drying of material at high temperatures. Our study showed that the investigated herbal products contained species not listed on the product labels, and this reveals a clear need for improved quality control. A novel analytical approach should eventually use a combination of advanced chemical methods and innovative high throughput sequencing to complement the standard ones recommended today. The findings of our study show that DNA metabarocoding is a promising tool for quality evaluation of herbal products and pharmacovigilance, and

a good candidate for an effective use as a regulatory tool to authenticate complex herbal products. However, standardization of protocols is necessary before DNA metabarcoding can be implemented as a routine analytical approach and approved by competent authorities for use in a regulatory framework.

SUPPORTING INFORMATION

Ion-Torrent sequencing data is deposited in Zenodo doi: 10.5281/zenodo.2548681.

AUTHOR CONTRIBUTIONS

GS, ACRM, HW, and HdB conceived the experiment. GS collected the material and carried out the molecular lab work and analysis together with ACRM. JA carried out high-throughput sequencing together with GS. GS wrote the manuscript together with HdB. All authors contributed to and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

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DNA metabarcoding authentication of Ayurvedic herbal products on the European market raises concerns of quality and fidelity

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Supplementary data

Supplementary Table S1. Ayurvedic herbal products used in the study.

Supplementary Table S2. Details of the plants species used as ingredients in the herbal products.

Supplementary Table S3. Details of total number sequencing reads per products.
Supplementary Table S4. Details of normalized sequencing reads per products.

Supplementary Table S5. Details of sum occurrences of a species in Ayurvedic herbal products used to generate heatmap.

Supplementary Table S6. Details of concentration of genomic DNA, nrITS amplicon concentration and success rate of sequence yield in herbal products after quality filtering of sequences between nrITS1 and nrITS2, and among replicates, and number of MOTUs in nrITS1 and nrITS2 after NCBI-BLAST identification.

Supplementary Table S7. Results of the DNA metabarcoding analysis of herbal products.

Supplementary Table S1. Ayurvedic herbal products used in the study.

Herbal product ID	Species on label	Product type	Scientific names of the plant ingredients as indicated on the product label	Plant family	Vernacular names on product label
1	7	Tablets	Acacia arabica (Lam.) Willd. (= Acacia nilotica (L.) Delile)	Leguminosae	
			Achyranthes aspera L.	Amaranthaceae	
			Bergenia ligulata Engl.	Saxifragaceae	
			Boerhaavia diffusa L.	Nyctaginaceae	
			Crateva nurvala Buch.-Ham	Capparaceae	
			Phyllanthus urinaria L.	Phyllanthaceae	
			Piper cubeba Bojer	Piperaceae	
2	11	Tablets	Acacia arabica (Lam.) Willd. (= Acacia nilotica (L.) Delile)	Leguminosae	
			Azadirachta indica A.Juss.	Meliaceae	
			Curcuma longa L.	Zingiberaceae	
			Enicostemma littorale Blume	Gentianaceae	
			Gymnema sylvestre (Retz.) R.Br. ex Sm.	Apocynaceae	
			Momordica charantia L.	Cucurbitaceae	
			Piper longum L.	Piperaceae	
			Pterocarpus marsupium Roxb.	Leguminosae	
			Syzygium cumini (L.) Skeels	Myrtaceae	
			Tinospora cordifolia (Willd.) Miers	Menispermaceae	
			Trigonella foenum-graecum L.	Leguminosae	
3	4	Tablets	Acacia arabica (Lam.) Willd. (= Acacia nilotica (L.) Delile)	Leguminosae	
			Asparagus racemosus Willd.	Asparagaceae	
			Chlorophytum arundinaceum Baker	Asparagaceae	
			Withania somnifera (L.) Dunal	Solanaceae	
4	4	Tablets	Eclipta prostrata (L.) L.	Compositae	
			Guma acacia $		
			Mandur bhasma $		
			Phyllanthus urinaria L.	Phyllanthaceae	
			Picrorhiza kurroa Royle ex Benth.	Plantaginaceae	
			Piper longum L.	Piperaceae	
5	1	Capsules	Withania somnifera (L.) Dunal	Solanaceae	
6	9	Tablets	Eclipta alba (L.) Hassk. (= Eclipta prostrata (L.) L.)	Compositae	
			Phyllanthus niruri L.	Phyllanthaceae	

				Family
			Rheum emodi L.*	Polygonaceae
			Capparis spinosa L.*	Capparaceae
			Tephrosia purpurea (L.) Pers.*	Leguminosae
			Picrorhiza kurroa Royle ex Benth.*	Plantaginaceae
			Aloe barbadensis Mill. (= Aloe vera (L.) Burm.f.)*	Xanthorrhoeaceae
			Cichorium intybus L. *	Compositae
			Boerhaavia diffusa L.*	Nyctaginaceae
7	6	Capsules	Allium sativum L.	Amaryllidaceae
			Pueraria tuberosa (Willd.) DC.	Leguminosae
			Trigonella foenum-graecum L.	Leguminosae
			Withania somnifera (L.) Dunal	Solanaceae
			Asparagus racemosus Willd.	Asparagaceae
			Leptadenia reticulata (Retz.) Wight & Arn.	Apocynaceae
8	2	Capsules	Albizia lebbeck (L.) Benth.	Leguminosae
			Curcuma longa L.	Zingiberaceae
9	2	Capsules	Valeriana officinalis L.	Caprifoliaceae
			Humulus lupulus L.	Cannabaceae
10	7	Capsules	Picrorhiza kurroa Royle ex Benth.	Plantaginaceae
			Eclipta alba (L.) Hassk. (= Eclipta prostrata (L.) L.)	Compositae
			Phyllanthus niruri L.	Phyllanthaceae
			Bacopa monnieri (L.) Wettst.	Plantaginaceae
			Boerhaavia diffusa L.	Nyctaginaceae
			Camellia sinensis (L.) Kuntze	Theaceae
			Piper longum L.	Piperaceae
11	5	Capsules	Azadirachta indica A.Juss.	Meliaceae
			Rubia cordifolia L.	Rubiaceae
			Curcuma longa L.	Zingiberaceae
			Terminalia chebula Retz.	Combretaceae
			Acacia catechu (L.f.) Willd.	Leguminosae
12	22	Tablets	Bacopa monnieri (L.) Wettst.*	Plantaginaceae
			Centella asiatica (L.) Urb. *	Apiaceae
			Withania somnifera (L.) Dunal*	Solanaceae
			Evolvulus alsinoides (L.) L.*	Convolvulaceae
			Nardostachys jatamansi (D.Don) DC.*	Caprifoliaceae
			Valeriana wallichii DC. (= Valeriana jatamansi Jones)*	Caprifoliaceae
			Embelia ribes Burm.f.*	Primulaceae

S.No	No.	Form	Botanical Name	Family	Local Name
			Prunus amygdalus Stokes*	Rosaceae	
			Acorus calamus L.*	Acoraceae	
			Tinospora cordifolia (Willd.) Miers *	Menispermaceae	
			Terminalia chebula Retz.*	Combretaceae	
			Emblica officinalis Gaertn. (= Phyllanthus emblica L.)*	Phyllanthaceae	
			Oroxylum indicum (L.) Kurz i*	Bignoniaceae	
			Celastrus paniculatus Willd.*	Celastraceae	
			Bacopa monnieri (L.) Wettst.	Plantaginaceae	
			Withania somnifera (L.) Dunal	Solanaceae	
			Mucuna pruriens (L.) DC.	Leguminosae	
			Elettaria cardamomum (L.) Maton	Zingiberaceae	
			Terminalia arjuna (Roxb. ex DC.) Wight & Arn.	Combretaceae	
			Anethum sowa Roxb. ex Fleming	Apiaceae	
			Ipomoea digitata L. (=Ipomoea cheirophylla O'Donell)	Convolvulaceae	
			Zingiber officinale Roscoe	Zingiberaceae	
13	16	Tablets	Capparis spinosa L.	Capparaceae	Himsra
			Cichorium intybus L.	Compositae	Kasani
			Solanum nigrum L.*	Solanaceae	Kakamachi
			Terminalia arjuna (Roxb. ex DC.) Wight & Arn.*	Combretaceae	Arjuna
			Cassia occidentalis L. (= Senna occidentalis (L.) Link)*	Leguminosae	Kasamarda
			Achillea millefolium L.*	Compositae	Biranjasipha
			Tamarix gallica L*	Tamaricaceae	Jhavuka
			Phyllanthus amarus Schumach. & Thonn.	Phyllanthaceae	
			Boerhaavia diffusa L.	Nyctaginaceae	
			Tinospora cordifolia (Willd.) Miers	Menispermaceae	
			Raphanus sativus L. (= R. raphanistrum subsp. sativus (L.) Domin)	Brassicaceae	
			Emblica officinalis Gaertn. (= Phyllanthus emblica L.)	Phyllanthaceae	
			Plumbago zeylanica L.	Plumbaginaceae	
			Embelia ribes Burm.f.	Primulaceae	
			Terminalia chebula Retz.	Combretaceae	
			Fumaria officinalis L.	Papaveraceae	
14	27	Tablets	Gymnema sylvestre (Retz.) R.Br. ex Sm.*	Apocynaceae	
			Pterocarpus marsupium Roxb.*	Leguminosae	
			Glycyrrhiza glabra L.*	Leguminosae	
			Casearia esculenta Roxb.*	Salicaceae	
			Eugenia jambolana Lam. (= Syzygium cumini (L.) Skeels)*	Myrtaceae	

		Botanical name	Family	Ayurvedic name
		Asparagus racemosus Willd.*	Asparagaceae	
		Boerhaavia diffusa L.*	Nyctaginaceae	
		Sphaeranthus indicus L.*	Compositae	
		Tinospora cordifolia (Willd.) Miers *	Menispermaceae	
		Swertia chirata Buch.-Ham. ex Wall.*	Gentianaceae	
		Tribulus terrestris L.*	Zygophyllaceae	
		Phyllanthus amarus Schumach. & Thonn.*	Phyllanthaceae	
		Gmelina arborea Roxb.*	Lamiaceae	
		Gossypium herbaceum L.*	Malvaceae	
		Berberis aristata DC.*	Berberidaceae	
		Aloe barbadensis Mill. (= Aloe vera (L.) Burm.f.)*	Xanthorrhoeaceae	
		Terminalia bellirica (Gaertn.) Roxb.	Combretaceae	
		Emblica officinalis Gaertn. (= Phyllanthus emblica L.)	Phyllanthaceae	
		Terminalia chebula Retz.	Combretaceae	
		Momordica charantia L.	Cucurbitaceae	
		Ocimum tenuiflorum L.	Lamiaceae	
		Abutilon indicum (L.) Sweet	Malvaceae	
		Rumex maritimus L.	Polygonaceae	
		Curcuma longa L.	Zingiberaceae	
		Piper nigrum L.	Piperaceae	
		Piper longum L.	Piperaceae	
		Zingiber officinale Roscoe	Zingiberaceae	
15	27	Capsules	Saraca asoca (Roxb.) Willd.	Leguminosae	Ashoka
			Aegle marmelos (L.) Corrêa	Rutaceae	Dashamoola #
			Premna integrifolia Willd. (= Premna serratifolia L.)	Lamiaceae	Dashamoola #
			Oroxylum indicum (L.) Kurz	Bignoniaceae	Dashamoola #
			Stereospermum suaveolens (Roxb.) DC. (= S. chelonoides (L.f.) DC.)	Bignoniaceae	Dashamoola #
			Gmelina arborea Roxb.	Lamiaceae	Dashamoola #
			Solanum indicum L.	Solanaceae	Dashamoola #
			Solanum xanthocarpum Schrad. & H. Wendl. (= S. virginianum L.)	Solanaceae	Dashamoola #
			Desmodium gangeticum (L.) DC.	Leguminosae	Dashamoola #
			Uraria picta (Jacq.) DC.	Leguminosae	Dashamoola #
			Tribulus terrestris L.	Zygophyllaceae	Dashamoola #
			Symplocos racemosa Roxb.	Symplocaceae	Lodhra
			Tinospora cordifolia (Willd.) Miers	Menispermaceae	Guduchi
			Solanum nigrum L.	Solanaceae	Kakamachi

No.	Count	Type	Scientific name	Family	Common name
16	8	Tablets	Boerhaavia diffusa L.	Nyctaginaceae	Punarnava
			Asparagus racemosus Willd.	Asparagaceae	Shatavari
			Aloe barbadensis Mill. (= Aloe vera (L.) Burm.f.)	Xanthorrhoeaceae	Kumari
			Santalum album L.	Santalaceae	Chandana
			Cyperus rotundus L.	Cyperaceae	Musta
			Justicia adhatoda L.	Acanthaceae	Vasaka
			Terminalia bellirica (Gaertn.) Roxb.	Combretaceae	Triphala #
			Emblica officinalis Gaertn. (= Phyllanthus emblica L.)	Phyllanthaceae	Triphala #
			Terminalia chebula Retz.	Combretaceae	Triphala #
			Piper nigrum L.	Piperaceae	Trikatu #
			Piper longum L.	Piperaceae	Trikatu #
			Zingiber officinale Roscoe	Zingiberaceae	Trikatu #
			Bombax ceiba L.	Malvaceae	Shalmali #
			Zingiber officinale Roscoe	Zingiberaceae	Ginger
			Didymocarpus pedicellata (Ait.) Ait. F.	Asclepiadaceae	Shilapuspha
			Saxifraga ligulata Murray (= Saxifraga stolonifera Curtis)	Saxifragaceae	Pasanabheda
			Rubia cordifolia L.	Rubiaceae	Indian madder
			Cyperus scariosus R.Br.	Cyperaceae	Umbrella's edge
			Achyranthes aspera L.	Amaranthaceae	Prickly chaff flower
			Onosma bracteatum Wall	Boraginaceae	Sedge
			Vernonia cinerea (L.) Less. (= Cyanthillium cinereum (L.) H.Rob.)	Compositae	Purple fleabane (Sahadevi) #
17	6	Tablets	Saraca indica L.	Leguminosae	Ashoka
			Asparagus racemosus Willd.*	Asparagaceae	Shatavari
			Terminalia chebula Retz.	Combretaceae	Haritaki
			Sida cordifolia L.	Malvaceae	Bala
			Glycyrrhiza glabra L.	Leguminosae	Yashtimadhu
			Centella asiatica (L.) Urb.	Apiaceae	Mandukaparni
18	6	Tablets	Boswellia serrata Roxb. ex Colebr.*	Burseraceae	Shallaki
			Commiphora wightii (Arn.) Bhandari*	Burseraceae	Guggul
			Alpinia galanga (L.) Willd.*	Zingiberaceae	Java galangal
			Glycyrrhiza glabra L.*	Leguminosae	Licorice
			Tribulus terrestris L.	Zygophyllaceae	Small caltrops
			Tinospora cordifolia (Willd.) Miers	Menispermaceae	Tinospora Gulancha
19	4	Tablets	Tribulus terrestris L.	Zygophyllaceae	Small caltrop
			Caesalpinia bonducella (L.) Fleming (= C. bonduc (L.) Roxb.)	Leguminosae	Bonduc nut

No.	No. of ingredients	Dosage form	Botanical name	Family	Common name
			Asparagus racemosus Willd.	Asparagaceae	Asparagus
			Crateva nurvala Buch.-Ham	Capparaceae	Three leaved caper
			Akik pishti$		Processed agate
20	6	Tablets	*Commiphora wightii* (Arn.) Bhandari	Burseraceae	Indian bedellium
			Tinospora cordifolia (Willd.) Miers	Menispermaceae	Gulancha tinospora
			Rubia cordifolia L.*	Rubiaceae	Indian madder
			Emblica officinalis Gaertn. (= *Phyllanthus emblica* L.)*	Phyllanthaceae	Indian gooseberry
			Moringa pterygosperma Gaertn.*	Moringaceae	Horse-radish tree
			Glycyrrhiza glabra L.*	Leguminosae	Licorice
21	5	Tablets	*Commiphora wightii* (Arn.) Bhandari*	Burseraceae	Guggulu
			Garcinia cambogia (Gaertn.) Desr. (= *G. gummi-gutta* (L.) Roxb.)*	Clusiaceae	
			Gymnema sylvestre (Retz.) R.Br. ex Sm.*	Apocynaceae	Meshashringi
			Terminalia chebula Retz.*	Combretaceae	
			Trigonella foenum-graecum L.*	Leguminosae	Medhika
22	7	Tablets	*Tinospora cordifolia* (Willd.) Miers	Menispermaceae	
			Zingiber officinale Roscoe	Zingiberaceae	
			Mentha arvensis L.	Lamiaceae	
			Moringa pterygosperma Gaertn.	Moringaceae	
			Carica papaya L.	Caricaceae	
			Citrus limon (L.) Osbeck	Rutaceae	
			Kaempferia galanga L.	Zingiberaceae	
23	4	Tablets	*Asteracantha longifolia* Nees (= *Hygrophila auriculata* (Schumach.) Heine)	Acanthaceae	
			Prunus amygdalus Batsch (= *Prunus dulcis* (Mill.) D.A.Webb)	Rosaceae	
			Crocus sativus L.	Iridaceae	
			Tribulus terrestris L.	Zygophyllaceae	
24	19	Extracts	*Commiphora wightii* (Arn.) Bhandari	Burseraceae	
			Vitis vinifera L.	Vitaceae	
			Ocimum tenuiflorum L.	Lamiaceae	
			Hyssopus officinalis L.	Lamiaceae	
			Tinospora cordifolia (Willd.) Miers	Menispermaceae	
			Justicia adhatoda L.	Acanthaceae	
			Myristica fragrans Houtt.	Myristicaceae	
			Glycyrrhiza glabra L.	Leguminosae	
			Onosma bracteatum Wall	Boraginaceae	
			Viola odorata L.	Violaceae	

No.	Form	Species	Family	Formulation
25	Extracts	Terminalia bellirica (Gaertn.) Roxb.	Combretaceae	Triphala #
		Emblica officinalis Gaertn. (= Phyllanthus emblica L.)	Phyllanthaceae	Triphala #
		Terminalia chebula Retz.	Combretaceae	Triphala #
		Piper nigrum L.	Piperaceae	Trikatu #
		Piper longum L.	Piperaceae	Trikatu #
		Zingiber officinale Roscoe	Zingiberaceae	Trikatu #
		Embelia ribes Burm.f.	Primulaceae	
		Solanum xanthocarpum Schrad. & H. Wendl. (= S. virginianum L.)	Solanaceae	
		Cinnamomum cassia (L.) J.Presl	Lauraceae	
		Commiphora wightii (Arn.) Bhandari	Burseraceae	
		Vitis vinifera L.	Vitaceae	
		Ocimum tenuiflorum L.	Lamiaceae	
		Hyssopus officinalis L.	Lamiaceae	
		Tinospora cordifolia (Willd.) Miers	Menispermaceae	
		Justicia adhatoda L.	Acanthaceae	
		Myristica fragrans Houtt.	Myristicaceae	
		Glycyrrhiza glabra L.	Leguminosae	
		Onosma bracteatum Wall	Boraginaceae	
19		Viola odorata L.	Violaceae	
26	Extracts	Terminalia bellirica (Gaertn.) Roxb.	Combretaceae	Triphala #
		Emblica officinalis Gaertn. (= Phyllanthus emblica L.)	Phyllanthaceae	Triphala #
		Terminalia chebula Retz.	Combretaceae	Triphala #
		Piper nigrum L.	Piperaceae	Trikatu #
		Piper longum L.	Piperaceae	Trikatu #
		Zingiber officinale Roscoe	Zingiberaceae	Trikatu #
		Embelia ribes Burm.f.	Primulaceae	
		Solanum xanthocarpum Schrad. & H. Wendl. (= S. virginianum L.)	Solanaceae	
		Cinnamomum cassia (L.) J.Presl	Lauraceae	
		Justicia adhatoda L.	Acanthaceae	
		Althaea officinalis L.	Malvaceae	
		Glycyrrhiza glabra L.	Leguminosae	
		Ocimum tenuiflorum L.	Lamiaceae	
		Piper longum L.	Piperaceae	
14		Ziziphus sativa Gaertn. (= Ziziphus jujuba Mill.)	Rhamnaceae	
		Alpinia galanga (L.) Willd.	Zingiberaceae	
		Hyssopus officinalis L.	Lamiaceae	

No.	Ingredients	Dosage form	Botanical name	Family
			Viola odorata L.	Violaceae
27	1	Capsules	*Zingiber officinale* Roscoe	Zingiberaceae
28	1	Capsules	*Piper nigrum* L.	Piperaceae
29	1	Capsules	*Curcuma longa* L.	Zingiberaceae
			Terminalia bellirica (Gaertn.) Roxb.	Combretaceae
			Mentha × *piperita* L.	Lamiaceae
30	3	Capsules	*Gymnema sylvestre* (Retz.) R.Br. ex Sm.	Apocynaceae
			Garcinia cambogia (Gaertn.) Desr. (= *G. gummi-gutta* (L.) Roxb.)	Clusiaceae
			Andrographis paniculata (Burm.f.) Nees	Acanthaceae
31	10	Tablets	*Emblica officinalis* Gaertn. (= *Phyllanthus emblica* L.)	Phyllanthaceae
			Terminalia chebula Retz.	Combretaceae
			Terminalia bellirica (Gaertn.) Roxb.	Combretaceae
			Ocimum tenuiflorum L.	Lamiaceae
			Curcuma longa L.	Zingiberaceae
			Emblica officinalis Gaertn. (= *Phyllanthus emblica* L.)	Phyllanthaceae
			Terminalia bellirica (Gaertn.) Roxb.	Combretaceae
			Terminalia chebula Retz.	Combretaceae
			Glycyrrhiza glabra L.	Leguminosae
			Zingiber officinale Roscoe	Zingiberaceae
32	6	Tablets	*Piper longum* L.	Piperaceae
			Piper nigrum L.	Piperaceae
			Cinnamomum zeylanicum Blume (= *C. verum* J.Presl)	Lauraceae
			Saxifraga granulata L.	Saxifragaceae
			Hemidesmus indicus (L.) R. Br. ex Schult.	Apocynaceae
			Sphaeranthus indicus L.	Compositae
33	6	Tablets	*Curcuma longa* L.	Zingiberaceae
			Santalum album L.	Santalaceae
			Carum copticum (L.) Benth. & Hook.f. ex C.B.Clarke (= *Trachyspermum ammi* (L.) Sprague)	Apiaceae
			Corallium rubrum $	
			Emblica officinalis Gaertn. (= *Phyllanthus emblica* L.)	Phyllanthaceae
			Asparagus racemosus Willd.	Asparagaceae
			Glycyrrhiza glabra L.	Leguminosae
			Mucuna pruriens (L.) DC.	Leguminosae
			Terminalia chebula Retz.	Combretaceae
			Zingiber officinale Roscoe	Zingiberaceae

No.	Form	Ingredient	Family
		Mytillus margaretiferous $	
34	Tablets	Glycyrrhiza glabra L.	Leguminosae
		Terminalia arjuna (Roxb. ex DC.) Wight & Arn.	Combretaceae
		Amomum subulatum Roxb.	Zingiberaceae
		Piper longum L.	Piperaceae
		Piper nigrum L.	Piperaceae
		Zingiber officinale Roscoe	Zingiberaceae
		Cinnamomum zeylanicum Blume (= C.verum J.Presl)	Lauraceae
		Iris × germanica L.	Iridaceae
35	Tablets	Boswellia serrata Roxb. ex Colebr.	Burseraceae
		Commiphora mukul (Hook. ex Stocks) Engl.	Burseraceae
		Curcuma longa L.	Zingiberaceae
		Allium sativum L.	Amaryllidaceae
		Zingiber officinale Roscoe	Zingiberaceae
		Piper nigrum L.	Piperaceae
		Piper longum L.	Piperaceae
		Piper chaba Hunter (= Piper retrofractum Vahl)	Piperaceae
		Apium graveolens L.	Apiaceae
		Sphaeranthus indicus L.	Compositae
36	Tablets	Terminalia chebula Retz.	Combretaceae
		Andrographis paniculata (Burm.f.) Nees	Acanthaceae
		Curcuma longa L.	Zingiberaceae
		Zingiber officinale Roscoe	Zingiberaceae
		Trigonella foenum-graecum L.	Leguminosae
		Swertia chirata Buch.-Ham. ex Wall.	Gentianaceae
		Carum copticum (L.) Benth. & Hook.f. ex C.B.Clarke (= Trachyspermum ammi (L.) Sprague)	Apiaceae
		Piper longum L.	Piperaceae
		Myristica fragrans Houtt.	Myristicaceae
		Lotus arabicus L.	Leguminosae
		Boswellia serrata Roxb. ex Colebr.	Burseraceae
		Cypraea moneta $	
		Terminalia bellirica (Gaertn.) Roxb.	Combretaceae
		Emblica officinalis Gaertn. (= Phyllanthus emblica L.)	Phyllanthaceae
37	Tablets	Commiphora mukul (Hook. ex Stocks) Engl.	Burseraceae
		Bauhinia variegata L.	Leguminosae

No.	Count	Form	Species	Family	Common name
38	12	Tablets	Hemidesmus indicus (L.) R. Br. ex Schult.	Apocynaceae	
			Curcuma longa L.	Zingiberaceae	
			Glycyrrhiza glabra L.	Leguminosae	
			Saxifraga granulata L.	Saxifragaceae	
			Hordeum vulgare L.	Poaceae	
			Santalum album L.	Santalaceae	
			Commiphora mukul (Hook. ex Stocks) Engl.	Burseraceae	
			Hibiscus rosa-sinensis L.	Malvaceae	
			Corallium rubrum $		
			Asparagus racemosus Willd.	Asparagaceae	
			Hemidesmus indicus (L.) R. Br. ex Schult.	Apocynaceae	
			Glycyrrhiza glabra L.	Leguminosae	
			Curcuma longa L.	Zingiberaceae	
			Emblica officinalis Gaertn. (= Phyllanthus emblica L.)	Phyllanthaceae	
			Terminalia bellirica (Gaertn.) Roxb.	Combretaceae	
			Terminalia chebula Retz.	Combretaceae	
			Zingiber officinale Roscoe	Zingiberaceae	
			Piper longum L.	Piperaceae	
			Piper nigrum L.	Piperaceae	
			Aloe mucilagines $		
39	6	Tablets	Bacopa monnieri (L.) Wettst.	Plantaginaceae	
			Alpinia galanga (L.) Willd.	Zingiberaceae	
			Glycyrrhiza glabra L.	Leguminosae	
			Emblica officinalis Gaertn. (= Phyllanthus emblica L.)	Phyllanthaceae	
			Santalum album L.	Santalaceae	
			Myristica fragrans Houtt.	Myristicaceae	
			Corallium rubrum $		
40	4	Capsules	Terminalia arjuna (Roxb. ex DC.) Wight & Arn.	Combretaceae	Arjun
			Cissus quadrangularis L.	Vitaceae	Harjor
			Phyllanthus emblica L.	Phyllanthaceae	Amalaki
			Ocimum gratissimum L.	Lamiaceae	Vana Tulsi
41	4	Capsules	Inula racemosa Hook.f.	Compositae	Pushkarmool
			Ocimum tenuiflorum L.	Lamiaceae	Krishna Tulsi
			Terminalia bellirica (Gaertn.) Roxb.	Combretaceae	Vibhitaki fruit
			Piper longum L.	Piperaceae	Pipali frukt

No.	Qty	Form	Scientific name	Family	Common name
42	1	Capsules	*Ocimum tenuiflorum* L.	Lamiaceae	Krishna Tulsi, Rama Tulsi, Vana Tulsi
43	3	Capsules	*Terminalia arjuna* (Roxb. ex DC.) Wight & Arn.	Combretaceae	Arjuna
			Sapindus trifoliatus L.	Sapindaceae	Reetha
			Moringa oleifera Lam.	Moringaceae	Sahijan blad
44	3	Capsules	*Coccinia indica* Wight & Arn. (= *Coccinia grandis* (L.) Voigt)	Cucurbitaceae	Bimbi blad
			Bougainvillea spectabilis Willd.	Nyctaginaceae	Bouginvellea blad
			Vinca rosea L. (= *Catharanthus roseus* (L.) G.Don)	Apocynaceae	Sadabahar blad
45	3	Capsules	*Aegle marmelos* (L.) Corrêa	Rutaceae	Bel blad
			Lepidium sativum L.	Brassicaceae	Chandrashoor frø
			Plantago ovata Forssk.	Plantaginaceae	Psyllium Husk
			Picrorhiza kurroa Royle ex Benth.	Plantaginaceae	Katuki
46	3	Capsules	*Ocimum tenuiflorum* L.	Lamiaceae	Krishna Tulsi
			Ocimum gratissimum L.	Lamiaceae	Vana Tulsi
			Phyllanthus niruri L.	Phyllanthaceae	Bhumyamalaki hel urt
47	3	Capsules	*Picrorhiza kurroa* Royle ex Benth.	Plantaginaceae	Katuki root
			Boerhaavia diffusa L.	Nyctaginaceae	Pumarnava root
48	1	Powder	*Althaea officinalis* L.	Malvaceae	Marshmallow root
49	1	Powder	*Hemidesmus indicus* (L.) R. Br. ex Schult.	Apocynaceae	
50	1	Powder	*Centella asiatica* (L.) Urb.	Apiaceae	
			Aegle marmelos (L.) Corrêa	Rutaceae	Bilva root
			Prenna integrifolia Willd. (= *P. serratifolia* L.)	Lamiaceae	Agnimantha root
			Oroxylum indicum (L.) Kurz	Bignoniaceae	Shyonaka root
			Stereospermum suaveolens (Roxb.) DC. (= *S. chelonoides* (L.f.) DC.)	Bignoniaceae	Patala root
51	10	Powders	*Gmelina arborea* Roxb.	Lamiaceae	Kashmari root
			Solanum indicum L.	Solanaceae	Bruhati root
			Solanum xanthocarpum Schrad. & H.Wendl. (= *S. virginianum* L.)	Solanaceae	Kantakari root
			Desmodium gangeticum (L.) DC.	Leguminosae	Shalaparni root
			Uraria picta (Jacq.) DC.	Leguminosae	Prushniparni root
			Tribulus terrestris L.	Zygophyllaceae	Gokshura root
52	1	Powder	*Eclipta alba* (L.) Hassk. (= *Eclipta prostrata* (L.) L.)	Compositae	Bringaraj
53	1	Powder	*Bacopa monnieri* (L.) Wettst.	Plantaginaceae	Brahmi
54	1	Powder	*Boerhaavia diffusa* L.	Nyctaginaceae	Punarnava
55	1	Powder	*Sida cordifolia* L.	Malvaceae	

No.	Count	Form	Scientific name	Family	Common name
56	1	Powder	*Rubia cordifolia* L.	Rubiaceae	Manjistha
57	1	Powder	*Gymnema sylvestre* (Retz.) R.Br. ex Sm.	Apocynaceae	Madhunashini
58	1	Powder	*Tinospora cordifolia* (Willd.) Miers	Menispermaceae	Guduchi
59	1	Capsules	*Garcinia indica* (Thouars) Choisy	Clusiaceae	
60	1	Capsules	*Terminalia arjuna* (Roxb. ex DC.) Wight & Arn.	Combretaceae	
61	1	Capsules	*Boerhaavia diffusa* L.	Nyctaginaceae	Punarnava
62	1	Powder	*Bacopa monnieri* (L.) Wettst.	Plantaginaceae	
63	3	Capsules	*Phyllanthus niruri* L.	Phyllanthaceae	Bhumiamalaki
			Tinospora cordifolia (Willd.) Miers	Menispermaceae	Guduchi
			Phyllanthus emblica L.	Phyllanthaceae	Amalaki
64	4	Capsules	*Bacopa monnieri* (L.) Wettst.	Plantaginaceae	Brahmi
			Centella asiatica (L.) Urb.	Apiaceae	Gotu Kola
			Convolvulus pluricaulis Choisy (= *C. prostratus* Forssk.)	Convolvulaceae	Shankpushpi
			Withania somnifera (L.) Dunal	Solanaceae	Ashwagandha
65	4	Capsules	*Cyperus rotundus* L.	Cyperaceae	Motha rhizome
			Withania somnifera (L.) Dunal	Solanaceae	Ashwagandha
			Tinospora cordifolia (Willd.) Miers	Menispermaceae	Guduchi
			Ocimum tenuiflorum L.	Lamiaceae	Rama Tulsi
66	6	Capsules	*Rubia cordifolia* L.	Rubiaceae	Manjit rot
			Pterocarpus santalinus L.f.	Leguminosae	Sandelträ
			Curcuma longa L.	Zingiberaceae	Gurkmeja
			Ocimum tenuiflorum L.	Lamiaceae	Rama Tulsi
			Azadirachta indica A.Juss.	Meliaceae	Neem
			Tinospora cordifolia (Willd.) Miers	Menispermaceae	Guduchi
67	4	Capsules	*Cyperus rotundus* L.	Cyperaceae	Motha
			Azadirachta indica A.Juss.	Meliaceae	Neem
			Curcuma longa L.	Zingiberaceae	Gurkmeja
			Ocimum tenuiflorum L.	Lamiaceae	Rama Tulsi
68	1	Capsules	*Glycyrrhiza glabra* L.	Leguminosae	Lakritsrot / Yashtimadhu
69	1	Capsules	*Saraca indica* L.	Leguminosae	
70	1	Capsules	*Aegle marmelos* (L.) Corrêa	Rutaceae	Bael
71	22	Tablets	Asphalatum $		Shilajit
			Withania somnifera (L.) Dunal	Solanaceae	Ashwagandha
			Gymnema sylvestre (Retz.) R.Br. ex Sm.	Apocynaceae	Gurmaar
			Azadirachta indica A.Juss.	Meliaceae	Nimba

No.	Form	Botanical name	Family	Vernacular name
		Terminalia chebula Retz.	Combretaceae	Harar choti
		Tinospora cordifolia (Willd.) Miers	Menispermaceae	Giloy
		Holarrhena antidysenterica (Roth) Wall. ex A.DC.(= *H. pubescens* Wall. ex G.Don)	Apocynaceae	Kutaj
		Tribulus terrestris L.	Zygophyllaceae	Gokhrudana
		Terminalia bellirica (Gaertn.) Roxb.	Combretaceae	Bahera
		Emblica officinalis Gaertn. (= *Phyllanthus emblica* L.)	Phyllanthaceae	Amala
		Aegle marmelos (L.) Corrêa	Rutaceae	Belpatra
		Curcuma zedoaria (Christm.) Roscoe	Zingiberaceae	Kachoor
		Justicia adhatoda L.	Acanthaceae	Vasa
		Ficus benghalensis L.	Moraceae	Badjata
		Acacia arabica (Lam.) Willd. (= *A. nilotica* (L.) Delile)	Leguminosae	Kikarfali
		Strychnos nux-vomica L.	Loganiaceae	Kuchla Shudh
		Centratherum anthelminticum (L.) Gamble (= *Baccharoides anthelmintica* (L.) Moench)	Compositae	Kaali jeeri
		Picrorhiza kurroa Royle ex Benth.	Plantaginaceae	Kutki
		Syzygium cumini (L.) Skeels	Myrtaceae	Jamun guthli
		Swertia chirata Buch.-Ham. ex Wall.	Gentianaceae	Chirayata
		Curcuma longa L.	Zingiberaceae	Haldi
		Trigonella foenum-graecum L.	Leguminosae	Methi
		Salacia chinensis L.	Celastraceae	Saptrangi
72	Tablets	*Bacopa monnieri* (L.) Wettst.	Plantaginaceae	
		Convolvulus pluricaulis Choisy (= *C. prostratus* Forssk.)	Convolvulaceae	
		Acorus calamus L.	Acoraceae	
		Onosma bracteatum Wall	Boraginaceae	
		Celastrus paniculatus Willd.	Celastraceae	
		Withania somnifera (L.) Dunal	Solanaceae	
		Tinospora cordifolia (Willd.) Miers	Menispermaceae	
		Praval pishti $		
		Mukta pishti $		
73	Tablets	*Nardostachys jatamansi* (D.Don) DC.	Caprifoliaceae	
		Rauvolfia serpentina (L.) Benth. ex Kurz	Apocynaceae	
		Oryza sativa L.	Poaceae	
		Saccharum munja Roxb. (= *Saccharum bengalense* Retz.)	Poaceae	
		Saccharum officinarum L.	Poaceae	
		Echinops echinatus Roxb.	Compositae	

No.	n	Form	Species	Family	Vernacular name
			Tinospora cordifolia (Willd.) Miers	Menispermaceae	
			Premna mucronata Roxb. (= *Premna mollissima* Roth)	Lamiaceae	
			Cassia fistula L.	Leguminosae	
			Sida cordifolia L.	Malvaceae	
			Asparagus racemosus Willd.	Asparagaceae	
			Pueraria tuberosa (Willd.) DC.	Leguminosae	
			Solanum surattense Burm. f.	Solanaceae	
			Solanum indicum L.	Solanaceae	
			Hordeum vulgare L.	Poaceae	
			Picrorhiza kurroa Royle ex Benth.	Plantaginaceae	
			Bacopa monnieri (L.) Wettst.*	Plantaginaceae	
			Convolvulus pluricaulis Choisy (= *C. prostratus* Forssk.)*	Convolvulaceae	
			Acorus calamus L.*	Acoraceae	
			Lavandula stoechas L.	Lamiaceae	
			Onosma bracteatum Wall	Boraginaceae	
74	9	Tablets	*Celastrus paniculatus* Willd.*	Celastraceae	
			Nardostachys jatamansi (D.Don) DC.	Caprifoliaceae	
			Foeniculum vulgare Mill.	Apiaceae	
			Withania somnifera (L.) Dunal	Solanaceae	
			Corallium rubrum$		
			Mytillus margaretiferous $		
			Tinospora cordifolia (Willd.) Miers	Menispermaceae	Giloy
75	3	Tablets	*Ocimum tenuiflorum* L.	Lamiaceae	Tulsi
			Azadirachta indica A.Juss.	Meliaceae	Neem
76	1	Powder	*Withania somnifera* (L.) Dunal	Solanaceae	Ashwagandha pulver
77	1	Powder	*Asparagus racemosus* Willd.	Asparagaceae	Raw Shatavari root
78	1	Powder	*Tribulus terrestris* L.	Zygophyllaceae	Raw Gokshura pulver
79	1	Powder	*Terminalia arjuna* (Roxb. ex DC.) Wight & Arn.	Combretaceae	Raw Arjuna Pulver

Notes. Ayurvedic herbal products 1–26 were purchased from pharmacies and herbal shops in Romania, 27–47 from Norway, and 48–79 purchased via e-commerce from Sweden. $ Non-plant ingredients. * Scientific names that indicate the use of various refined/standardized herbal substances within the product; whereas, all others indicate the use of not extracted plant material, including simply processed and comminuted plant material, within the product. # These vernacular names indicate that these species have more scientific names, and Ayurvedic pharmacopoeia of India was used to choose the correct plant species name. All other plant ingredients listed on the product label, are provided with both the scientific and vernacular name.

Supplementary Table S2. Details of the plants species used as ingredients in the herbal products.

Scientific names as indicated on the product label*	Plant family	Habit	Source#	Conservation status#	nrITS sequences in GenBank (NCBI)$
Abutilon indicum (L.) Sweet	Malvaceae	Shrub	Wild	Least Concern	Yes
Acacia arabica (Lam.) Willd. (= Acacia nilotica (L.) Delile)	Leguminosae	Tree	Wild	Least Concern	Yes
Acacia catechu (L.f.) Willd.	Leguminosae	Tree	Wild	Least Concern	Yes
Achillea millefolium L.	Compositae	Herb	Wild/Cultivation	Least Concern	Yes
Achyranthes aspera L.	Amaranthaceae	Herb	Wild	Least Concern	Yes
Acorus calamus L.	Acoraceae	Herb	Wild/Cultivation	Least Concern	Yes
Aegle marmelos (L.) Corrêa	Rutaceae	Tree	Wild	Vulnerable	Yes
Albizia lebbeck (L.) Benth.	Leguminosae	Tree	Wild	Least Concern	Genus is represented
Allium sativum L.	Amaryllidaceae	Herb	Cultivation	Least Concern	Yes
Aloe barbadensis Mill. (= Aloe vera (L.) Burm.f.)	Xanthorrhoeaceae	Herb	Cultivation	Least Concern	Yes
Alpinia galanga (L.) Willd.	Zingiberaceae	Herb	Wild/Cultivation	Least Concern	Yes
Althaea officinalis L.	Malvaceae	Herb	Wild	Least Concern	Yes
Amomum subulatum Roxb.	Zingiberaceae	Herb	Cultivation	Least Concern	Yes
Andrographis paniculata (Burm.f.) Nees	Acanthaceae	Herb	Wild/Cultivation	Least Concern	Yes
Anethum sowa Roxb. ex Fleming	Apiaceae	Herb	Cultivation	Least Concern	Genus is represented
Apium graveolens L.	Apiaceae	Herb	Cultivation	Least Concern	Yes
Asparagus racemosus Willd.	Asparagaceae	Shrub	Wild/Cultivation	Least Concern	Yes
Asteracantha longifolia Nees (= Hygrophila auriculata (Schumach.) Heine)	Acanthaceae	Herb	Wild	Least Concern	Yes
Azadirachta indica A.Juss.	Meliaceae	Tree	Wild/Cultivation	Least Concern	Yes
Bacopa monnieri (L.) Wettst.	Plantaginaceae	Herb	Wild/Cultivation	Least Concern	Yes
Bauhinia variegata L.	Leguminosae	Tree	Wild/Cultivation	Least Concern	Yes
Berberis aristata DC.	Berberidaceae	Tree	Wild	Vulnerable	Yes
Bergenia ligulata Engl.	Saxifragaceae	Herb	Wild	Least Concern	Yes
Boerhaavia diffusa L.	Nyctaginaceae	Herb	Wild	Least Concern	Yes
Bombax ceiba L.	Malvaceae	Tree	Wild	Least Concern	Yes
Boswellia serrata Roxb. ex Colebr.	Burseraceae	Tree	Wild	Vulnerable	Genus is represented

Species	Family	Habit	Source	Conservation Status	Endemic
Bougainvillea spectabilis Willd.	Nyctaginaceae	Woody Vine	Wild/Cultivation	Least Concern	Yes
Caesalpinia bonducella (L.) Fleming (= *Caesalpinia bonduc* (L.) Roxb.)	Leguminosae	Herb	Wild/Cultivation	Least Concern	Yes
Camellia sinensis (L.) Kuntze	Theaceae	Shrub	Cultivation	Least Concern	Yes
Capparis spinosa L.	Capparaceae	Shrub	Wild/Cultivation	Least Concern	Yes
Carica papaya L.	Caricaceae	Tree	Cultivation	Least Concern	Yes
Carum copticum (L.) Benth. & Hook.f. ex C.B.Clarke (= *Trachyspermum ammi* (L.) Sprague)	Apiaceae	Herb	Cultivation	Least Concern	Yes
Casearia esculenta Roxb.	Salicaceae	Shrub	Wild	Least Concern	Genus is represented
Cassia fistula L.	Leguminosae	Tree	Wild/Cultivation	Least Concern	Yes
Cassia occidentalis L. (=of *Senna occidentalis* (L.) Link)	Leguminosae	Herb	Wild	Least Concern	Yes
Celastrus paniculatus Willd.	Celastraceae	Woody Liana	Wild/Cultivation	Vulnerable	Yes
Centella asiatica (L.) Urb.	Apiaceae	Herb	Wild/Cultivation	Least Concern	Yes
Centratherum anthelminticum (L.) Gamble (= *Baccharoides anthelmintica* (L.) Moench)	Compositae	Herb	Wild/Cultivation	Least Concern	Yes
Chlorophytum arundinaceum Baker	Asparagaceae	Herb	Wild	Endangered	Yes
Cichorium intybus L.	Compositae	Herb	Wild/Cultivation	Least Concern	Yes
Cinnamomum cassia (L.) J.Presl	Lauraceae	Tree	Import	Least Concern	Yes
Cinnamomum zeylanicum Blume (= *Cinnamomum verum* J.Presl)	Lauraceae	Tree	Wild/Cultivation	Least Concern	Yes
Cissus quadrangularis L.	Vitaceae	Vine	Wild/Cultivation	Least Concern	Yes
Citrus limon (L.) Osbeck	Rutaceae	Tree	Cultivation	Least Concern	Yes
Coccinia indica Wight & Arn. (= *Coccinia grandis* (L.) Voigt)	Cucurbitaceae	Vine	Cultivation	Least Concern	Yes
Commiphora mukul (Hook. ex Stocks) Engl.	Burseraceae	Shrub	Wild	Least Concern	Yes
Commiphora wightii (Arn.) Bhandari	Burseraceae	Shrub	Wild	Endangered	Yes
Convolvulus pluricaulis Choisy (= *Convolvulus prostratus* Forssk.)	Convolvulaceae	Herb	Wild	Least Concern	Yes
Crateva nurvala Buch.-Ham	Capparaceae	Tree	Wild/Cultivation	Least Concern	Genus is represented
Crocus sativus L.	Iridaceae	Herb	Cultivation	Least Concern	Yes
Curcuma longa L.	Zingiberaceae	Herb	Cultivation	Least Concern	Yes
Curcuma zedoaria (Christm.) Roscoe	Zingiberaceae	Herb	Wild/Cultivation	Least Concern	Yes

Species	Family	Habit	Source	Conservation Status	Genus Represented
Cyperus rotundus L.	Cyperaceae	Herb	Wild	Least Concern	Yes
Cyperus scariosus R.Br.	Cyperaceae	Herb	Wild	Least Concern	Genus is represented
Desmodium gangeticum (L.) DC.	Leguminosae	Herb	Wild	Least Concern	Yes
Didymocarpus pedicellata (Ait.) Ait. F.	Asclepiadaceae	Herb	Wild	Endangered	Genus is represented
Echinops echinatus Roxb.	Compositae	Herb	Wild	Least Concern	Yes
Eclipta alba (L.) Hassk. (= Eclipta prostrata (L.) L.)	Compositae	Herb	Wild	Least Concern	Yes
Elettaria cardamomum (L.) Maton	Zingiberaceae	Herb	Cultivation	Least Concern	Yes
Embelia ribes Burm.f.	Primulaceae	Woody Climber	Wild	Vulnerable	Yes
Enicostemma littorale Blume	Gentianacae	Herb	Wild	Least Concern	No (other genera are represented)
Evolvulus alsinoides (L.) L.	Convolvulaceae	Herb	Wild	Least Concern	Yes
Ficus benghalensis L.	Moraceae	Tree	Wild	Least Concern	Yes
Foeniculum vulgare Mill.	Apiaceae	Herb	Cultivation	Least Concern	Yes
Fumaria officinalis L.	Papaveraceae	Herb	Wild/Cultivation	Least Concern	Yes
Garcinia cambogia (Gaertn.) Desr. (= Garcinia gummi-gutta (L.) Roxb.)	Clusiaceae	Tree	Wild/Cultivation	Least Concern	Yes
Garcinia indica (Thouars) Choisy	Clusiaceae	Tree	Wild/Cultivation	Least Concern	Yes
Glycyrrhiza glabra L.	Leguminosae	Herb	Cultivation	Least Concern	Yes
Gmelina arborea Roxb.	Lamiaceae	Tree	Wild	Least Concern	Yes
Gossypium herbaceum L.	Malvaceae	Shrub	Cultivation	Least Concern	Yes
Gymnema sylvestre (Retz.) R.Br. ex Sm.	Apocynaceae	Herb	Wild	Vulnerable	Yes
Hemidesmus indicus (L.) R. Br. ex Schult.	Apocynaceae	Herb	Wild	Least Concern	Yes
Hibiscus rosa-sinensis L.	Malvaceae	Shrub	Cultivation	Least Concern	Yes
Holarrhena antidysenterica (Roth) Wall. ex A.DC.(= Holarrhena pubescens Wall. ex G.Don)	Apocynaceae	Tree	Wild	Least Concern	Yes
Hordeum vulgare L.	Poaceae	Herb	Cultivation	Least Concern	Yes
Humulus lupulus L.	Cannabaceae	Climber	Cultivation	Least Concern	Yes
Hyssopus officinalis L.	Lamiaceae	Herb	Wild	Vulnerable	Yes
Inula racemosa Hook.f.	Compositae	Herb	Wild	Least Concern	Yes
Ipomoea digitata L. (=Ipomoea cheirophylla O'Donell)	Convolvulaceae	Herb	Wild	Least Concern	Genus is represented
Iris germanica L.	Iridaceae	Herb	Import/Cultivation	Least Concern	Yes
Justicia adhatoda L.	Acanthaceae	Shrub	Wild/Cultivation	Least Concern	Yes

Species	Family	Habit	Source	Status	Native
Kaempferia galanga L.	Zingiberaceae	Herb	Cultivation	Least Concern	Yes
Lavandula stoechas L.	Lamiaceae	Shrub	Import	Least Concern	Yes
Lepidium sativum L.	Brassicaceae	Herb	Cultivation	Least Concern	Yes
Leptadenia reticulata (Retz.) Wight & Arn.	Apocynaceae	Shrub	Wild	Endangered	Yes
Lotus arabicus L.	Leguminosae	Herb	Wild	Least Concern	Yes
Mentha piperita L.	Lamiaceae	Herb	Cultivation	Least Concern	Yes
Mentha arvensis L.	Lamiaceae	Herb	Wild/Cultivation	Least Concern	Yes
Momordica charantia L.	Cucurbitaceae	Vine	Cultivation	Least Concern	Yes
Moringa oleifera Lam.	Moringaceae	Tree	Cultivation	Least Concern	Yes
Moringa pterygosperma Gaertn.	Moringaceae	Tree	Cultivation	Least Concern	Yes
Mucuna pruriens (L.) DC.	Leguminosae	Climber	Wild	Endangered	Yes
Myristica fragrans Houtt.	Myristicaceae	Tree	Wild/Cultivation	Least Concern	Yes
Nardostachys jatamansi (D.Don) DC.	Caprifoliaceae	Herb	Wild	Vulnerable	Yes
Ocimum gratissimum L.	Lamiaceae	Herb	Wild/Cultivation	Least Concern	Yes
Ocimum tenuiflorum L.	Lamiaceae	Herb	Cultivation	Least Concern	Yes
Onosma bracteatum Wall	Boraginaceae	Herb	Wild	Least Concern	Genus is represented
Oroxylum indicum (L.) Kurz	Bignoniaceae	Tree	Wild	Vulnerable	Yes
Oryza sativa L.	Poaceae	Herb	Cultivation	Least Concern	Yes
Phyllanthus amarus Schumach. & Thonn.	Phyllanthaceae	Herb	Wild	Least Concern	Yes
Phyllanthus emblica L.	Phyllanthaceae	Tree	Wild/Cultivation	Vulnerable	Yes
Phyllanthus niruri L.	Phyllanthaceae	Herb	Wild	Least Concern	Yes
Phyllanthus urinaria L.	Phyllanthaceae	Herb	Wild	Least Concern	Yes
Picrorhiza kurroa Royle ex Benth.	Plantaginaceae	Herb	Wild	Endangered	Yes
Piper chaba Hunter (= *P. retrofractum* Vahl)	Piperaceae	Vine	Cultivation	Least Concern	Yes
Piper cubeba Bojer	Piperaceae	Vine	Cultivation	Least Concern	Yes
Piper longum L.	Piperaceae	Vine	Cultivation	Least Concern	Yes
Piper nigrum L.	Piperaceae	Vine	Cultivation	Least Concern	Yes
Plantago ovata Forssk.	Plantaginaceae	Herb	Cultivation	Least Concern	Yes
Plumbago zeylanica L.	Plumbaginaceae	Climber	Wild	Vulnerable	Yes
Premna integrifolia Willd. (= *P. serratifolia* L.)	Lamiaceae	Shrub	Wild	Least Concern	Yes
Premna mucronata Roxb. (= *P. mollissima* Roth)	Lamiaceae	Shrub	Wild	Least Concern	Yes
Prunus amygdalus Batsch (= *P. dulcis* (Mill.) D.A.Webb)	Rosaceae	Tree	Wild	Least Concern	Yes
Pterocarpus marsupium Roxb.	Leguminosae	Tree	Wild	Endangered	Yes

Species	Family	Habit	Source	IUCN Status	Included
Pterocarpus santalinus L.f.	Leguminosae	Tree	Wild	Endangered	Yes
Pueraria tuberosa (Willd.) DC.	Leguminosae	Climber	Wild	Vulnerable	Yes
Raphanus sativus L. (= Raphanus raphanistrum subsp. sativus (L.) Domin)	Brassicaceae	Herb	Cultivation	Least Concern	Yes
Rauvolfia serpentina (L.) Benth. ex Kurz	Apocynaceae	Shrub	Wild	Endangered	Yes
Rheum emodi L.	Polygonaceae	Herb	Wild	Endangered	Yes
Rubia cordifolia L.	Rubiaceae	Climber	Wild	Vulnerable	Yes
Rumex maritimus L.	Polygonaceae	Herb	Wild	Least Concern	Genus is represented
Saccharum munja Roxb. (= Saccharum bengalense Retz.)	Poaceae	Shrub	Wild/Cultivation	Least Concern	Yes
Saccharum officinarum L.	Poaceae	Shrub	Cultivation	Least Concern	Yes
Salacia chinensis L.	Celastraceae	Climber	Wild	Least Concern	Yes
Santalum album L.	Santalaceae	Tree	Wild	Endangered	Yes
Sapindus trifoliatus L.	Sapindaceae	Tree	Wild	Least Concern	Yes
Saraca asoca (Roxb.) Willd.	Leguminosae	Tree	Wild	Endangered	Yes
Saraca indica L.	Leguminosae	Tree	Wild	Least Concern	Yes
Saxifraga granulata L.	Saxifragaceae	Herb	Wild	Least Concern	Yes
Saxifraga ligulata Murray (= Saxifraga stolonifera Curtis)	Saxifragaceae	Herb	Wild	Least Concern	Yes
Sida cordifolia L.	Malvaceae	Herb	Wild	Least Concern	Yes
Solanum indicum L.	Solanaceae	Herb	Wild	Least Concern	Yes
Solanum nigrum L.	Solanaceae	Herb	Wild	Least Concern	Yes
Solanum surattense Burm. f.	Solanaceae	Herb	Wild	Least Concern	Yes
Solanum xanthocarpum Schrad. & H. Wendl. (= Solanum virginianum L.)	Solanaceae	Herb	Wild	Least Concern	Yes
Sphaeranthus indicus L.	Compositae	Herb	Wild	Least Concern	Yes
Stereospermum suaveolens (Roxb.) DC. (= Stereospermum chelonoides (L.f.) DC.)	Bignoniaceae	Tree	Wild	Vulnerable	Yes
Strychnos nux-vomica L.	Loganiaceae	Tree	Wild	Vulnerable	Yes
Swertia chirata Buch.-Ham. ex Wall.	Gentianaceae	Herb	Wild	Endangered	Yes
Symplocos racemosa Roxb.	Symplocaceae	Shrub	Wild	Vulnerable	Yes
Syzygium cumini (L.) Skeels	Myrtaceae	Tree	Wild/Cultivation	Least Concern	Yes
Tamarix gallica L	Tamaricaceae	Shrub	Wild	Least Concern	Yes
Tephrosia purpurea (L.) Pers.	Leguminosae	Herb	Wild/Cultivation	Least Concern	Yes
Terminalia arjuna (Roxb. ex DC.) Wight & Arn.	Combretaceae	Tree	Wild	Least Concern	Yes

Scientific name	Family	Habit	Source	Conservation status	nrITS
Terminalia bellirica (Gaertn.) Roxb.	Combretaceae	Tree	Wild	Least Concern	Yes
Terminalia chebula Retz.	Combretaceae	Tree	Wild	Least Concern	Yes
Tinospora cordifolia (Willd.) Miers	Menispermaceae	Climber	Wild/Cultivation	Least Concern	Yes
Tribulus terrestris L.	Zygophyllaceae	Herb	Wild	Least Concern	Yes
Trigonella foenum-graecum L.	Leguminosae	Herb	Cultivation	Least Concern	Yes
Uraria picta (Jacq.) DC.	Leguminosae	Herb	Wild	Endangered	Yes
Valeriana officinalis L.	Caprifoliaceae	Herb	Wild	Least Concern	Yes
Valeriana wallichii DC. (= *Valeriana jatamansi* Jones)	Caprifoliaceae	Herb	Wild	Vulnerable	Yes
Vernonia cinerea (L.) Less. (= *Cyanthillium cinereum* (L.) H.Rob.)	Compositae	Herb	Wild	Least Concern	Yes
Vinca rosea L. (= *Catharanthus roseus* (L.) G.Don)	Apocynaceae	Herb	Cultivation	Least Concern	Yes
Viola odorata L.	Violaceae	Herb	Cultivation	Least Concern	Yes
Vitis vinifera L.	Vitaceae	Climber	Cultivation	Least Concern	Yes
Withania somnifera (L.) Dunal	Solanaceae	Shrub	Wild/Cultivation	Least Concern	Yes
Zingiber officinale Roscoe	Zingiberaceae	Herb	Cultivation	Least Concern	Yes
Ziziphus sativa Gaertn. (= *Ziziphus jujuba* Mill.)	Rhamnaceae	Tree	Wild/Cultivation	Least Concern	Yes

Notes. *Scientific names as indicated on the product label, whereas scientific names within the parenthesis are the accepted names according to the Plant List (http://www.theplantlist.org/). $Plant source and conservation status was categorized based on the database ENVIS centre for medicinal plants, National Medicinal Plants Board, India (http://envis.frlht.org/), and from the reports by published by National Medicinal Plants Board, Ministry of AYUSH, Government of India (Demand and supply of medicinal plants in India, 2007; and Medicinal Plants in India: An Assessment of their Demand and Supply, 2017). #The availability of nrITS sequences in Genbank was checked on July 27, 2017.

Supplementary Table S3. Details of total number sequencing reads per products.

Species	Family
Phyllanthus amellus Roth	Phyllanthaceae
Piper longum L.	Piperaceae
Piper nigrum L.	Piperaceae
Piper retrofractum Vahl	Piperaceae
Pisum sativum L.	Leguminosae
Plantago alba L.	Plantaginaceae
Plantago major L.	Plantaginaceae
Plantago ovata Forssk.	Plantaginaceae
Pleurospermum diversum Hook. f.	Amaranthaceae
Podostemum madagascariense Lundin	Euphorbiaceae
Polygonatum verticillatum (L.) Bixf	Planntaceae
Pseudoelephantopus spicatus (B.Juss.ex Aubl.)Rohr	Scrophulariaceae
Punica granatum L.	Lythraceae
Pyrus malus subsp. mitis Desf.	Rosaceae
Euphorbia hirta L.	Brassicaceae
Rauvolfia serpentina (L.) Benth. ex Kurz	Apocynaceae
Robinia pseudoacacia L.	Leguminosae
Rosa rugosa Thunb.	Rosaceae
Salvia hispanica L.	Lamiaceae
Salvia tomentosa Schltdl.	Lamiaceae
Senna alexa (Roxb.) Willd.	Leguminosae
Senna occidentalis L.	Fabaceae
Sida japonicum Siebold ex Miq.	Crassulaceae
Senna alexandrina Mill.	Leguminosae
Senna auriculata (L.) Roxb.	Leguminosae
Senna occidentalis (L.) Link	Leguminosae
Senna tora (L.) Roxb.	Leguminosae
Sesamum indicum L.	Pedaliaceae
Salvia sclarea Benth.	Malvaceae
Sida cordifolia L.	Malvaceae
Silene aristata (Rupr.) Bocquet	Caryophyllaceae
Solanum lycopersicum L.	Solanaceae
Solanum nigrum L.	Solanaceae
Solanum virginianum L.	Solanaceae
Sorghum bicolor (L.) Moench	Poaceae
Spondias tuberosa Arruda	Anacardiaceae
Stenocereus chrysocarpus Sánchez-Mej.	Cactaceae
Strychnos nux-vomica L.	Loganiaceae
Swertia chirayita (Roxb.) Buch.-Ham. ex C.B.Clarke	Gentianaceae
Tamarindus indica L.	Leguminosae
Taraxacum officinale (L.) Weber ex F.H.Wigg.	Compositae
Terminalia arjuna (Roxb. ex DC.) Wight & Arn.	Combretaceae
Terminalia bellirica (Gaertn.) Roxb.	Combretaceae
Terminalia chebula Retz.	Combretaceae
Thalia plucnceana (Mart.) Eichler	Apiaceae
Dysoxylum latifolium (Roxb. ex Hornem.) Honda	Poaceae
Trichosanthes cucurdifolia (Willd.) Miers	Menispermaceae
Trachyspermum ammi (L.) Sprague	Apiaceae
Trianthema portulacastrum L.	Aizoaceae
Tribulus terrestris L.	Zygophyllaceae
Tinospora malagnoy F.Muell.	Molluginaceae
Tinctorella foenum-graecum L.	Leguminosae
Triticum aestivum L.	Poaceae
Veronica alternans R.Br.	Plantaginaceae
Vigna mungo (L.) Hepper	Leguminosae
Vigna radiata (L.) R.Wilczek	Leguminosae
Vigna unguiculata (L.) Walp.	Leguminosae
Vitex negundo L.	Lamiaceae
Withania somnifera (L.) Dunal	Solanaceae
Xanthium strumarium (L.) D.F.Austin & Stuples	Convolvulaceae
Xerococcus alliaceus W.R.B.Oliv.	Nyctaginaceae
Zea mays L.	Poaceae
Zanthoxylum (Rutaceae) Roxb.	Zanthoxylaceae

Total number of species within the product: 188

Supplementary Table S4. Details of normalized total number of sequencing reads per products.

Name of the species detected in the herbal product ID

Relative abundance of nrITS1 and nrITS2 reads (Replicate 1+2+3)

Supplementary Table S5. Details of num occurrences of a species in Ayurvedic herbal products used in generic healings

Supplementary Table S6. Details of genomic DNA concentration, nrITS amplicon concentration and success rate of sequence yield in herbal products after filtering the raw sequences between nrITS1 and nrITS2, and number of MOTUs in nrITS1 and nrITS after NCBI-BLAST identification

Table header structure (column groups):

- Herbal products code
- Herbal product type
- Total genomic DNA concentration measured using Qubit 2.0 Fluorometer (ng/µl)
- nrITS1 - Amplicon concentration of triplicates using qPCR*
  - nrITS1 amplicon concentration of replicate 1 (ng/µl)
  - nrITS1 amplicon concentration of replicate 2 (ng/µl)
  - nrITS1 amplicon concentration of replicate 3 (ng/µl)
- nrITS2 - Amplicon concentration of triplicates using qPCR*
  - nrITS2 amplicon concentration of replicate 1 (ng/µl)
  - nrITS2 amplicon concentration of replicate 2 (ng/µl)
  - nrITS2 amplicon concentration of replicate 3 (ng/µl)
- nrITS1 - Sequence yield in triplicates after filtering
  - No. of reads in replicate1 (MOTU's)
  - No. of reads in replicate2 (MOTU's)
  - No. of reads in replicate3 (MOTU's)
- nrITS2 - Sequence yield in triplicates after filtering
  - No. of reads in replicate1 (MOTU's)
  - No. of reads in replicate2 (MOTU's)
  - No. of reads in replicate3 (MOTU's)
- nrITS1 - No. of MOTU's in triplicates after NCBI-BLAST identification
  - No. of MOTU's in replicate1 (MOTU's)
  - No. of MOTU's in replicate2 (MOTU's)
  - No. of MOTU's in replicate3 (MOTU's)
- nrITS2 - No. of MOTU's in triplicates after NCBI-BLAST identification
  - No. of MOTU's in replicate1 (MOTU's)
  - No. of MOTU's in replicate2 (MOTU's)
  - No. of MOTU's in replicate3 (MOTU's)
- nrITS1 - No. of MOTU's in replicate1 (MOTU's)
- nrITS2 - No. of MOTU's in replicate2 (MOTU's)

Notes. *Values that correspond to '0' in amplicon concentration represents that the qPCR measured as '0' which implies that it has no amplification. In such cases, the complete volume from PCR well were taken for normalization and sequencing. The sample represented as 'Not Applicable' is that qPCR results have not given any values for those samples, in such cases Gel images were compared and the samples were normalized based on the intensity of the DNA band in the Gel.

Supplementary Table S7. Results of the DNA metabarcode analysis of herbal products