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‘BIOCHEMICAL ANALYSIS OF COMMON MACROPHYTES AND THEIR POTENTIAL USES’

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Submitted By:

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CANDIDATE'S DECLARATION

I, Prerna Bora, Roll no 2K16/IBT/07 of M. Tech (Industrial Biotechnology), hereby declare that the project Dissertation titled "Biochemical Analysis of Common Macrophytes and Their Potential Uses" which is submitted by me to the Department of Biotechnology, Delhi Technological University, Delhi in partial fulfillment of the requirement for the award of the degree of Master of Technology, is original and not copied from any source without proper citation. This work has not previously formed the basis for the award of any Degree, Diploma Associateship, Fellowship or other similar title or recognition.

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CERTIFICATE

I hereby certify that the project dissertation titled "Biochemical Analysis of Common Macrophytes and Their Potential Uses" which is submitted by Prerna Bora, 2K16/IBT/07 (Department of Biotechnology), Delhi Technological University, Delhi in partial fulfillment of the requirements for the award of the degree of Master of Technology, is a record of the project work carried out by the student by my permission and supervision. To the best of my knowledge this work has not been submitted in part or in full for any Degree or Diploma to this University or elsewhere.

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ABSTRACT

A major constraint to livestock production in developing countries is the scarcity and fluctuating quantity and quality of the year-round feed supply. Providing adequate good quality feed to livestock to raise and maintain their productivity is a major challenge to agricultural scientists all over the world. The increase in population and rapid growth in world economies will lead to an enormous increase in demand for animal products, a large part of which will be from developing countries. Future hopes of feeding millions of people and ensuring food security will depend on the enhanced and efficient utilization of alternative feed resources. Forage-based economical feeding strategies are required to reduce production cost of quality livestock products; as feed alone constitutes 60-70% of production cost. At present, the country faces a net deficit of 35.6 per cent of green fodder, 26 per cent of dry-crop residues and 41 per cent of concentrate feed ingredients. This can be compensated using macrophyte meals. With proper research, this idea can be perfected and with proper application, this can prove to be a lucrative option for animal husbandry.

Even though several authors have worked on the AA profile, few have studied the fatty acid profiles of these plant. Data on fatty acid content in terms of dry weight from literature is difficult to find and even inconclusive, therefore this project mainly targets fatty acid profiling. The present study has concluded that these macrophytes are rich in linolenic acid and palmitic acid with moderate amounts of polyunsaturated fatty acids like arachidonic acid which are essential amino acid for growth for pigs and poultry.

Crude protein estimations also showed satisfying results. Amino acid profile of *A. pinnata* show high amounts of lysine, an essential amino acid, critical for the growth of livestock and fish.

This report concludes that due to its excellent nutritional properties, *L. minor, S. polyrhiza* and *A. pinnata* can efficiently serve as alternate feed sources for fish, poultry and pigs. It is favorable to replace a portion of the regular feed with macrophyte meal instead of providing 100% macrophyte meals. This could prove to be a lucrative option, as cost of feed is expected to decrease with macrophyte addition.

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PRERNA BORA

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LIST OF ABBREVIATIONS

EAA	ESSENTIAL AMINO ACID
EFA	ESSENTIAL FATTY ACID
GC-MS	GAS CHROMATOGRAPHY- MASS SPECTROSCOPY
CP	CRUDE PROTEIN
CF	CRUDE FAT
HPLC	HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

CHAPTER 1. INTRODUCTION

The current scenario of this world demands a redemption. The aftermath of industrialization has put us in the danger zone in terms of environmental pollution, over-exploitation of earth's natural resources and deteriorating health of the urban population. The ramifications of ill-advised advancement in science has led to many upsetting conclusions. All these circumstances have cumulatively lowered the living standards of people, especially the aspect of food and nutrition. India, an aspiring country constantly dealing with over-population issue often find herself, in the crisis of food shortage, or prejudicial food in terms of inadequate nutritional profiles and excessive adultery. Since the population has crossed the billion mark, it has gotten even harder to provide good quality and quantity of food across the masses. This has led to a nation of malnutrition stricken population, and it doesn't limit to humans only. Naturally, feed quality standards for the livestock has significantly degraded as well. One of the most important function of domestic animal production is to provide high protein meat for human consumption. To fulfil this role, it is important that the animals too receive a high quality source of feed and a decent nutritional diet [1]. The world's population is estimated to cross the 9 billion mark in the next thirty years, and correspondingly the demand for protein to feed is expected to rise as well. Therefore, it is now of utmost important that we explore for an all-natural source of protein and use it as sparingly and wisely as possible. In this context, sustainable agriculture doesn't only limit to animal production but also extends to everything that incorporates diet and the source of feed for the animals in question [2]. In a country struggling with malnutrition and low-grade food sources, it is of utmost importance that an efficient alternative is constructed that is both economic and biologically sound. Researchers in India have found out some underutilised plant-based food sources with amino acid compositions that sufficiently meet animal feed standards and human consumption standards with some necessary processing. These food sources contain mainly of non-conventional weeds and inedible plant sources like macrophytes [3].

Macrophytes by definition are aquatic plants i.e. which can grow on or near a water source and which are large enough to be seen with the naked eye. They are found growing

naturally pretty much worldwide. They are commonly seen as a blanket of a dense population of tiny plants which typically dominates wetland, shallow lakes, brackish waters and slow-moving streams. Macrophytes can be flowering plants (e.g. Ferns) or large algal plants (e.g. Duckweeds). [4] They include the botanical families of Amaranthaceae, Araceae, Azollaceae, Lemnaceae and many more, given in table 1.

Table 1: List of macrophytes commonly found in India [5]

Sl no.	**Family**	**Number of species**	**Species**
1.	Pontederiaceae	1	*Eichornia crassipes*
2.	Hydrocharitaceae	3	*Hydrilla verticillata* *Najas indica* *Vallisneria spiralis*
3	Lemnaceae	3	*Lemna perpusilla* *Wolffia arrhiza* *Lemna minor*
4.	Poaceae	1	*Cynodondactylon*
5.	Characeae	1	*Charaspecies*
6.	Araceae	2	*Pistia stratioides* *Spirodella polyrhiza*
7.	Polygonaceae	1	*Polygonum glabrum*
8.	Ceratophyllaceae	1	*Ceratophyl lumdemersum*
9.	Cyperaceae	1	*Cyperus rotundus*
10.	Commelinaceae	1	*Commelina benghalensis*
11.	Convolvulaceae	2	*Ipomoea aquatica* *Typhaanguistata*
12.	Azollaceae	1	*Azolla pinnata*
13.	Trapaceae	1	*Trapanatans*
14.	Nymphaeaceae	1	*Nymphea mtuberosa*
15.	Marsileaceae	1	*Marselia minuta*

Macrophytes can be majorly categorised as:

i) Submerged, Emergent and Free- floating

ii) Rooted and Un-rooted.

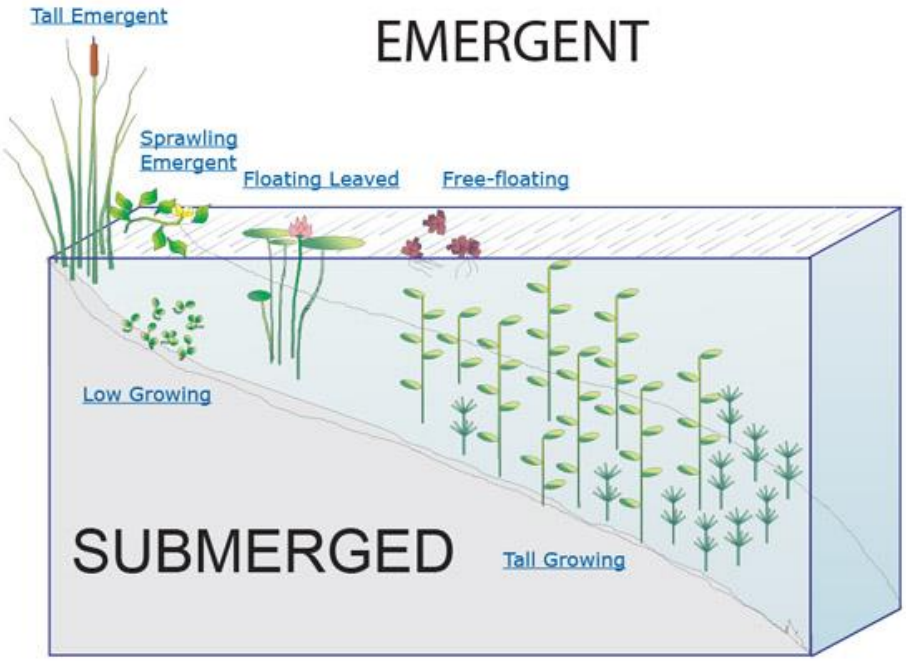


Fig 1: Classification of macrophytes [6]

This thesis investigates 3 different species of macrophytes: viz. *Lemna minor*, *Spirodela polyrhiza*, *Azolla pinnata*. They belong to the unrooted, free- floating category of macrophytes. Water with high amounts for nitrogen, phosphorous and potassium can foster better growth of these macrophytes. Macrophytes can easily synthesized protein of high quality by utilising these nutrient and concentrating minerals. Existing literature suggest a nutritional profile of crude protein 9-30%, crude fat 2.2-5%, carbohydrate 9.5-35%, ash 8-25%, and crude fibre 15-25%, moisture 85-95%, dry matter 4-16%, with caloric content of 2.5-4 kcal/g [7]. If nutrient availability and sunlight are optimum, macrophytes can increase their biomass by almost one fold. Also, these plants possess a unique feature i.e., its nutritional profile significantly changes along with its substrate water. Different substrate sources used in macrophyte production play a pivotal role in determining the ultimate fatty acid and amino acid profiles. It is almost as if these profiles can be designed according to need, use, type of feed to be synthesized and the animal to which the feed is being provided. Research show macrophytes (especially duckweed)

grown on water with 10-30 mg NH_3-N/litre have a high protein content (around 40%) of high biological value, comparable to animal protein [8] Traditional fodder and feed given to poultry, pigs and fish are typically manufactured from agro-industrial by-products and crop residues, hence are critically deficit in nutrients crucial for suitable growth and optimum meat production. Moreover, the aquaculture and poultry industries are dependent on commercially manufactured feed which is overpriced and scarcely available in local areas. Because of its versatile properties, these plants have been gaining popularity. Farmers in Africa and Asia have harvested naturally grown aquatic plants for purposes including fodder, manure and even for human consumption. [9]. Azolla, a member of the fern family, grows extensively in association with nitrogen fixing bacteria. This allows it to grow in waters with low nitrogen but substantial amounts of phosphorus. Very recently, a commonly occurring aquatic plant, "duckweed" (Lemna, Spirodela), has become prominent, because of its phytoremediation abilities. Although some have been explored in the past decade, its uses were not applied in an industrial/commercial level due to lack of technical knowledge and poor financing. [10].

This project aims to give an overview of the biochemical properties and nutritional quality of the aforementioned macrophyte species. It encompasses qualitative and quantitative analysis of its protein, amino acid, fatty acid and sterol profiles employing advanced bio-instrumentation. It goes on to draw a comparative study of these 3 plants, comprehend its ecological and economic role, and further deduce its potential use as feed source primarily.

These plants include several fundamental properties that can be exploited to serve the needs of humans and animals, viz., the plants naturally contain high amounts of protein and can be used as alternative food/feed source, these plants have great potential for phytoaccumulation hence making them promising candidates for phytoremediation, they can also be used as organic fertilizer. As a step forward, it could also be designed for human consumption after additional processing steps such as removal of toxic or unwanted compounds and careful determination of its nutritive composition and its effects. Legal issues are also to be taken care of prior to any large scale involvement to make this concept more acceptable to the general public. In the past decade, we have seen a blooming population all over the world, especially in developing countries where most of the current population lives, this has begun a movement to investigate a more sustainable form of agriculture. It is essential to maintain people in agriculture and simultaneously produce an increasing amounts of good quality food to cater to the

growing population. Particularly some places in Asia and Africa, where people have been suffering with polluted, limited or mis-used water resources, macrophyte cultivation can serve more than just feed-stock. By employing the water cleansing abilities of macrophytes, one can generate potable water in semi-arid conditions, provided certain safe-guards are applied in place. This is possible in small farmer systems without any heavy machinery or labour cost.

1.1 Importance of macrophytes

Macrophytes can used as indicators of health of a water body. Several water bodies have been excessively polluted due to anthropogenic activities and natural resources. Rapid industrialization, urbanization, and increase in population have increased the amount of water pollutants. Such unprecedented pollution in aquatic ecosystems needs eco-friendly cost-effective remediation technology. Many of the macrophytes are known to accumulate and concentrate heavy metals from water sources. Phytoremediation, a plant based green technology, includes several processes namely, phytoextraction, phytodegradation, rhizofiltration, phytostabilization and phytovolatilization. These aquatic plants act as natural filters that take up water-soluble toxic compounds mainly through roots and temporarily immobilize them with-in its living system. It is seen that these plants grow rather vigorously where the nutrient load is high. Macrophytes absorb primarily nitrogen, phosphorus, calcium, sodium and heavy metals such as lead, mercury, arsenic and tin. [11] Several researchers around the globe have studied macrophytes as agents of phytoremediation. Several macrophytes have been investigated remediation of waste-water containing Cu, Hg, As, Pb and Cd. *L. minor* has shown effective results with Pb and Ni [12] [13]. Azolla species have been investigated for Arsenic uptake. [14] [15]. *S. polyrhiza* has been studied for arsenic uptake under laboratory conditions and further researched for phosphorous and iron. [16] [17].

Table 2: Literature of heavy metal uptake by *L. minor, A. pinatta, S. polyrhiza.*

Scientific name	**Trace elements**	**Reference**
Lemna minor	As, Zn, Hg	[18, 19]

Azolla pinatta	As, Hg, Cd	[20, 21]
Spirodela polyrhiza	As, Cr, Co, Cu	[22, 23]

1.2 OBJECTIVE

i. Study and analyse the nutritional composition of *Lemna minor, Spirodela polyrhiza and Azolla pinnata*.

ii. Qualitative and quantitative analysis of the Fatty acid profiles of *Lemna minor, Spirodela polyrhiza and Azolla pinnata*.

iii. Calculation of crude protein levels in *Lemna minor, Spirodela polyrhiza and Azolla pinnata*.

iv. Amino acid analysis of *Azolla pinnata.*

v. Sterol analysis of *Lemna minor, Spirodela polyrhiza and Azolla pinnata*.

vi. Creation of a comparative study of the three macrophytes and evaluation of their potential to serve as feedstock for fish, poultry and pigs.

CHAPTER 2: REVIEW OF LITERATURE

The process of turning an inedible plant into feed source can have many scientific and legal implications. Macrophytes like already mentioned are versatile plants and happens to be hyperaccumulators, because of this nature of the plant, large-scale production could be tedious. For an item to be labelled food grade, good amount of research is required. In order to formulate a feed source using these macrophytes, following data is vital:

i. To qualitatively and quantitively derive the concentration of protein, amino acid, fatty acids, carbohydrates, secondary metabolites, sterols, biologically active compounds, medicinal compounds etc.
ii. To design a substrate sources that fosters optimum production and quality of feed.
iii. To know how different water sources (substrate) affect the final nutritional profile of the plant, and which nutrient can beneficially affect its composition.
iv. To deduce what type of waste-water (domestic or agricultural) can be used to for its production
v. To learn and calculate the concentration bio-accumulated elements (heavy metals) within the plant
vi. To analyse if the plant is safe to consume, if not to find a way to remove the toxic or unwanted compounds making it fit to be used as feed again.

Pursuing a solution for the short-comings, this project aims to better understand these plant's nutritional properties, construct its best use and concurrently map its effects on the environment and ecosystem.

2.1 Overview of the plants

L. minor, S. polyrhiza and A. pinnata are small, fragile, free floating aquatic plants. *L. minor, S. polyrhiza* fall into a sub-class of macrophytes known as 'duckweed' while *A.*

pinnata belong to the fern family. *L. minor* is commonly known as the lesser duckweed *while S. polyrhiza* goes by the name the greater duckweed. *A. pinnata* is a heterosporous free-floating freshwater ferns that live symbiotically with *Anabaena azollae*, a nitrogen-fixing blue-green algae, which enables them to rapidly grow on nitrogen deficit water and commonly known as water fern, mosquito fern etc [24] (Islam and Haque, 1986). These plants grow floating on the surface of a water body with submerged roots and usually don't depend on water depth or soil. Their mode of reproduction is primarily vegetative. Reproduction is rapid when nutrient densities are optimum [25].

Fig 2: Overview of *L. minor, S. polyrhiza and A. pinnata*

Table 3: Macrophyte divisions and representative genera [26]

Kingdom	**Macrophyte Divisions**	**Representative Freshwater Macrophyte Genera**
Monera	Cyanobacteria	*Oscillatoria, Lyngbya*
Protista	Chlorophyta	*Chara, Nitella, Cladophora, Enteromorpha*
	Rhodophyta	*Lemanea, Batrachospermum*
	Xanthophyta	*Vaucheria*
Plantae	Bryophyta	*Fontinalis, Riella, Ricciocarpus*
	Pteridophyta	*Azolla, Salvinia, Isoetes*
	Spermatophyta	*Lemna, Spirodela, Eichhornia*

Table 4: Scientific classification [27]

Lemna minor	*Spirodela ploryhiza*	*Azolla pinnata*
Kingdom: Plantae (unranked): Angiosperms Order: Alismatales Family: Lemnaceae	Kingdom: Plantae Class: Angiosperms Order: Arales Family: Lemnaceae	Kingdom: Plantae Class: Polypodiopsida/Pteridopsida (disputed) Order: Salviniales Family: Salviniaceae

2.1.1 Morphology and Anatomy

Duckweeds are monocotyledons, consists of a single leaf or frond. Lemna minor consists of a minute thallus each bearing a single root under the surface. Duckweeds have circular to elliptic or obovate thallus. The roots are 1-2 cm long and adventitious. The leaves are oval, 1-8 mm long and 0.6-5 mm broad, light green, and small air spaces to assist flotation. Spirodela have the largest fronds (20 mm across), while Lemna has intermediate fronds. Needle like raphides made of Calcium oxalate are typically present in Lemna. The upper epidermis in the Lemna is highly cutinized. Stomata are on the upper side. S. polyrhiza is a smooth, round, ½-1 cm wide [28]. Azolla consists of a main stem growing at the surface of the water. Roots are adventitious roots and are found at regular intervals along the stem. Plant diameter ranges from 1-2.5 cm. Azolla usually forms triangular or polygonal frond [29].

2.1.2 Habitat and Distribution

They are adapted to a wide variety of geographic and climatic zones. They can survive extreme temperatures are naturally found in almost every part of the world except for waterless deserts and permanently frozen areas, however tropical and temperate zones are best suited for their proliferation. Duckweeds are rare in places with extremely low or high rainfall. Duckweeds are most commonly seen on the surface of fresh or brackish water, sheltered from wind and wave action. They cannot tolerate water current more than.3 m/second or un-sheltered water sources. This particular quality prevents them from becoming weeds in water ways. These particular species of macrophytes are found in several bioregions with highest occurrence in highest in the Neotropics, Oriental, and Afrotropics, lower in the Australasia, and Pacific Oceanic Islands, and lowest in the Antarctic region [30] [31]. It is a naturally occurring all over India, abundant in NCR (National Capital Region).

Fig 3: *Lemna minor* cultivation in Ghaziabad

Fig 4: Natural duckweed formation in Hapur

Fig 5: *A. pinnata* in a holding tank in Uttarakhand

2.1.3 Environmental Requirements

The plants can grow over a temperature range of 6 to 33°C. Optimum temperature for duckweed's maximum growth lies between 17.5 and 30 ºC and it is seen that the growth rate starts to decline below 17 ºC (Culley et al., 1981). Duckweeds has a pH range of 5 to 9, however optimum pH for *L. minor* is 6.1-6.7 and for *S. polyrhiza* is 6.5-8.5 [32] [33]. Duckweeds prefer ammonia nitrogen (NH_4-N) as a source of nitrogen and will remove ammonia preferentially, even in the presence of relatively high nitrate concentrations. Phosphorus is a major limiting nutrient after nitrogen. Optimum P concentrations were estimated from 6 to 154 mg/l [34]. The family Lemnaceae as a whole requires potassium in the range of 0.5 – 100 mg/l, Ca 0.1 – 365 mg/l, Mg 0.1– 230 mg/l and Na 1.3 – >1000 mg/l [35]. Growth rate of *A. pinnata* is severely affected by the lack of water and phosphorous concentrations. Azolla can survive a water pH ranging from 3.5-10, reported optimum growth occurring at pH 4.5-7.0. *Azolla* can withstand a salinity up to 300mg/l but growth decreases as salinity increases higher than 380mg/l [36]. *Azolla* grows in 50-100% sunlight, and quickly decreases under heavy shade. It requires a minimum P concentration of 0.06mg/l/day and an optimum concentration of 20mg/l [37].

2.2 Nutritional Assessment

2.2.1. Chemical compositions

A. pinnata has a higher crude protein content, fitting for animal feed. Crude protein content is estimated at 19-30% DM basis during the optimum conditions and 20-22% under normal conditions. Its carotene content ranged from 206 to 619 mg / kg on a dry matter basis. Lignin was reported to vary between 9.27 and 28.24% and silica content varies between 4.8 and 16% [38]. Pillai et al. 2002 reported that Azolla is very rich vitamins (vitamin A, vitamin B12 and Beta-carotene) and growth promoter intermediaries [39]. Freshly harvested duckweed contains 91-95% water and very less fibre of < 9%. Duckweed grown in under nutrient-poor water have CP in between 9-20 percent, while the nutrient rich waters give a varying protein content of 24-41% DM. Similarly, the lipid content for duckweed grown in nutrient poor water was about 1.8-3% while in nutrient rich water it was 3-7% DM. Duckweeds also has β-carotene and xanthophyll. Duckweeds store varying amounts of calcium as calcium oxalate crystals in the raphides [40]. Duckweeds contain numerous biologically active compounds, for example: aromatic

alcohols (menthol), olefins, phytosterols (campesterol, stigmasterol, -sitosterol) and flavonoid as luteolin-7-glucoside [41].

Table 5: Chemical compositions

Species	**Minerals (% DM)**			**Moisture (%)**	**C.F (% DM)**	**C.P (% DM)**	**E.E (% DM)**	**Ash (% DM)**	**C.F* (% DM)**	**References**
	N	P	Ca							
A. pinnata	4-5	0.5-0.9	0.4-1.0	93.5	12.7	21.4	2.7	16.2	3-3.36	[42, 43]
L. minor	-	0.7	0.6	98.3	10	20.3-23.5	1.9	12.1	4.4	[44, 45]
S. polyrhiza	-	1.28	2.10	91	18.7	23.8	3.8	18.3	4	[46]

CF: Crude fibre; CP: Crude protein; EE: Ether extract; CF*: Crude fat

2.2.2 Protein and Amino acid Composition

Crude protein estimation is done by Kjeldahl Method, developed by Johan Kjeldahl in 1883. It enables us to quantify total nitrogen in an organic compound plus the inorganic nitrogen (ammonia, ammonium). The main principle is to oxidised nitrogen by sulphuric acid in presence of a catalyst of liberate ammonium sulphate. This process in practice is largely automated. It is widely used to assay waste water, soil, fertilizers and even certain food sources. TNK (Total Kjeldahl Nitrogen) is often used as substitute for protein for food sources. However, it is fairly easy to derive the protein concentrations, it is done by multiplying total nitrogen with a conversion factor which are assigned for different foods [47].

For amino acid profiles, HPLC has been the long standing golden standard. Since amino acids are non-volatile, HPLC gives the most sensitive and reliable results, however, over the years number of modifications have been done. Sample preparation usually follows with hydrolysis with HCl at a high temperature overnight. Analysis of sulphur containing compounds and proline require additional step in sample preparation. Detection is most done using UV. Absorption of the carboxyl group (-COOH) in seen in the range of 200 to 210 nm while some amino acids with benzene rings can also be detected in the 250 to 280 nm range. Since amino acids are non-volatile, derivatization is carried out for better detection. This can be done in two ways: separation of underivatized amino acids by ion exchange HPLC and subsequent detection by post-column derivatization, or pre-column derivatization and subsequent separation of amino acid derivatives by reverse phase HPLC. Some common examples of pre-column derivatizing reagents are phenyl isothiocyanate (PITC), o-phthalaldehyde, and fluorescamine [48]. The most commonly used column for separation is cation exchange. As amino acids are zwitter ions, using the cation exchange method efficiently separates multiple amino acids as it provides selectivity and adequate retention. Amino acids can also be analysed using LC-MS; however, it cannot be applied to free amino acids. Capillary electrophoresis (CE) is a new separation technique uses UV fluorophores to provide better resolution and sensitivity [49]. Amino acids analysers are HPLC unit with an ion-exchange column which ae dedicated for amino acids only. It commonly uses post-column derivatization with ninhydrin [50].

Several authors have analysed and quantified the amino acid profile of these plants, a few are tabulated below.

Table 6: Amino acid compositions (values expressed in g/100g of Crude protein) (from literature)

Amino acid	***L. minor* [51]**	***S. polyrhiza* [51]**	***A. pinnata* [52]**
CYS	0.9	0.8	0.9
MET	1.6	1.6	1.5
ASP	8.2	7.8	8.3
THR	4.0	4.2	4.0
SER	4.1	4.1	4.0

GLU	9.8	9.6	9.6
GLY	4.6	4.3	5.2
ALA	5.1	5.4	5.8
VAL	4.6	4.4	5.1
ILEU	3.7	3.3	4.2
LEU	7.3	6.8	7.7
TYR	3.1	3.1	4.0
PHE	4.4	3.97	4.6
LYS	5.0	4.2	3.8
HIS	1.5	1.6	1.6
ARG	4.8	4.7	5.0
PRO	3.8	3.5	4.0

2.2.3 Fatty Acid Composition

The technique of gas chromatography (GC) revolutionized the study of lipids and currently an indispensable technique for detection and quantification of fatty acids. Most common sample preparation includes conversion of fatty acid components of lipids into its simplest convenient volatile derivative, usually methyl esters. This can be done in two ways: base- or acid-catalysed esterification. Base catalysis is a less harsh process compared to acid catalysis. It commonly used methanolic KOH/NaOH. However, in presence of FFA, it results in soap formation. Acid catalysis is done with HCl, attributed to its comparably mild nature. BF_3 is a commonly used acid catalyst for methylation but is highly corrosive in nature. Present-day GC methods with high-quality capillary columns allow sensitive and reproducible fatty acid analyses, along with characterization of individual FAMEs. Conventional detectors include flame ionization detector (FID), electron capture detector (ECD), flame photometric detector (FPD) and flame thermionic detector (FTD or NPD). The flame-ionization detector is preferred. Detection is sensitive and accurate, it also provides a huge range of detection [53]. Although GC sounds synonymous with FA analysis, HPLC is also used. A major advantage of HPLC is that it operates at closer to room temperature, this can be important in maintain the integrity of sensitive functional groups. Reversed-phase HPLC enables fatty acids to be separated on the basis of chain length and degree of saturation. Excellent separations of positional isomers of fatty acids have been reported using HPLC. However, drawbacks such as

difficulties in recognising the order of elution of different components has somewhat limited its use. [54]

Existing literature provides the fatty acid profile of *L. minor* and *S. polyrhiza*, which are tabulated below. However, values for *A. pinnata* are disputed.

Table 7: Fatty acid compositions of *L. minor* and *S. polyrhiza.* (in % of total FAME)

Fatty Acid	***L. minor***	***S. polyrhiza***
SFA	27.9	39.8
MUFA	4.63	4.4
PUFA	48.1	55.7
C-16:0 **Palmitic acid**	21.3	29.6
C-18:1c9 **Oleic acid**	1.67	1.71
C-18:2c9, c12 **Linoleic acid**	20.11	1.59
γC-18:3c6, c9, c12 **γ-Linolenic acid**	0.52	0.01
αC-18:3c9, c12, c15 **α-Linolenic acid**	44.47	43.86
C-18:4c6, c9, c12, c15 **Stearidonic acid**	1.16	0.00
C-22:0 **Behenic acid**	0.37	0.42
C-24:0 **Lignoceric acid**	1.32	3.0
C-26:0 **Cerotic acid**	0.46	0.56

Values are expressed as % of total FAME

2.2.4 Sterol Composition

Phytosterols are found in plant cells. They are cholesterol-like compound belonging to the family of lipids, broadly classified either as 4-desmethylsterols, 4-methylsterols, or 4,4'-dimethylsterols. Sitosterol, campesterol, and stigmasterol (4-desmethylsterols group) are found in abundance in most of the plants. Phytochemical screening of *A. pinnata* show presence of phytosterol along with secondary metabolites, however it doesn't confirm the individual phytosterol qualitatively or quantitatively [55]. Literature suggests that *L. minor* contains a wide range of sterols (campesterol, stigmasterol, -sitosterol) and are present in quantity of 52.8 mg/kg of dry weight of the plant.

Gas Chromatographic-Flame Ionization Detection (GC-FID) is widely used in the analysis of phytosterols. Most preferred methods for detection of phytosterol is GC-FID. Typical sample preparation includes extraction followed by alkaline hydrolysis (saponification). Since most sterols contain hydroxyl group, they can be derivatized using silylating agents such as BSFTA+TMCS. Derivatization gives trimethylsilyl (TMS) ethers, which improves their volatility, peak shape, and response factors [56].

2.3 Nutritional requirement of fish, poultry and pigs

2.3.1 Protein and amino acid requirements

Animal diets doesn't require specific crude protein but certain amino acids which are essential for protein synthesis and more. Different combinations of proteogenic amino acids make up all the proteins. When proteins are digested, they are broken down to individual amino acids which are then absorbed by the blood stream. These amino acids are incorporated to form proteins according to the demand of the body. Certain amino acids are essential i.e. it can't be synthesized by the body and therefore are supposed to be acquired from diet. If a diet lacks any particular essential amino acid, protein synthesis will cease as soon as the EAA is exhausted. This is called "limiting amino acid". Naturally if a diet is enriched with the limiting amino acid, it will result in higher production of that protein whose limiting amino acid was externally supplemented. Additionally, if supply of amino acids is not in the proper, or ideal, ratio, then amino acids in excess of the least limiting amino acid will be de-aminated and will end up being used as a source of energy and not as a protein synthesis precursor. This will finally result in higher nitrogenous

excretions and give rise to soil or water toxicity. Reductions in nitrogen level in excreta is observed when non-essential amino acid pool is lowered, coupled with higher amounts of “ideal” amino acid Therefore, diets should be formulated in a way that it contains optimum amounts of essential amino acids, with a corresponding amount of non-essential amino acids to prevent the conversion of essential amino acids into energy source. Growth of an animal cannot be fully understood or attributed to the crude protein content in diet, hence it should no longer be used to evaluate the quality of feed [57]. Unfortunately, there is a wide-spread belief that whenever crude protein concentrations are lowered, performance is negatively affected [58] [59].

Essential amino acids are indispensable and has to be acquired through feed, for fish they are: methionine, arginine, threonine, tryptophan, histidine, isoleucine, lysine, leucine, valine and phenylalanine. Most common limiting amino acids are methionine and lysine and are usually added as supplements in feed. Favourable CP levels in aquaculture feeds average for marine shrimp is at around 20%, for catfish at 30%, 32-38% for tilapia at 35%, for hybrid striped bass at 40%. Protein requirements are higher for carnivorous fish than for herbivorous and omnivorous fish. Also, protein requirements decrease as the fish as they grow larger. A diet containing more than 30% nitrogen results in exceeding amounts of nitrogen in excreta, produced 90% through gills and rest as solid excreta. This leads to accelerated eutrophication and is a major concern for water quality management for fish farmers. Effective feeding and waste management practices are essential to protect downstream water quality [60].

The essential amino acids for poultry are: arginine, glycine, histidine, leucine, isoleucine, lysine, methionine, cystine, phenylalanine, threonine, tryptophan and valine. Out of these, the ones critical in practical diets are arginine, lysine, methionine, cystine and tryptophan. The type of poultry feed used directly affects the quality of eggs or meat they produce therefore its important that the requirements are met. [61]

Table 8: Dietary amino acid (% of diet) requirements for poultry

Animal (At 3 weeks)	**Nutrients %**								**Reference**
	CP	TS AA	Met	Lys	Thr	Trp	Arg	Val	
Broiler	23	0.9	0.5	1.1	0.8	0.2	1.2	0.9	[62]

Laying hen	-	0.8	0.5	1.2.	0.7	-	1.3	0.9	[63]
Turkey	28	1.0	0.5	1.6	1.0	0.26	1.6	1.2	[64]

The essential amino acids for swine are: lysine, threonine, tryptophan, methionine (and cystine), isoleucine, histidine, valine, arginine, and phenylalanine (and tyrosine). Most feed sources are limiting in lysine, tryptophan, and threonine. Protein sources for swine can be classified into two major categories: animal (tankage, meat and bone meal, fish meal, or dried skim milk) and plant (soybean meal, cotton seed meal, or corn gluten meal). Soybean meal is usually the most economical source of high quality protein used. An ideal protein should provide exactly 100% of the recommended level of each amino acid. Standard diets are usually formulated to meet the pig's requirement for lysine (the most limiting amino acid), however excess of many other amino acids may exist. Here lysine is used as a base value with other amino acids expressed as the percentage of the lysine requirement. [65]

Table 9: Amino acid ratio recommendations for pigs

Amino Acid	**Piglet (Body weight 4-20 kg)**	**Growing Swine (Body weight 20-40 kg)**
Lys	100	100
Thr	62	63
Met	28	29
Met+ Cys	58	58
Trp	17	16
Ile	55	55
Val	65	65
Arg	42	40
His	32	32
Leu	100	100
Phe+ Tyr	94	94
Phe	60	60

2.3.2 Fatty acid Requirements

Lipids are high energy nutrients that can be used as substitutes for protein in aquaculture feed. They provide almost twice as much as energy as carbohydrates and proteins and serve as transporters for fat soluble vitamins. An ideal fish diet should contain about 15% lipid. However higher amounts of lipid may result in fat deposition in the liver of the fish. Fish typically require fatty acids of the omega 3 and 6 (n-3 and n-6) families. n-3 HUFA is essential for optimal growth and health for marine fish, its typical concentration should be from 0.5-2.0% of dry diet. The two major EFA of this group are eicosapentaenoic acid (EPA: 20:5n-3) and docosahexaenoic acid (DHA: 22:6n-3). On the other hand, freshwater require 18 carbon n-3 fatty acid, linolenic acid (18:3-n-3), suitable quantity range from 0.5 to 1.5% of dry weight of diet. Tilapia require fatty acids of the n-6 family, while still others, such as carp or eels, require a combination of n-3 and n-6 fatty acids [66]

Poultry feed should contain long-chain unsaturated fatty acids. Essential fatty acids (EFA) in poultry nutrition consist of linoleic acid and arachidonic acid. Research shows and high mortality rates and retarded growth in chicks which were fed fat-free purified diet [Reiser 1950a]. Linoleic acid reserve of the young chick was very important, diets without Linoleic acid resulted in chicks with enlarged liver and increased liver fat content. It is observed that vegetable oils high in linoleic acid were most effective in improving egg size. 20-80 mg of linoleic acid/hen/day had a significant effect on egg production [67]. Although the optimum concentration of linolenic acid is disputed, studies suggest a range of 0.15-1.0% of dry weight [68].

Animals are unable to synthesise n-6 or and n-3 polyunsaturated fatty acids (PUFAs). Therefore, these must be supplied in the diet either in their long-chain form- linoleic acid (LA 18:2n- 6) or as their precursors- α-linolenic acid (ALA 18:3n-3) in relative proportions. Although the role of ‘essential’ fatty acids were long known, limited research has been conducted on the essential fatty acid requirements for swine and its dietary intake values are somewhat vague. NRC (1998) recommendations only provide optimum concentration of Linolenic Acid at 0.1% of the diet. Some authors suggest that reproduction may be optimised at levels of 3.3% linoleic and 0.45% linolenic acid [69]

2.4 Potential use as feed source

2.4.1 *A. pinnata* as feed

Ali et al. 1995 fed male broilers rice polishings, Azolla meal and snail meal at concentrations 200, 100 and 80 g/kg of diet respectively for 14 days and then compared weight gain to broiler feeding on soybean meal. The body weight, weight gain and feed/gain for birds fed Azolla and snail meal were similar to those seen in birds fed the maize-soybean meal diet [70].

Study performed by T. Sujatha et al. Azolla was fed, at the rate of 200 g per chick per day for 15 weeks and compared with chicks which were fed basal diet. End results show no difference in weight gain, immunocompetence, egg quality. Hence, it was concluded that is a good feed additive for Nicobari fowl and profitable due to savings on feed cost.

Alalade et al. 2007 designed a study where birds were fed 0, 5, 10 and 15% of AZM (Azolla meal) till 18 weeks of age then switched to corn-soybean meals. Results indicate a benefit from AZM at low level of supplementation and up to 15% AZM can be incorporated in diets of growing pullets without jeopardizing health and subsequent laying performance [71].

Duran (1994) reported that the aquatic Azolla can replace up to 20 per cent of the soya bean protein with no adverse effect on the performance of growing and fattening pigs.

Almazan et al. (1986) fed *A. pinnata* to Nile tilapia (Oreochromis niloticus) fingerlings and adult males in concentrations of 10-90%. Results show a lowering of growth rate in all Azolla incorporated diets.

In a feeding experiment with Tilapia nilotica, Nobuyuki Shiomi et al. 2000 reported a diet containing 20.7, 34.4, and 48.2% of the total weight of dried Azolla, given for 3 weeks, led to a decrease of the fish weight gain of 5.2, 16.8, 17.1% when compared with the control, respectively [72].

2.4.2 Duckweeds as feed

Nolan et al., 1997 conducted a study with where birds were changed from conventional diet to diets with *S. polyrhiza* at an increasing concentration of 10, 30, 50, 80, 120 and 200g/kg of feed replacing both grain and soybean meal. Diets containing higher duckweed content showed lowered egg mass production.

Hassan and Edwards, 1992, Hajra and Tripathi, 1985 concluded that meals with duckweed were efficiently utilized by common carp, Indian major carps (rohu and mrigal), silver carp, and in some cases (grass carp) growth was rapid than meals without duckweed [73].

Fasakin, Balogum and Fasuru (1999) reported that *Spirodela polyrhiza* meal can go up to 30 percent of the total diet for Nile tilapia without significantly affecting growth performance, compared to a control without duckweed [74].

Rodriguez and Preston (1996b) fed pigs, a duckweed diet along with cane juice as major source of energy. Results show mediocre utilization of duckweed protein and slightly less weight gain when compared to pigs which were fed normal diet.

CHAPTER 3: MATERIALS AND METHODS

A brief account of experimental procedures and analytical techniques adopted during study are presented below.

3.1 Materials

3.1.1 Sample

i. *Lemna minor*
ii. *Spirodela polyrhiza*
iii. *Azolla pinnata*

Fig 6: Dried Samples of *Lemna minor*, *Spirodela polyrhiza*, *Azolla pinnata*

3.1.2 Instruments

i. Centrifuge (Fig 7)

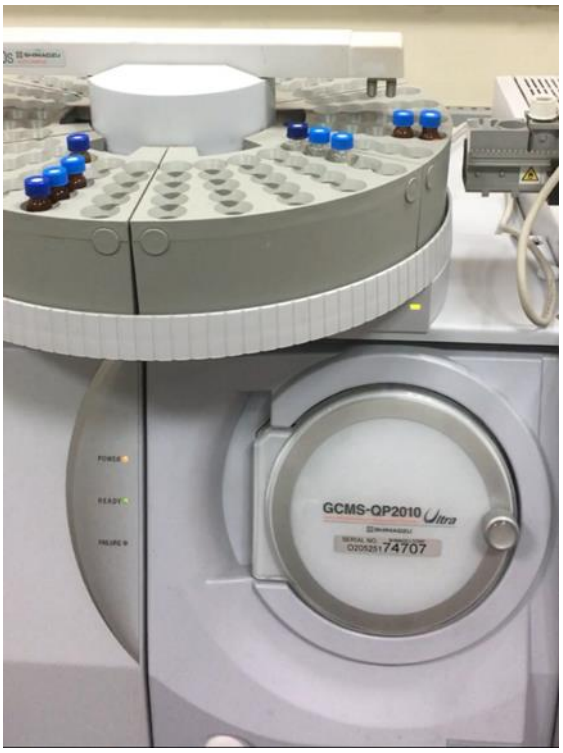
ii. Water bath

iii. Incubator

iv. Nitrogen Evaporator

v. Gas Chromatography

vi. Gas Chromatography- Mass spectroscopy (Fig 8)



vii. Kelplus Classic DX VA, Kjeldahl Apparatus, Pelican Instruments (Fig 9)

viii. Amino Acid Analyser (Fig 10)

Fig 11: Inside view of Amino Acid Analyzer

3.2 Methods

3.2.1 Plant cultivation and preparation

The plants were grown in a freshwater pond in University of Delhi. Pondwater was supplemented mustard oil cake and cow dung. Plants leaves were sieved out, air-dried and grinded in a mixer-grinder. Dried samples were stored in a vacuum sealed container and stored at 4°C for further use.

Fig 12: Cultivation of *A. pinnata* in University of Delhi,
Dept. of Zoology, Aqua Lab.

Fig 13: Air-dried *L. minor* in University of Delhi, Dept. of Zoology, Aqua Lab

3.2.2 Fatty acids Analysis

FA analysis was done in two different methods: Qualitative Analysis by GC-MS and Quantitative Analysis by GC using standards.

3.2.2.1 Qualitative Analysis by GC-MS

a) Reagents Used: Chloroform, Methanol, Potassium hydroxide, n-Heptane, Nitrogen Stream for drying, Milli-Q water. (All chemicals used were AR grade or GC grade)

b) Lipid Extraction (Folch's extraction method)

200mg of plant sample was weighed and mixed with 3 ml of extraction solvent (2:1 solution of chloroform and methanol). Solution was vortexed and allowed to mix in room temperature for 10 minutes. Solution was then centrifuged at 2070 rcf for 15 minutes at 10°C. Supernatant was pipetted out into another test tube. 1ml of extraction solvent was added to the pellet are centrifuged again at the same parameters and supernatant was pipetted out and collected in the same test tube as the previous supernatant. This step was repeated 2 more times. The total collected supernatant was filtered using No 1 Whatman paper. 2ml of Milli-pore water was added to the supernatant was centrifuged at 2070 rcf for 5 minutes. The aqueous

phase along with the interface was pipetted out and remaining the organic layer was dried under a nitrogen stream. (19)

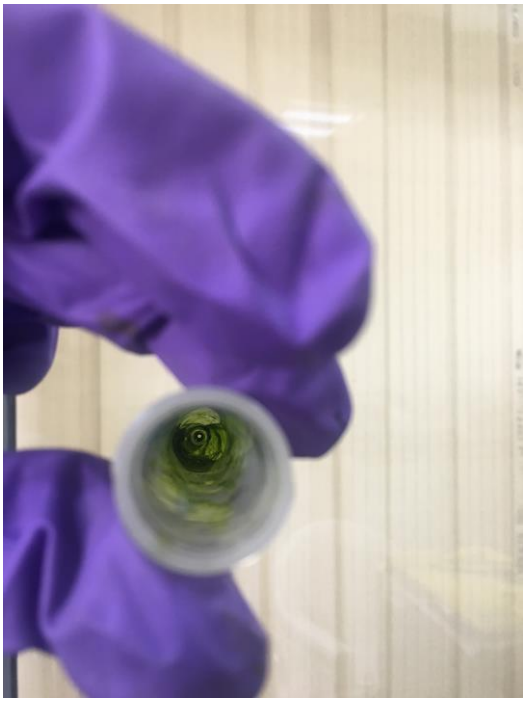


Fig 14: Plant extract after drying with N_2 stream

c) Transesterification to FAME (Base catalysis) (20)

10ml of n-heptane was added to the dried plant extract and vortexed till properly mixed. 4ml of freshly prepared 3.5% Methanolic KOH was added and vortexed. Solution was sealed tight and put in a water path maintained at 70°C for 2 minutes and vortexed again, this step was repeated till colour change in the upper layer was noticed. The test tubes were allowed to cool down then, upper layer (n-heptane mixed with the methyl esters) was pipetted out to a fresh test tube and dried under a nitrogen stream till methyl esters were found sticking to the walls of the test tube. 1.5ml of n-heptane was added along the walls of the test tube to dissolve all the methyl ester and vortexed to ensure proper mixing. Samples were labelled are examined in GC-MS.

Figure 15: Nitrogen dried methyl ester

Figure 16: ME re-dissolved in n-heptane

d) Gas chromatography and Mass spectroscopy.

The methyl esters formed were separated and detected by a Gas Chromatography (GC-2010 Plus) from Shimadzu, Japan. Helium was used as the carrier gas at a flow rate of 1.2ml/min with a FID (Flame Ionisation Detector). The temperature profiles were as follows: Initial temperature = 140°C (hold time 4 minutes); heating rate = 4°C/min; final temperature =260°C; ion source temperature = 260°C. Fatty acids separated were identified using mass spectroscopy (GCMS-

QP2010 Ultra) from Shimadzu, Japan. Column used was Rxi-5Sil (30 m). Mass spectra for every individual compound was generated and compared to the NIST and Wiley library which gives top five hits with decreasing value of similarity index. Individual fatty acids were expressed as percentage of total fatty acids detected.

3.2.2.2 Quantitative Analysis of FA by Gas Chromatography

a) Steps from lipid extraction to transesterification were followed as mentioned earlier.

b) Gas chromatography

Supelco 37-Component FAME Mix 1X1ml (external standard of varied concentration) was procured from Sigma-Aldrich. 3 different concentrations of 1mg/ml, 5mg/ml and 10mg/ml were made by adding calculated volumes of dichloromethane. 3 different concentrations of standard were run on GC along with FAME samples prepared from the dried plant. Instrument used was Gc-QP20C0 Ultra from Shimadzu, Japan. Analytical conditions were maintained as specified with standard. Column used was Rxi-5Sil (100 m x 0.25mm x 0.2µm). Detector type used was FID at 260°C. Inlet temperature was 250°C. Carrier gas was Helium at 20cm/s flow rate. Make up gas was Nitrogen at 30ml/min. Column temperature was at 100°C with a hold of 5 mins then ramped up to 240°C at 4°C/min. Final hold at 240°C was for 30 mins. Run time was 50 mins.

3.2.3 Protein and Amino acid analysis

3.2.3.1 Kjeldahl Method for Crude protein estimation

The protein content of the samples was determined by micro-Kjeldahl method using an automated nitrogen estimating system (Pelican Instruments, Chennai, India). In this system there were three consecutive steps: digestion, distillation and titration. The first step digestion was performed taking 250 - 300 mg of sample in the digestion tube. After that concentrated sulfuric acid (10ml) was added in presence of catalyst mixture (potassium sulphate: copper sulphate, 5:1). Digestion started as preheated at 350°C for 30 min and then the temperature was raised to 420°C for maintained 2 h.

After 2 h, the samples were allowed to cool on a stand and then 10 ml of distilled water was added to the digested sample. The sample was loaded in the distillation. Excess base (10 ml) NaOH was added to the digestion product to convert

ammonium (NH^{+}_{4}) to ammonia (NH_3) and it was collected in boric acid (4%). Distillation process was conducted on an automated digestion unit (Kelplus Classic DX VA, Pelican Instruments, Chennai, India) and this whole process took 10 min.

Titration is based on the principle that it quantifies the amount of ammonia in the receiving solution (2.5% Boric acid solution with mixed indictor methyl red and bromocresol green, 2:1). The amount of nitrogen in a sample was calculated from the quantified amount of ammonia ion in the received solution. Titration was carried out in an Autotitrator (pH-STAT 902 Titrando, Metrohm, Switzerland). The distillate was collected and then titrated against 0.1 N HCl. The dosing unit of the autotitrator which accurately detected the sharp shift in pH as the end point of the titration. The percent of nitrogen and protein for each sample were analysed using pre-installed programme software (TIAMO 2.2-81, Metrohm, Switzerland).

3.2.3.2 Amino Acid Analysis of *A. pinnata*

Sample preparation was performed separately for tryptophan.

a) All amino acid except tryptophan

30mg of sample (equals to 9mg pf CP at 30% of dry weight) was taken in a hydrolysis tube and 10ml of 6NHCl was added (sample to acid ratio should maintained at 3gm:1ml). Reaction mixture was exposed to nitrogen gas for 3-4 mins (for evaporation of oxygen and making the condition inert). The tube was closed tightly to ensure that there is no air leakage from the tube. Tube is kept the tube in Oven at 110 °C for 22 h for hydrolysis. After 22h, tubes were brought to room temperature. Sample was transfer to a volumetric flask and 25ml of distilled water was added. Sample was filtered with Whatman filter paper (110 mm, Cat No. 10001-110). 5ml of the sample was kept in the nitrogen Evaporator (18 – 20 psi, 60 – 70 °C) till it was completely dried to eliminate HCl. Sample was washed with 5 ml of distilled water 3 times to remove residual acid. Dry sample was re-dissolved in 8.4ml of 0.02N HCL (pH 3) to obtain sample. Finally, it was filtered with syringe filter Whatman 0.20 µm PES Filter (Cat.No. 6794 – 2512). 1.5ml of the final sample was taken in the chromatography glass vial and labelled and put

on the autosampler. It is important that the final concentration of protein the sample is les than 0.5mg/ml.

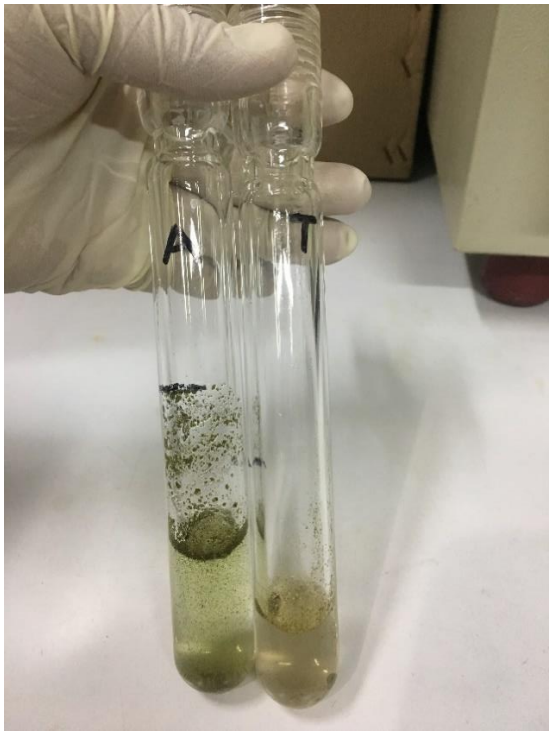


Fig 17: Digestor Tube A= All amino acids; Tube T= Tryptophan

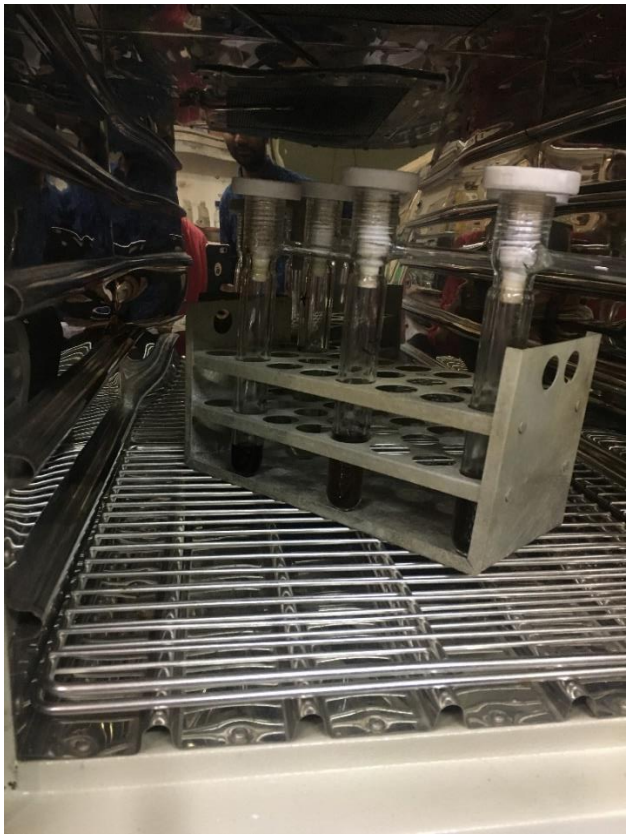


Fig 18: Digestor tubes containing sample and acid in incubator

b) Tryptophan

15mg of sample (equals to 4.5g of CP at 30% of dry weight) was weighed and added to the digestion tube. To it, 10ml of 4 N methanesulfonic acid with 0.2 % 3-(2-aminoethyl) indole was added. Reaction mixture was exposed to nitrogen gas for 3-4 mins (for evaporation of oxygen and making the condition inert). The tube was closed tightly to ensure that there is no air leakage from the tube. Tube is kept the tube in Oven at 110 °C for 22 h for hydrolysis. After 22h, tubes were brought to room temperature. X ml of 4N NaOH was added to bring the pH around 2 (X=4ml). pH was checked after adding each 1ml of NaOH using pH test paper. Final concentration of protein in sample was brought to 0.5mg/ml by adding 5ml of 0.02N HCl. Finally, it was filtered with syringe filter Whatman 0.20 µm PES Filter (Cat.No. 6794 – 2512). 1.5ml of the final sample was taken in the chromatography glass vial and labelled and put on the autosampler.

c) Specification of Amino Acid Analyser and Analytical conditions

Instrument used was HITACHI L-8900 automatic amino acid analyser (Hitachi Co. Ltd., Tokyo, Japan). Separation of free amino acid was done by an Ion-Exchange chromatography (Cation exchange column) reacted with Ninhydrin colouring solution and analysed by UV-VIS spectrophotometer at 440 and 570nm. Deamination column: 4.6 mm × 40 mm, #2650. Separation column: 4.6 mm × 60 mm (packed with Hitachi custom ion exchange resin #2622, 3 µm), lithium type. Sample injection volume: 20 µL. Separation buffer: MCI Buffer L-8500-PF Kit (Mitsubishi Chemical Corporation). Flow rate: 0.35 mL min-1. Column temperature: 30-70 °C. Reaction column: 4.6 mm × 40 mm, built-in inert silicon carbide particles. Reaction agent: Ninhydrin colouring solution kit for HITACHI (Wako Pure Chemical Industries, Limited). Reaction temperature: 135 °C. Ninhydrin flow rate: 0.30 mL min-1. Standard solution used were Amino Acids Mixture Standard Solution, Type B (Wako Pure Chemical Industries, Limited), including arginine, histidine, lysine, ornithine, δ-hydroxylysine, 1-methylhistidine, 3-methylhistidine, anserine, carnosine, γ-aminobutyric acid (GABA), ethanolamine; and Amino Acids Mixture Standard Solution, Type AN-2 (Wako Pure Chemical Industries, Limited), including aspartic acid, serine,

proline, glutamic acid, glycine, alanine, valine, cystine, methionine, isoleucine, leucine, tyrosine, phenylalanine, threonine, hydroxyproline, citrulline, β-alanine, α-aminoadipic acid, α-aminobutyric acid, β-aminoisobutyric acid, cystathionine, o-phosphoserine, o-phosphoethanolamine, taurine, sarcosine.

3.2.4 Sterol Analysis

3.2.4.1 Organic extraction

300mg of sample was taken in a glass vial with cap. 3.75ml of chloroform and methanol in the ratio 1:2 was added and vortexed vigorously. Mixture was centrifuged at 5000rpm for 10 mins at 10°C. Supernatant was collected in another tube. 1.25ml of chloroform was added to the pellet and centrifuged again at same conditions for 5 mins. Supernatant was added to the same tube as the previous step. 2ml of dH_2O was to the total supernatant and vortexed well. Mixture was again centrifuged in same conditions for 10 mins. Bottom organic water was carefully pipetted out into a fresh tube. Solution was dried under a nitrogen stream till the chloroform was completely evaporated.

3.2.4.2 Alkaline hydrolysis

500 µl of 6% methanolic KOH (w/v) was added to the dried residue and incubated at 85ºC for ½ -1 hrs. To it, half the volume i.e. 250 µl of dH2O was added and then equal volume i.e. 750 µl of n-heptane was added and vortexed well. The mixture was allowed to stand till the layers get separated. Upper phase was transfer to a fresh tube. Previous step was repeated twice. Samples were dried under a nitrogen stream till all the n-heptane was evaporated.

3.2.4.3 Derivatization

To the dried residue, 150 µl of derivatization reagent (BFSTA+ 1% TMCS) was added and incubated in a water bath at 65ºC for 1 hr. Mixture was again evaporated under nitrogen stream and re-dissolved in 2 ml of n-heptane. Final samples were filtered through a 0.2µm Nylon filter and collected in GC glass vial. Stored at -20°C till analysis.

Fig 19: Filtering of sterol sample through 0.2 micron nylon filter (left), Final sterol samples in GC glass vials (right).

3.2.4.3 Gas chromatography and Mass spectroscopy.

The silytated sterols were separated and detected by a Gas Chromatography (GC-2010 Plus) from Shimadzu, Japan. Helium was used as the carrier gas at a flow rate of 1.2ml/min with a FID (Flame Ionisation Detector). The temperature profiles were as follows: Initial temperature = 140°C (hold time 4 minutes); heating rate = 4°C/min; final temperature =260°C; ion source temperature = 260°C. Fatty acids separated were identified using mass spectroscopy (GCMS-QP2010 Ultra) from Shimadzu, Japan. Column used was Rxi-5Sil (30 m). Mass spectra for every individual compound was generated and compared to the NIST and Wiley library which gives top five hits with decreasing value of similarity index. Individual phytosterols were expressed as percentage of total sterols detected.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Fatty Acid GC-MS Results

The fatty acid compositions are analysed from its mass spectra profiles obtained from NIST and Wiley library. Each peak on the chromatogram obtains 5 most similar compounds, out of which the compound with the highest similarity index is selected and tabulated.

4.1.1. *L. minor*

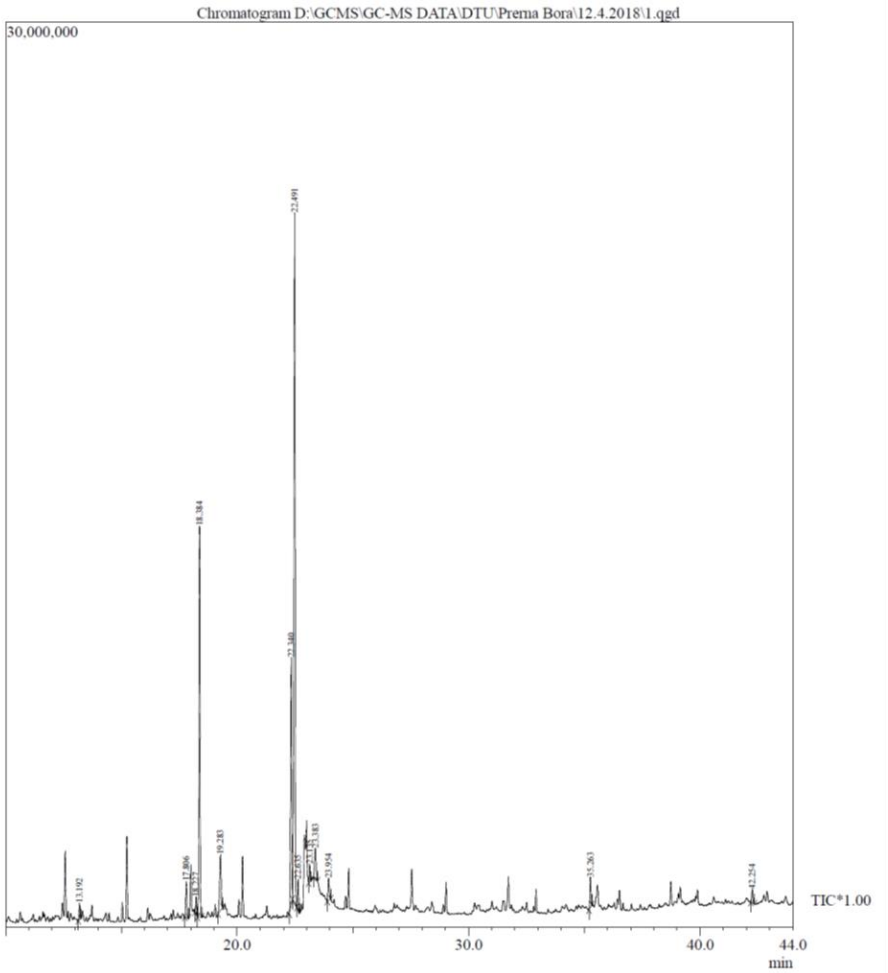


Fig 20: GC- MS FAME chromatogram for *L. minor*

Table 10: Fatty acid concentrations in *L. minor*

Sl no.	Retention Time	Compound Name	Lipid Number	Percentage of FA of Total lipid content (%)
1.	13.9	Myristic acid	14:0	0.8

		(1-tetradecanoic acid)		
2.	17.8	Palmitoleic acid (hexadec-9-enoic acid)	16:1	3.2
3.	18.3	Palmitic Acid (hexadecanoic acid)	16:0	27.3
4.	22.3	Linoleic acid (octadeca-9,12-dienoic acid)	18:2	15.5
5.	22.6	Linolenic acid (9,12,15-Octadecatrienoic acid)	18:3 (n-3)	47
6.	23.1	Oleic acid (Octadecenoic acid)	18:1 cis 9	1.4
7.	23.3	Stearic Acid (octadecanoic acid)	18:0	2.2
8.	35.2	Lignoceric acid (Tetracosanoic acid)	24:0	1.6
9.	42.2	Montanic acid (Octacosanoic acid)	-	1

4.1.2 *S. polyrhiza*

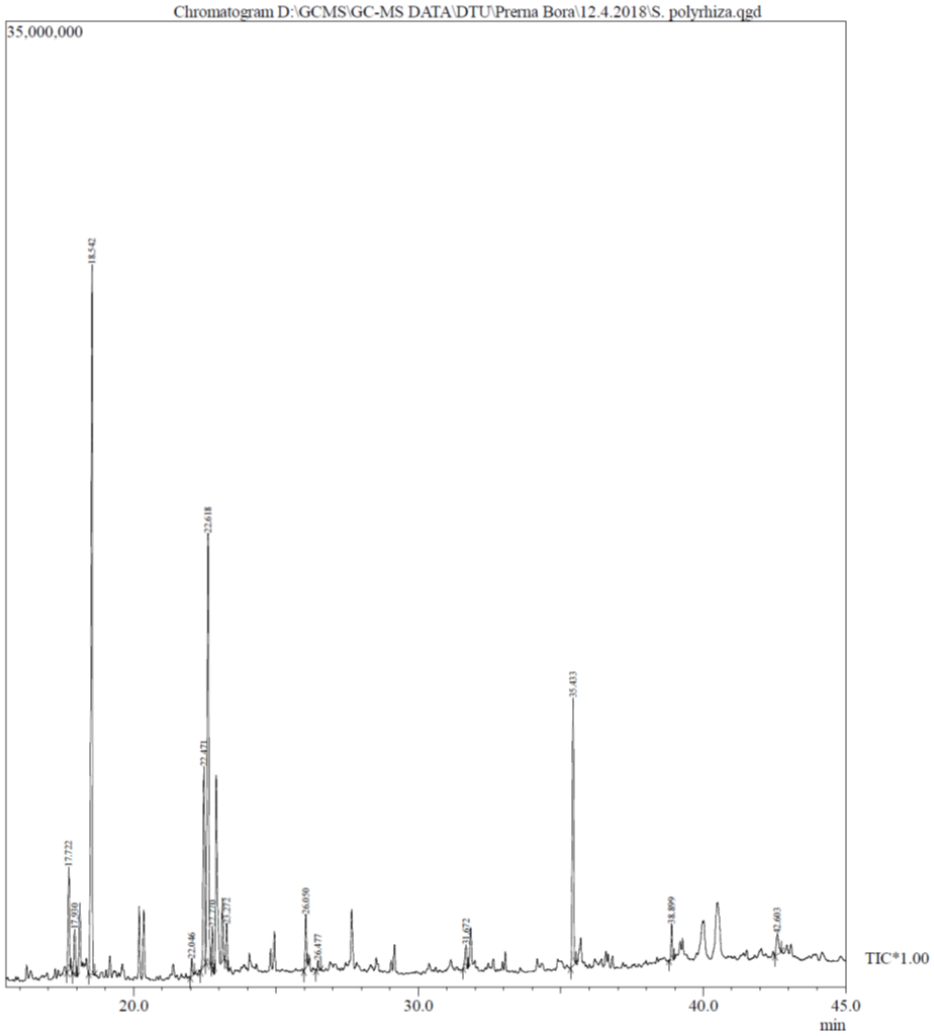


Fig 21: FAME chromatogram for *S. polyrhiza*

Table 12: Fatty acid concentrations in *S. polyrhiza*

Sl no.	Retention Time	Compound Name	Lipid Number	Percentage of FA of Total lipid content (%)
1.	17.9	Palmitoleic acid (hexadec-9-enoic acid)	16:1	2.28
2.	18.5	Palmitic Acid (hexadecanoic acid)	16:0	36.08
3.	22.4	Linoleic acid (octadeca-9,12-dienoic acid)	18:2	12.51

4.	22.6	Linolenic acid (9,12,15-Octadecatrienoic acid)	18:3 (n-3)	28.95
5.	23.2	Stearic acid (octadecanoic acid)	18:0	1.82
6.	26.0	Arachidonic acid (5,8,11,14-Eicosatetraenoic acid)	20:4	2.87
7.	31.6	Behenic acid (Docosanoic acid)	22:0	1.36
8.	35.4	Lignoceric acid (Tetracosanoic acid)	24:0	12.46
9.	38.8	Cerotic acid (Hexacosanoic acid)	26:0	1.58

4.1.3 *A. pinnata*

Table: Fatty acid concentrations in *S. polyrhiza*

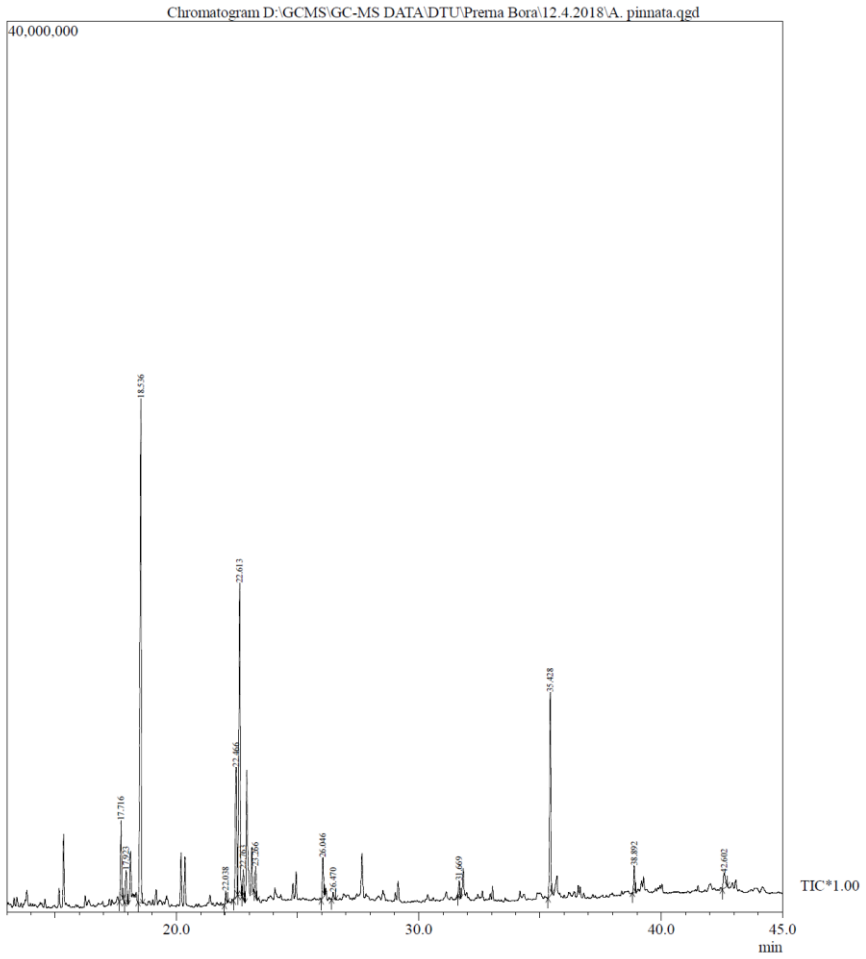


Fig 22: FAME chromatogram for *A. pinnata*

Table 13: Fatty acid concentrations in *A. pinnata*

Sl no.	Retention Time	Compound Name	Lipid Number	Percentage of FA of Total lipid content (%)
1.	17.9	Palmitoleic acid (hexadec-9-enoic acid)	16:1	3.8
2.	18.5	Palmitic Acid (hexadecanoic acid)	16:0	35.8
3.	22.4	Linoleic acid (octadeca-9,12-dienoic acid)	18:2	9.8
4.	22.6	Linolenic acid	18:3 (n-3)	30.1

		(9,12,15-Octadecatrienoic acid)		
5.	23.2	Stearic acid (octadecanoic acid)	18:0	1.82
6.	26.0	Arachidonic acid (5,8,11,14-Eicosatetraenoic acid)	20:4	2.94
7.	31.6	Behenic acid (Docosanoic acid)	22:0	1.8
8.	35.4	Lignoceric acid (Tetracosanoic acid)	24:0	12.64
9.	38.8	Cerotic acid (Hexacosanoic acid)	26:0	1.3

4.2 GC results

Chromatograms of 1mg/ml, 5mg/ml and 10mg/ml standard along with chromatograms of *L. minor*, *S. polyrhiza* and *A. pinnata* are given below.

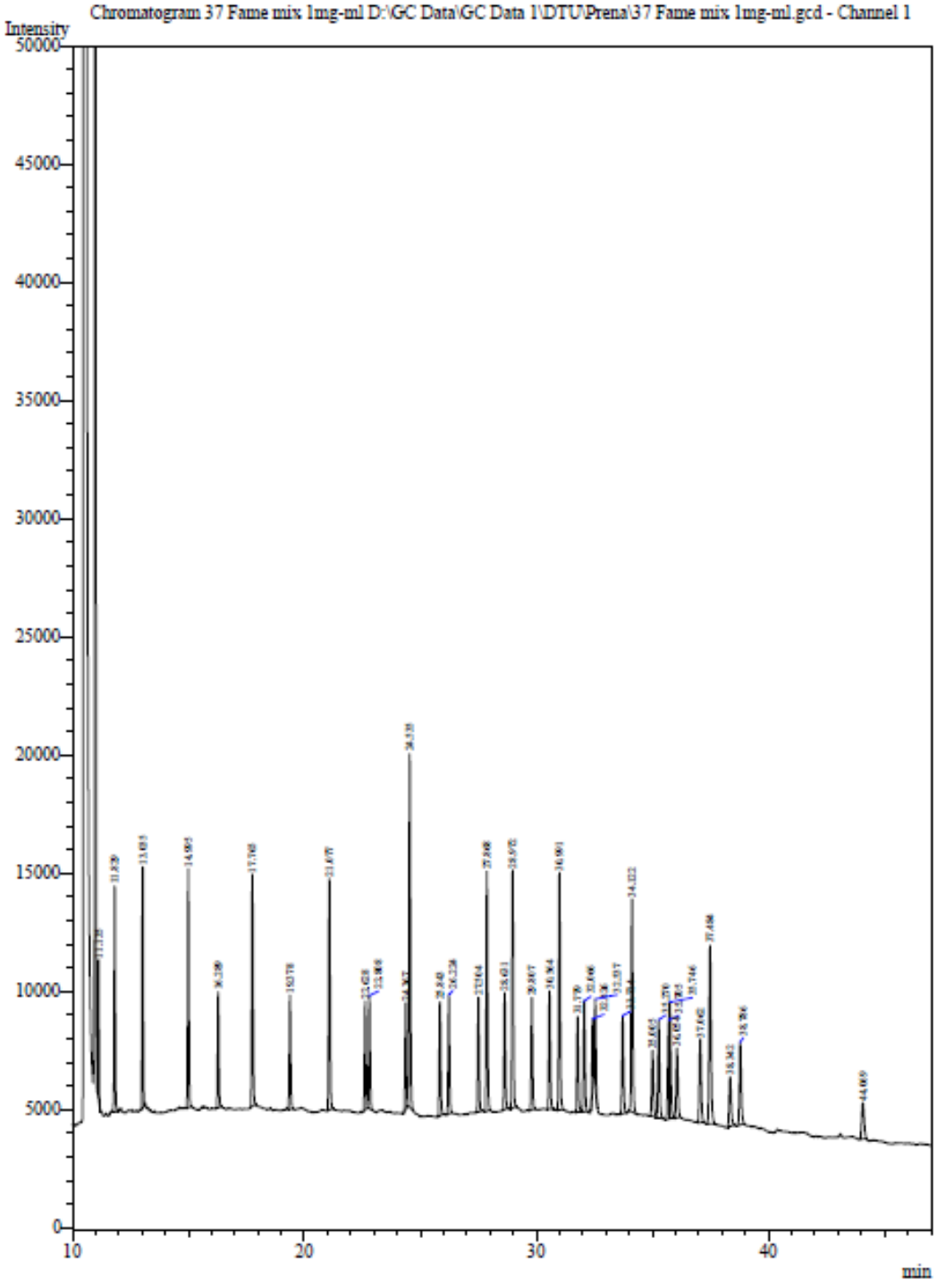


Fig 23: GC Chromatogram for 1mg/ml of Standard

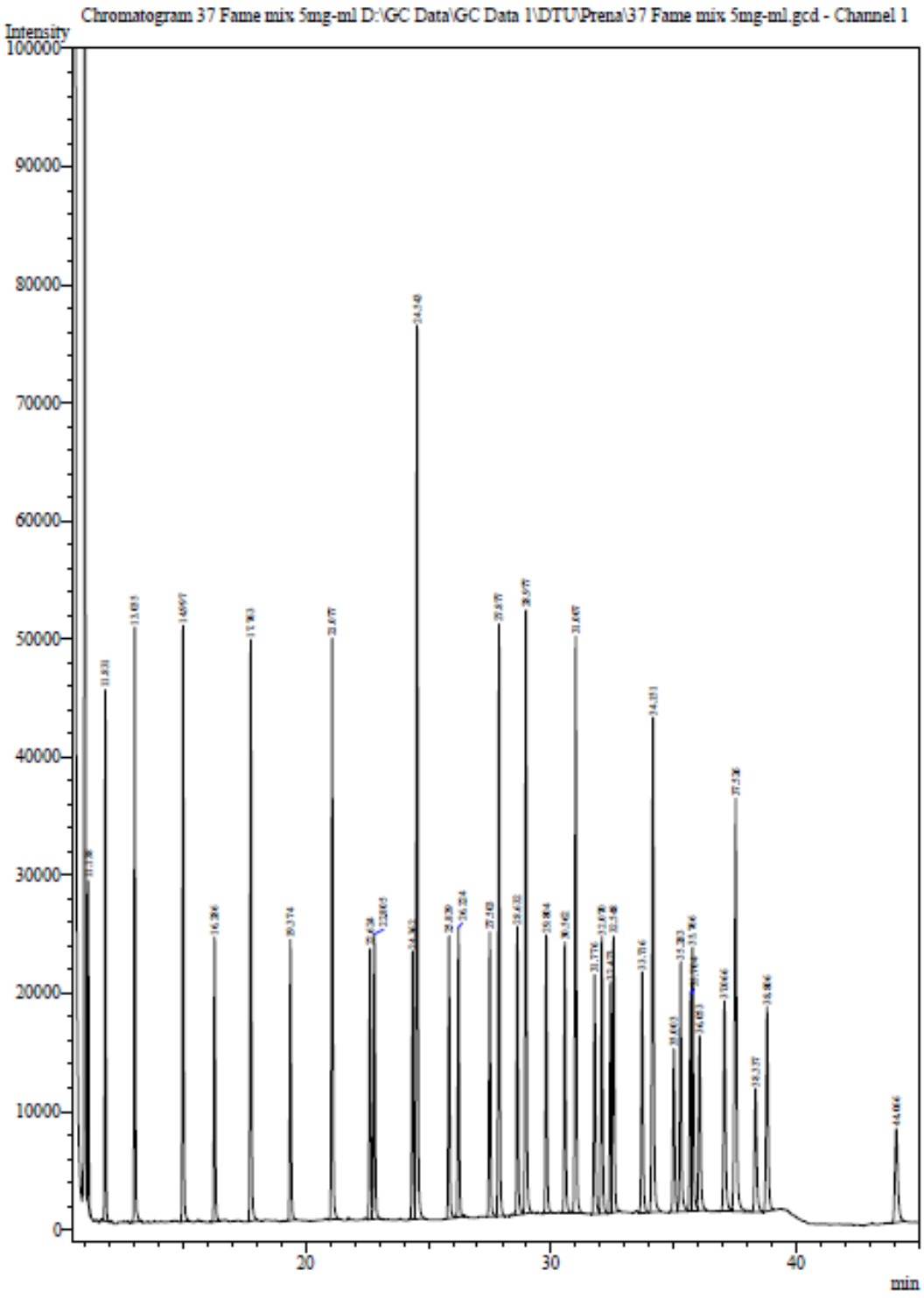


Fig 24: Chromatogram for 5mg/ml of Standard

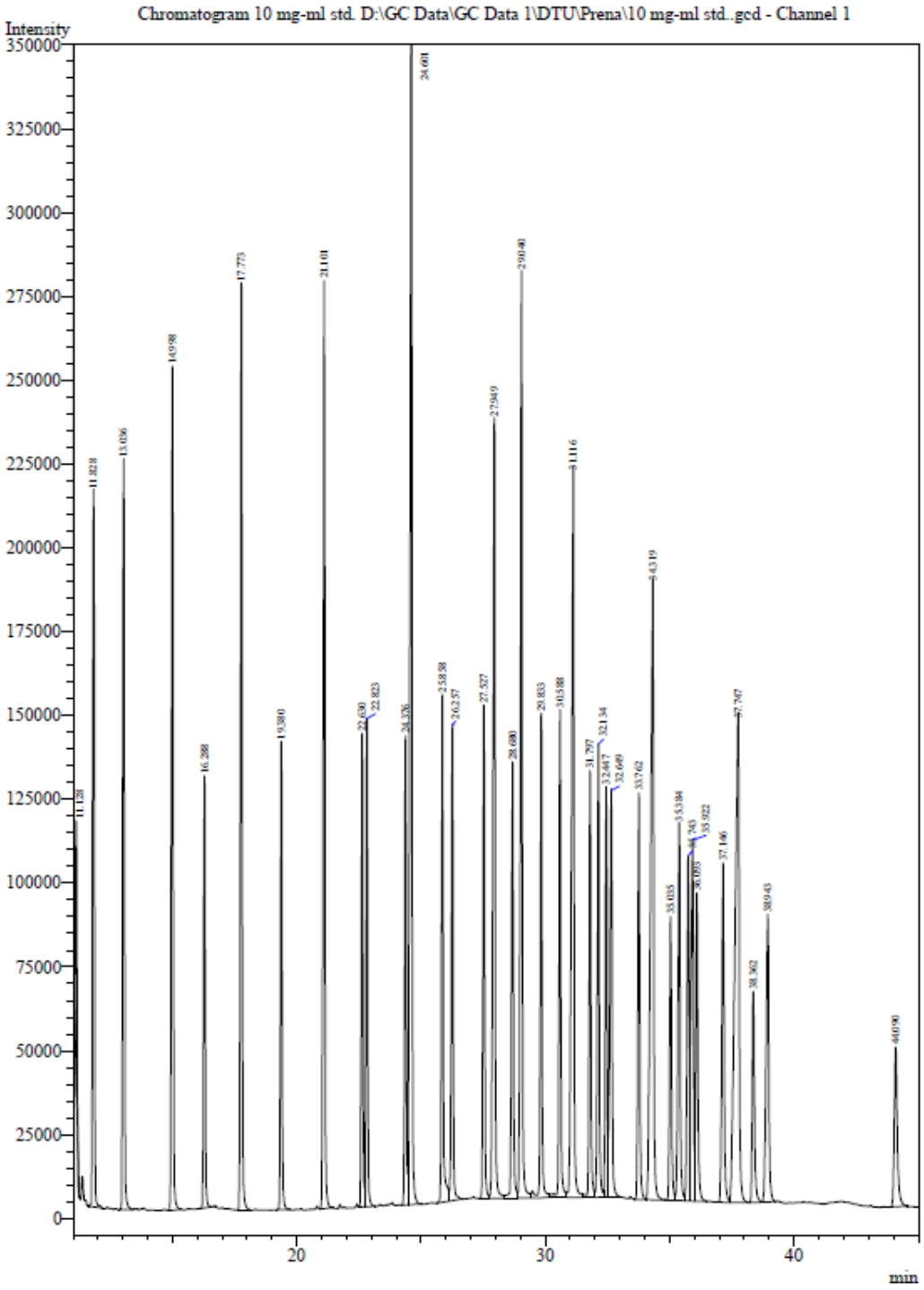


Fig 25: Chromatogram for 10mg/ml of Standard

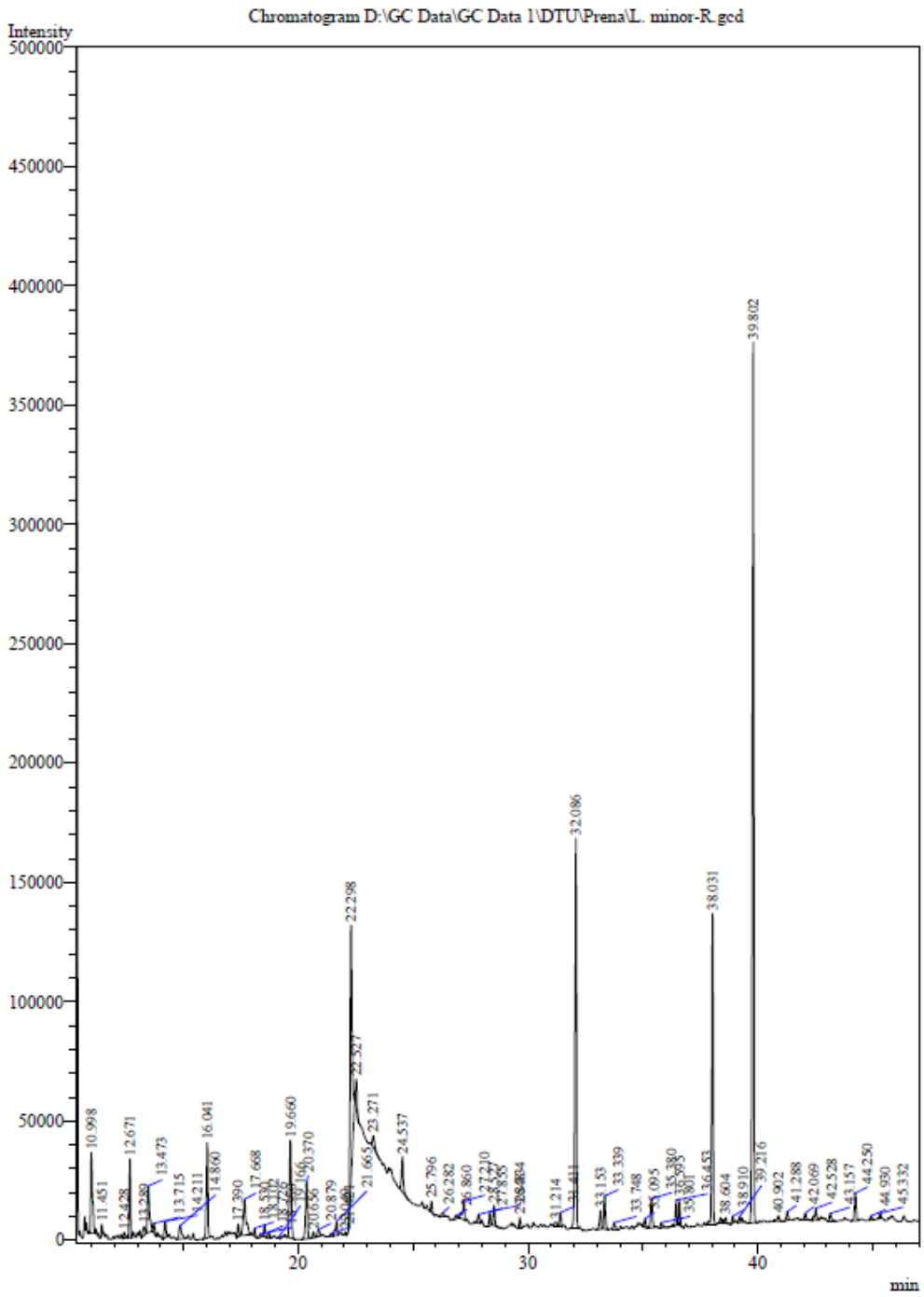


Fig 26: GC Chromatogram for *L. minor*

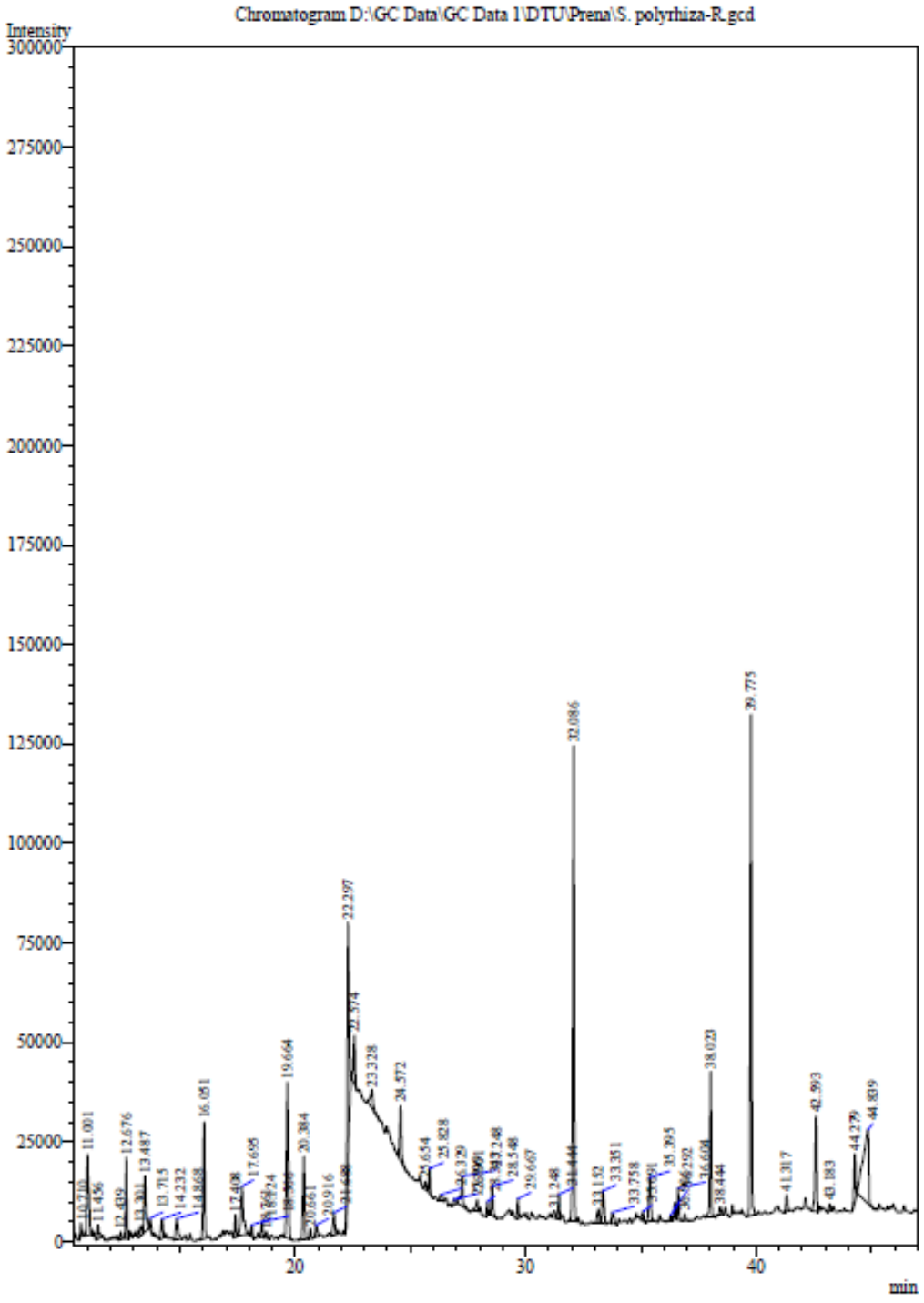


Fig 27: GC Chromatogram for *S. polyrhiza*

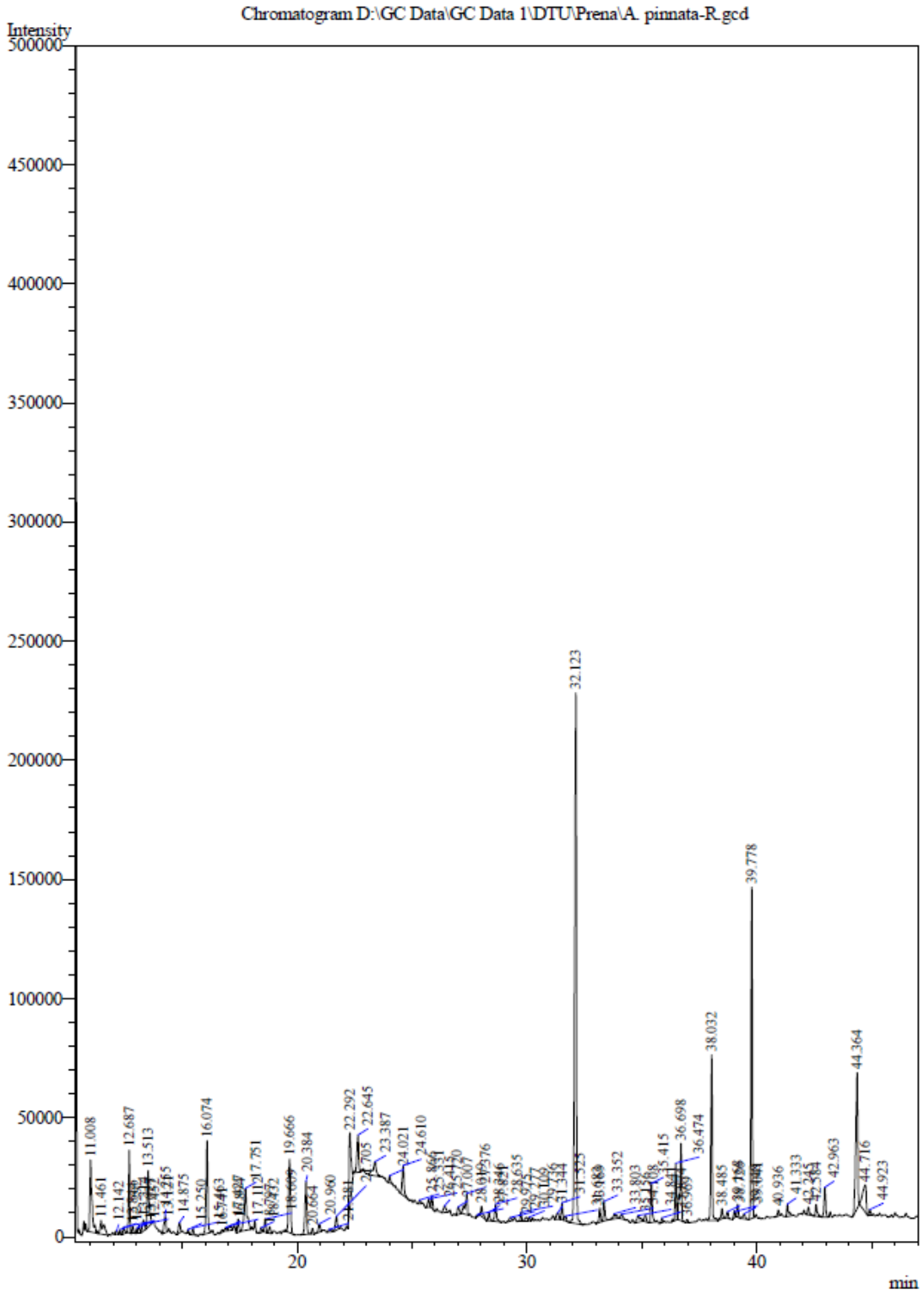


Fig 28: GC Chromatogram for *A. pinnata*

For quantification, the following steps were followed.

Step 1: Quantity of individual FAME were calculated (in mg) in injection volume (20µl) from the concentration chart given along the standard.

Step 2: 3-point calibration graph was constructed using values from 1mg/ml, 5mg/ml and 10mg/ml. Calibration graphs were plotted each individual FAME components. Area was plotted on the y axis and concentration on the x axis. Area obtained from chromatograms and its known concentrations were plotted on the graph and a linear trendline was constructed. A line equation for each trendline was generated.

Step 3: Now area of a particular FAME of a plant sample was put into its corresponding line equation and unknown concentrations were acquired. Quantities of a particular methyl ester directly co-relates to its corresponding fatty acid.

Step 4: Values derived are quantities of FA present in only 20 µl of total sample. Now, quantities of FAME were back-calculated to acquire the total quantity of FA present in 2ml (in mg).

Step 5: Loss percentage from sample preparation steps wherever needed were added to obtain the final quantitative value of individual FA present in the plant sample as mg/g of dry weight.

Table 13: Fatty acid compositions in plant samples in mg/g of dry weight

Retention time (in min)	**FA**	**Lipid No.**	***L. minor***	***S. polyrhiza***	***A. pinnata***
24.601	Palmitic Acid (hexadecanoic acid)	16:0	7.4	6.54	6.89
25.858	Palmitoleic acid (hexadec-9-enoic acid)	16:1	2.1	2.3	0.84
27.949	Stearic acid (octadecanoic acid)	18:0	0.39	0.8	--
30.588	Linoleic acid	18:2	6.5	4.2	4.8

	(octadeca-9,12-dienoic acid)				
32.447	Linolenic acid (9,12,15-Octadecatrienoic acid)	18:3 (n-3)	16.2	15.8	15.9
34.319	Behenic acid (Docosanoic acid)	22:0	--	--	0.23
35.922	Arachidonic acid (5,8,11,14-Eicosatetraenoic acid)	20:4	--	0.50	0.54
37.747	Lignoceric acid (Tetracosanoic acid)	24:0	0.36	0.12	--

4.2 Protein and Amino acid

4.2.1 Kjeldahl Method

The principle equation for calculating %N is given below.

$$\% \text{ Nitrogen} = \frac{[(\text{ml standard acid} \times \text{N of acid}) - (\text{ml blank} \times \text{N of base})] - (\text{ml standard base} \times \text{N of base}) \times 1.4007}{\text{weight of sample in grams}}$$

However, like already mentioned the process was largely automated, therefore the outputs were already calculated.

Table 14: Crude protein (%)

Substrate	**%N**	**Conversion factor**	**Crude Protein (%)**
A. pinnata	4.8	6.25	30
L. minor	4.32	6.25	27
S. polyrhiza	3.68	6.25	23

Conversion rate of 6.25 is used for food item such as meat, beans and rice. Since these plants are treated as potential feed sources, conversion rate of 6.25 applies here justly.

4.2.2 Results of Amino Acid Analyser

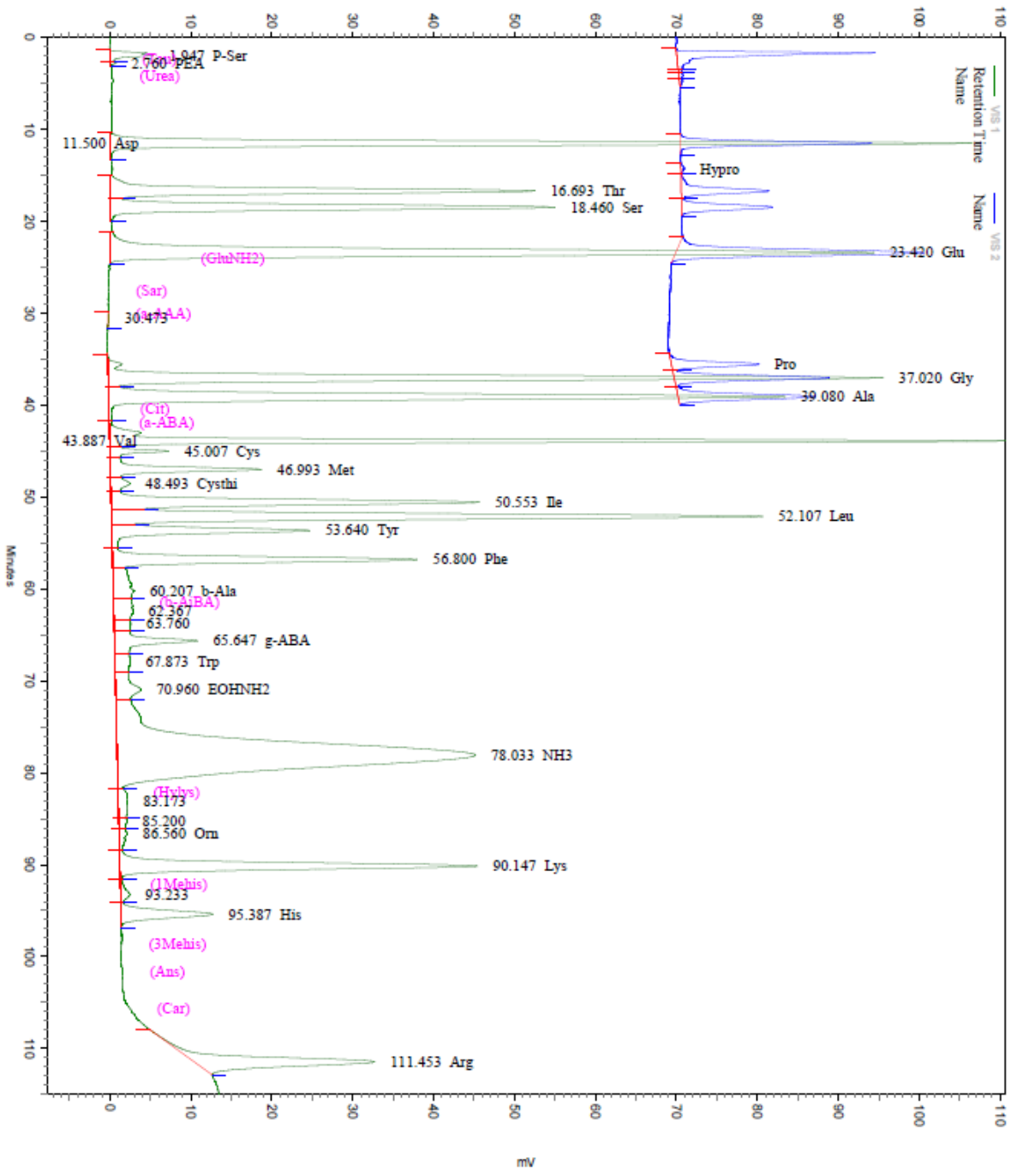


Fig 29: Chromatogram for All amino acid except tryptophan

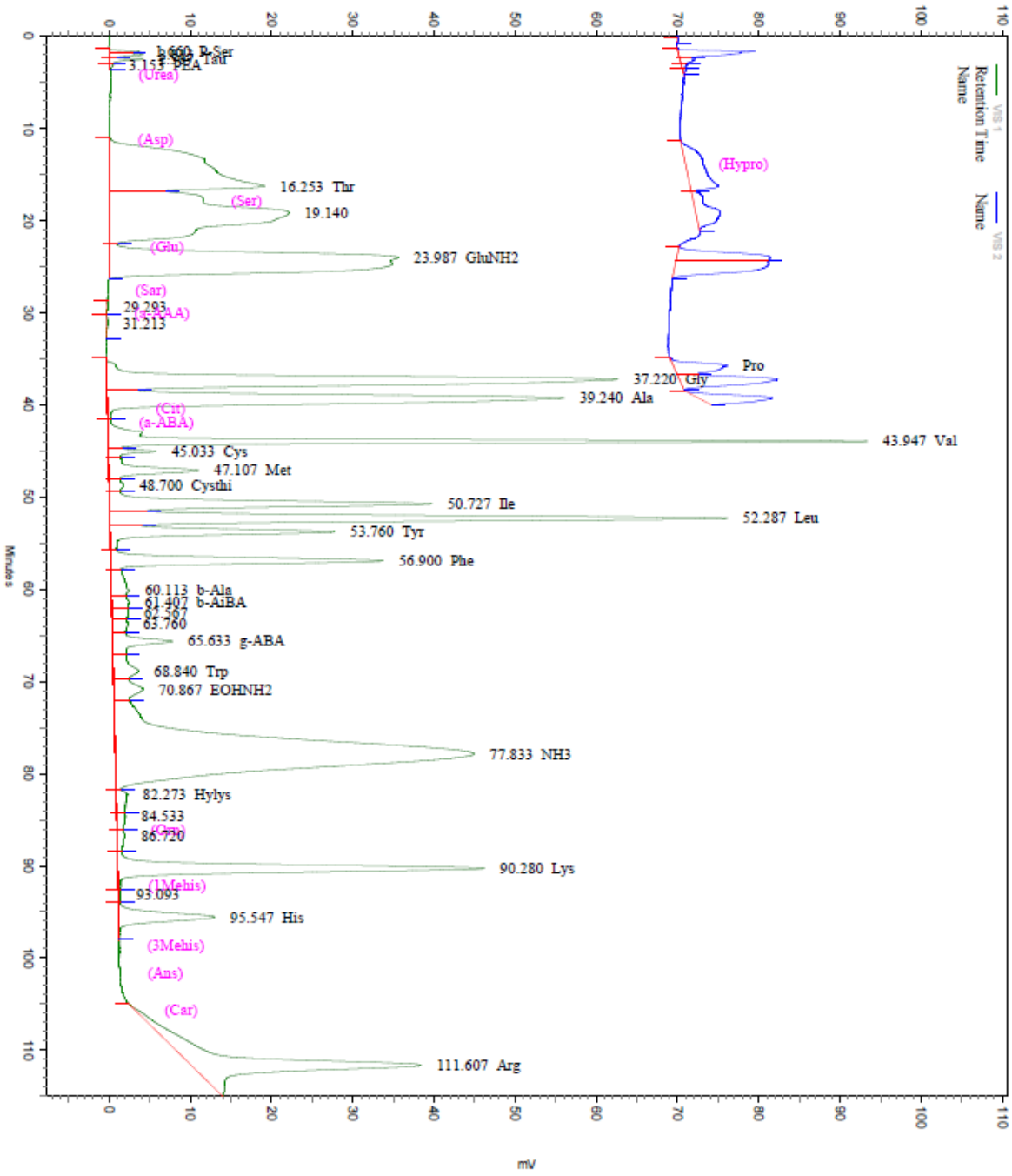


Fig 30: Chromatogram for tryptophan analysis

Amino acid Analyser already provides the concentrations in ng/µl which are back-calculated to obtain the quantity of individual amino acids in mg/100g of dry weight of plant sample.

	Amino Acids	Concentration (g/100g)
Essential		
	Histidine (His)	0.720 ± 0.043
	Isoleucine (Ile)	1.638 ± 0.051
	Leucine (Lue)	3.02 ± 0.079
	Lysine (Lys)	2.086 ± 0.088
	Methionine (Met)	0.650 ± 0.064
	Phenylalanine (Phe)	1.959 ± 0.083
	Threonine (Thr)	1.767 ± 0.010
	Tryptophan (Trp)	0.325 ± 0.078
	Valine (Val)	2.125 ± 0.063
Non- Essential		
	Alanine (Ala)	2.151 ± 0.063
	Arginine (Arg)	2.060 ± 0.010
	Asparatate (Asp)	3.458 ± 0.030
	Cysteine (Cys)	0.297 ± 0.060
	Glutamic Acid (Glu)	5.573 ± 0.143
	Glycine (Gly)	2.012 ± 0.056
	Proline (Pro)	1.499 ± 0.019
	Serine (Ser)	1.673 ± 0.017
	Tyrosine (Tyr)	1.503 ± 0.108
Non- Proteinogenic		
	Phosphoserine (p- Ser)	0.190 ± 0.090
	Taurine (Tau)	0.067 ± 0.00
	Phospho ethanol amine (PEA)	0.028 ± 0.003
	α Amino adipic acid (a- AAA)	0.008 ± 0.001
	Cystathionine (Cysthi)	0.171 ± 0.022
	β -Alanine (b-Ala)	0.286 ± 0.050
	β -Amino isobutyric acid (b-AiBA)	0.269 ± 0.081
	ϒ –Amino- n- butyric acid (g- ABA)	0.308 ± 0.040
	Ethanol amine (EOHNH2)	0.182 ± 0.004
	Hydroxylysine (Hylys)	0.181 ± 0.014
	Ornithine (Orn)	0.070 ± 0.003
	1 Methylhistidine (1 Mehis)	0.060 ± 0.038
	Hydroxy proline (Hypro)	0.073 ± 0.010

Table 15: Amino acid content in g/100g of dry sample. Values are expressed as mean ± S.D.

4.4 Results of Sterol Analysis

GC-MS Chromatograms for the 3 plants are given below. Identification of phytosterols were done using Mass Spectroscopy. Individual phytosterols are compared with the NIST and Wiley library.

4.4.1 *L. minor*

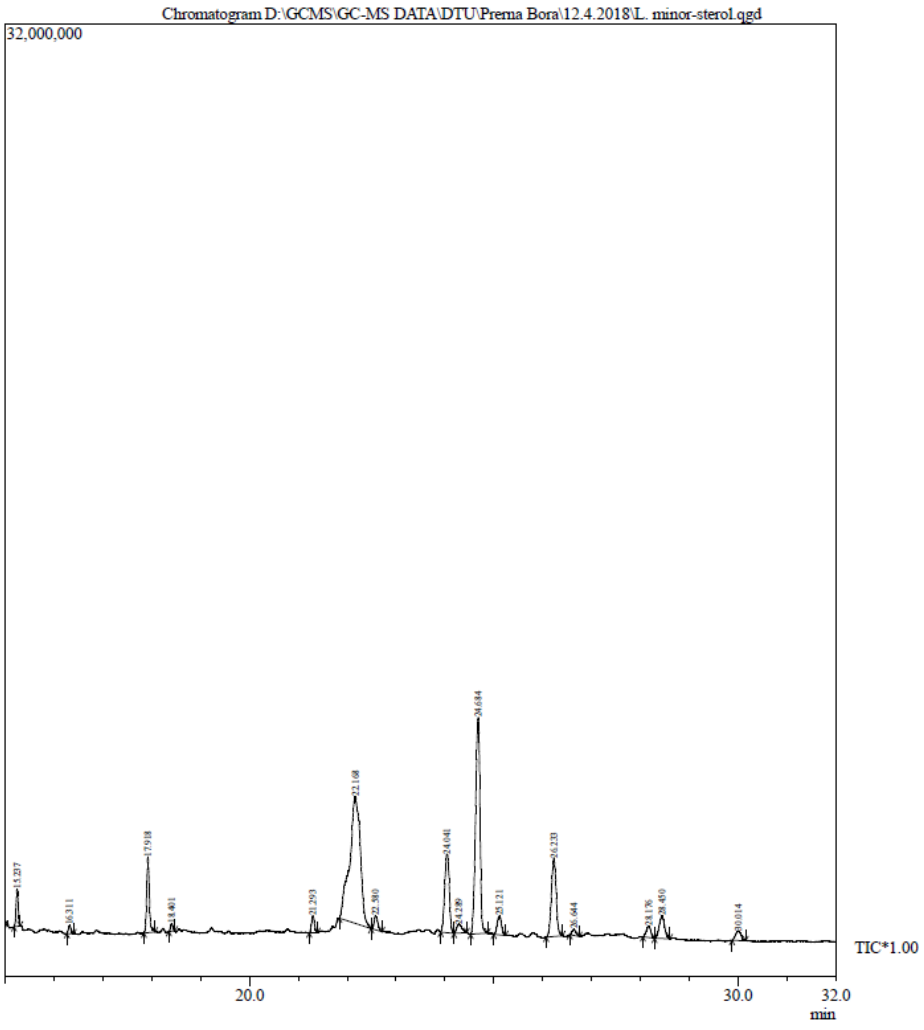


Figure 31: GC Chromatogram for sterol in *L. minor*

Table 16: List of phytosterol present in *L. minor*

Phytosterol	**Retention Time (in min.)**	**Molecular Formula**
1-Hexacosanol	18.405	$C_{32}H_{68}OSi$
Cycloartenol		$C_{33}H_{58}OSi$
Ergosta-5,22-dien-3-ol (Brassicasterol)	22.592	$C_{28}H_{46}O$
Campesterol	24.050	$C_{31}H_{56}OSi$
Stigmasterol	24.691	$C_{29}H_{48}O$

4.4.2 *S. polyrhiza*

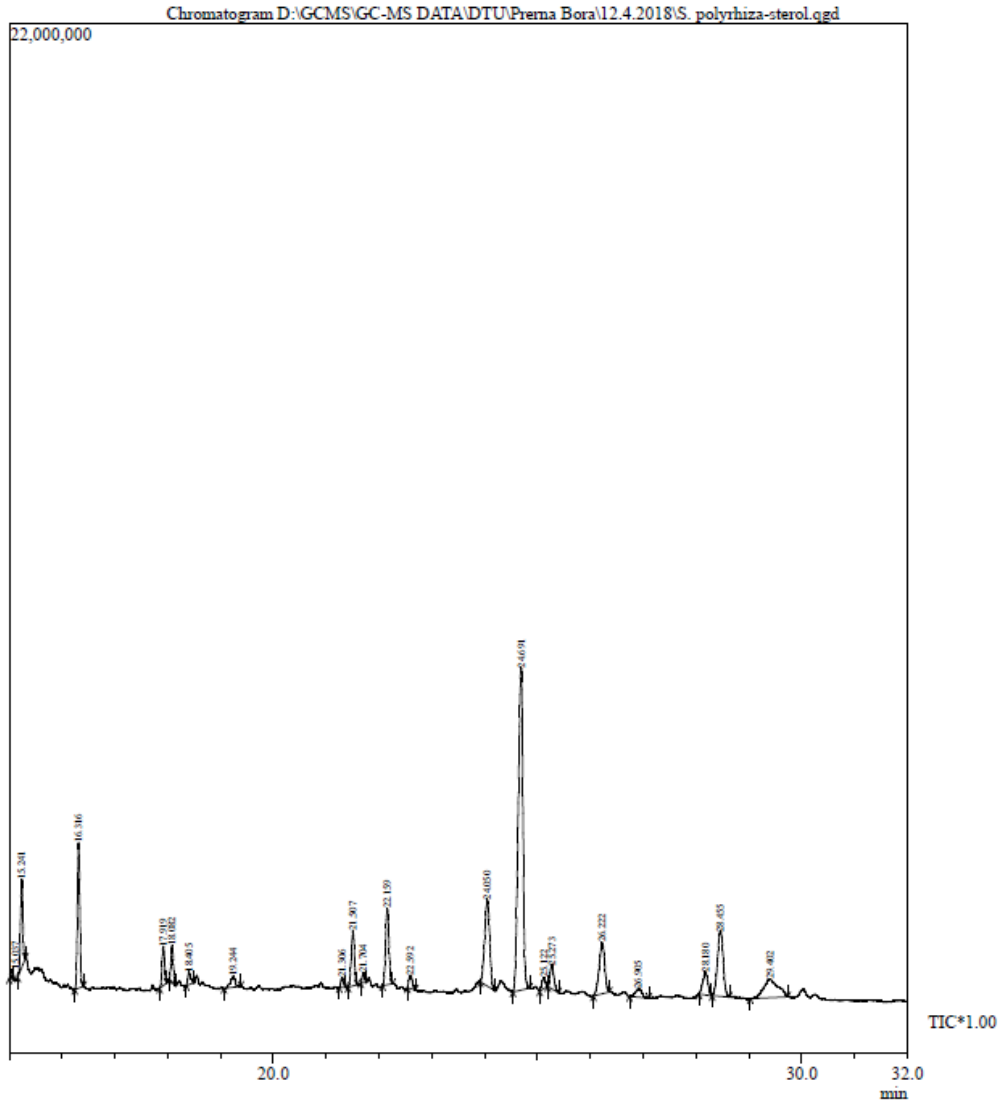


Figure 32: GC-MS Chromatogram for sterol in *S. polyrhiza*

Table 17: List of phytosterol present in *S. polyrhiza*

Phytosterol	**Retention time (min.)**	**Molecular Formula**
1-Tetracosanol	15.241	$C_{27}H_{58}OSi$
1-Hexacosanol	18.405	$C_{32}H_{68}OSi$
Ergosta-5,22-dien-3-ol (Brassicasterol)	22.592	$C_{28}H_{46}O$
Campesterol	24.050	$C_{31}H_{56}OSi$
Stigmasterol	24.691	$C_{29}H_{48}O$
beta-Sitosterol	26.222	$C_{32}H_{58}OSi$
Lanostan-3-beta-ol	26.905	$C_{30}H_{54}O$

4.4.3 *A. pinnata*

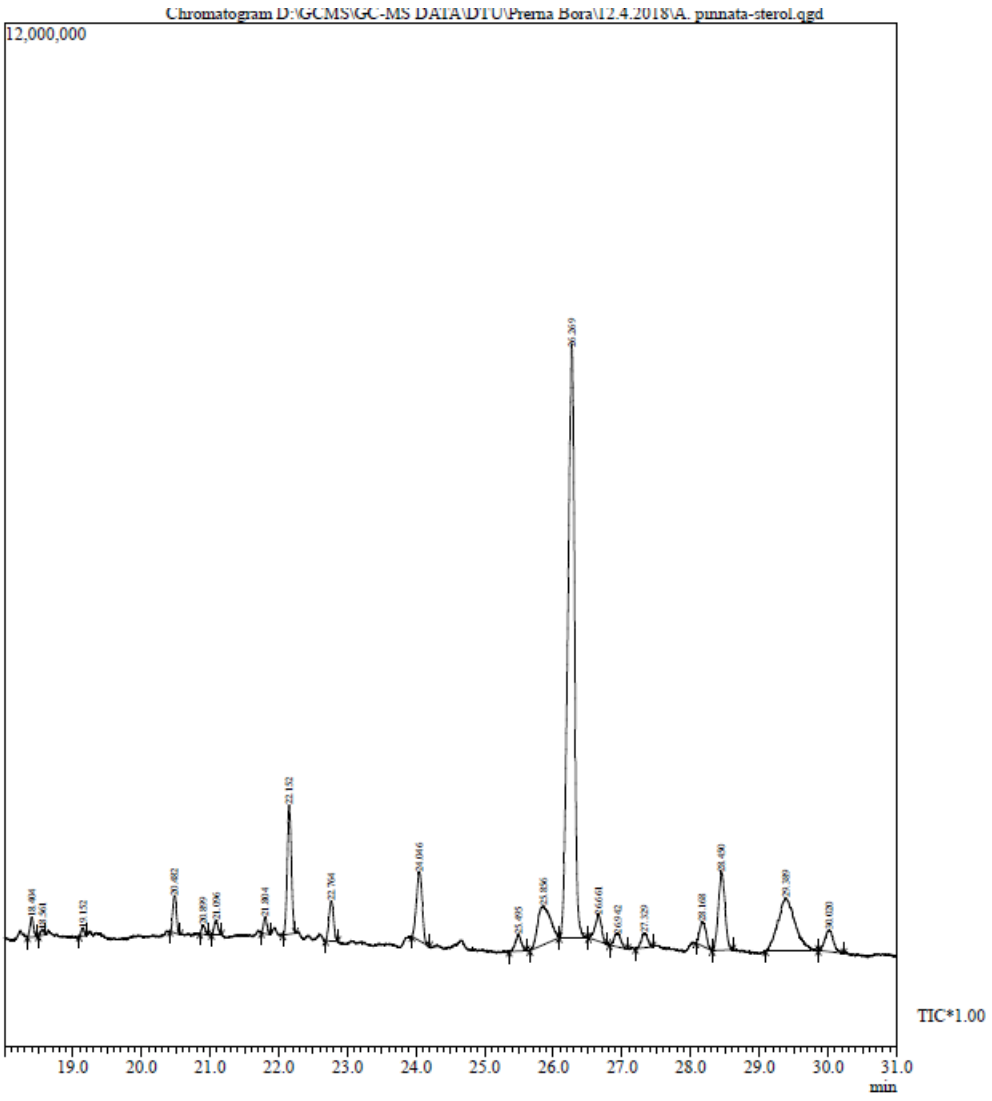


Figure 33: GC-MS Chromatogram for sterol in *A. pinnata*

Table 18: List of phytosterol present in *A. pinnata*

Phytosterol	**Retention time (min.)**	**Molecular Formula**
1-Hexacosanol	18.405	$C_{32}H_{68}OSi$
1-Heneicosanol	20.482	$C_{24}H_{52}OSi$
1-Decanol	22.764	$C_{13}H_{30}OSi$
Campesterol	24.050	$C_{31}H_{56}OSi$
gamma-Sitosterol	25.856	$C_{29}H_{50}O$
9,19-Cyclolanostan-3-ol	26.942	$C_{30}H_{52}O$
Ergosta-5,22-dien-3-ol	27.329	$C_{28}H_{46}O$

4.5 Discussion

Along with human population, livestock population is also expected to rise. India being one of the leading producers of livestock, suffers a productivity loss due to primarily inadequate feed supply and poor health management. This is due to a considerable decline in forest area originally used to produce feed and fodder. Fodder availability from certain crops are also negatively affected by high yielding dwarfs. This shortage in feed is usually met by commercially manufactured feed, which is rather costly, ultimately making animal husbandry a lucrative profession.

Ensured year around supply of good quality feed is a major concern for scientists all over the globe. With fluctuating feed generation and rapidly increasing population, food safety is often speculated. Safe-guarding of food will now completely depend on efficient utilization of alternate feed sources.

The present study has obtained the different fatty acid compositions and its quantities in terms of dry weight, crude protein compositions in %, amino acid profile (proteogenic and non-proteogenic) of *Azolla pinnata* in terms of dry weight and identified different phytosterols present in the samples. These macrophytes are rich in linolenic acid and palmitic acid with moderate amounts of polyunsaturated fatty acids like arachidonic acid. Linolenic acid is an important EAA in poultry and pigs and hence these macrophytes can efficiently serve as alternate feed provided formulation are carefully constructed according to the age of animal. There are some reports that show fatty acid deposition in the liver with macrophyte feed. This calls for further correction in quantities of feed given to the animal, however as long as nutritional properties of macrophytes are concerned, its

FA profile is exceedingly fitting and wholesome. On the other hand, fish require polyunsaturated FA for growth which is also present in these macrophytes. Lipids can also be used as energy source and act as a surrogate for carbohydrate.

Crude protein levels in these macrophytes is sufficient. Changes can be made in the composition of substrate water to further increase the crude protein level. Amino acid profile of *A. pinnata* show high amounts of lysine, an EAA, critical for the growth of livestock and fish. *A. pinnta's* amino acid profile are comparable to that of a meat source. However, exceeding amounts of amino acids might give rise digestibility issues and high nitrogen concentration in excreta. This can be fixed by studying the animals demand for individual amino acids and formulating a diet accordingly.

These macrophytes also show the presence of phytosterol and biologically active compounds. While, they have no nutritional properties and are usually don't contribute in any way, high amounts of it can be toxic in some conditions. It is suggested that role and effects of phytosterols in animals are studied.

This report concludes that due to its excellent nutritional properties, *L. minor*, *S. polyrhiza* and *A. pinnata* can efficiently serve as alternate feed sources for fish, poultry and pigs. It is favourable to replace a portion of the regular feed with macrophyte meal instead of providing 100% macrophyte meals. This could lead to a lucrative option, as cost of feed is expected to decrease with macrophyte addition.

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