Chapter 2

Chemical Investigation on Astavarga Plants and Study of their Anti-oxidant Property

2.1 Introduction

Since the ancient times we are using plants to cure diseases. Progress in botanical studies during the nineteenth century has had a revolutionary influence on utilizing plants for new drugs. However, from the 1956 proper documentation of this was started.[1] The term "*Astavarga*" was found in the ancient text "*Paryayaratnamala"* and provide its herbal description.[2] *Astavarga* is a group of eight medicinal plants in ayurvedic medicine, namely, Jeevak (*Malaxis acuminata*), Rishvak (*Malaxis muscifera*), Vriddhi (*Habenaria.edgeworthii*), Ridhi (*Habenaria intermedia*), Meda (*Polygonatum ver-ticillatum*), Mahameda (*Polygonatum cirrhifolium*), Kshirakakoli (*Lilium polyphyllum*) and Kakoli (*Roscoea procera*),[3,4] distributed in small patches of North West Himalayan region with an altitudinal range of 1600–4000 m asl. In ancient time this group of plants were invented by Rishi Chyvan and developed a formulation called Chyvanprash for rejuvenat-ing and restoring youthfulness. These plants are considered as a very good chemistry with rejuvenating and health-promoting properties. *Astavarga* plants are also used as a antioxidant and reported to restore health immediately.[5,6] *Astavarga* plants have several medicinal activities. Due to its diversified medicinal activity *Astavarga* plants are used in different forms, e.g., Churana (powder), Taila (oil), Ghritam (medicated clarified butter) and formulations in the traditional medical system (TMS) including *Chyavanprasha*, a health-promotive and disease-preventive tonic. Thorough literature search by us have revealed that there is no report of the active chemical constituents of most of the *Astavarga* plants. Chemical investigation of some *Ashtvarga* plants like *H. intermedia, Habenaria edgeworthii, Roscoea proceraand and Polygonatum verticillatum* with regards to flavonoid, polyphenol, riboflavin, thiamine and mineral contents, etc.[7] has been carried out but the details studies on the anti-ageing and

antioxidant properties of the group of *Astavarga* plants are lacking. Therefore, the present investigation was carried out for (i) identifying chemical compounds and (ii) Check the antioxidant activity. The result of this study was helpful for further chemical investigation of *Astavarga* plants, helpful for development of antioxidant property and promoting the group of plants for conservation and cultivation.

2.2 Astavarga plants

Himalaya is a natural garden of various flora and fauna. From the aesthetic, medicinal and nutritional points of view Himalayan region is important for natural vegetation. Currently numbering more than 35,000 plant species are used in medicine worldwide[8,9,10] and in India more than 1,600 species of medicinal plants have been traditionally used.[11] Indian Himalayan region is one of the most important area for biodiversity that harbors near about 8,000 species of flowering plants including 25.3% regional ones.[12,13,14,15,16,17] In the North-West Himalayan region in India, each one of these plants has its specific territory.

Figure 1: Astavarga

Table 1: Members of Astavarga Plants[18,19]

Sl no	Local name	Botanical name	Family	Distribution in Himalaya
1	Kakoli	*Roscoea purpurea*	*Zingiberaceae*	Eastern himalaya and sikkim
2	Kshirakakoli	*Lilium polyphyllum D. Don*	*Orchidaceae*	Jammu & Kashmir, Uttarakhand and Himachal Pradesh
3	Jeevak	*Crepidium acuminatum (D. Wear) Szlach*	*Orchidaceae*	Himachal Pradesh, Uttarakahand [18]
4	Rishbhak	*Malaxis muscifera (Lindl)Kuntze*	*Orchidaceae*	Sikkim, Himachal Pradesh, Jammu & Kashmir and Uttarakhand [18]
5	Meda	*Polygonatum verticillatum (Linn.)*	*Liliaceae*	Kashmir, Sikkim, Himachal Pradesh and Uttarakhand
6	Mahameda	*Polygonatum cirrhifolium (Wall.) Royle*	*Liliaceae*	Himalayas ,Himachal Pradesh, Sikkim [18]
7	Riddhi	*Habenaria intermedia D. Wear*	*Orchidaceae*	Temperate Himalaya To Kashmir To Sikkim, Uttarakhand and Himachal Pradesh[18]
8	Vriddhi	*Habenaria edgeworthii*	Orchidaceae	Himachal Pradesh, Uttarakhand To North West Himalaya [18]

2.3 Plant collection

Himalayan region holds most of the medicinal plants compared to rest of India. The availability *Astavarga* plants and their uses in 21 diverse localities were found across various parts of Garhwal: Mussorrie, Dhanaulti, Chamba, Dayara, Devban, Gangotri, Gangnani, Gaurikund, Kedarnath, Khirsu, Madmaheshwar, Mandal, Pauri, Rainthal, Rudranath, Tungnath, Yamnotri, the Valley of Flowers, etc at altitudes from 1600 to 3900 m asl. We made several expeditions in various parts of Himalaya especially Dhanaulti and Barlowganj, Uttarakhand (India) regions for the collection of *Astavarga* plants. From this region we could collect four of eight plants namely Kakoli, Jeevak, Riddhi and Vriddhi.[20] A group of scientist from Patanjali research foundation helped us to identify the plants and the region where the plants grow. Botanical identity of each species was authenticated by the botanist of Patanjali Research Foundation and Department of Botany and Forestry, Vidyasagar University.

2.4 General description and chemical constituents of Astavarga plants

Natural distribution range of astavarga plants, followed by plant description and chemical constituents are given in Table 2.

Table 2: Natural distribution range of Astavarga plants

Plant species	Vernacular name	Distribution range	Plant description	Chemical constituents
Roscoea purpurea	*Kakoli*	2400–3900 m	Herb, Leaves linear lanceolate, flower yellow-green (June–July)	Alkaloids: peimine, peiminine, peimisine, peimitidine, peimiphine, peimidine (CSIR 1966) sipeimine (Jiang et al. 2001)
Lilium polyphyllum D. Don	*Kshirakakoli*	1500–3300 m	Herb 30–90 cm, Leaves narrow lanceolate, flower pendulous creamish white, speckled pink (June–July)	NA
Crepidium acuminatum (D. Wear) Szlach	*Jeevak*	1800–2300 m	Herb 10–25 cm, Leaves ovate lanceolate, flower pale green tinged purple (August–October)	Erucic acid
Malaxis muscifera (Lindl)Kuntze	*Rishbhak*	1800–3500 m	Herb 30–50 cm, Leaves ovate lanceolate, flowers, yellowish–green (July–September)	NA
Polygonatum verticillatum (Linn.)	*Meda*	2000–3600 m	Herb 60–120 cm, Leaves linear lanceolate, flower white tinged purple or green (July–August)	Digitalis glucoside (CSIR 1966) Steroidal saponin, Ethanol (55%) (Kuo et al. 2002)
Polygonatum cirrhifolium (Wall.) Royle	*Mahameda*	2000–3600 m	Herb 80–150 cm, Leaves linear lanceolate, flower white (June–September)	Steroidal saponins, lectins (Antoniuk 1993), polysaccharides.
Habenaria intermedia D. Wear	*Riddhi*	800–2800 m	Herb 20–25 cm, Leaves alternate, ovate lanceolate, flowers white, greenish (June–July)	NA
Habenaria edgeworthii	*Vriddhi*	800–2500 m	Herb, flower yellow (June–July)	NA

2.5 Isolation of Stigmasterol from kakoli

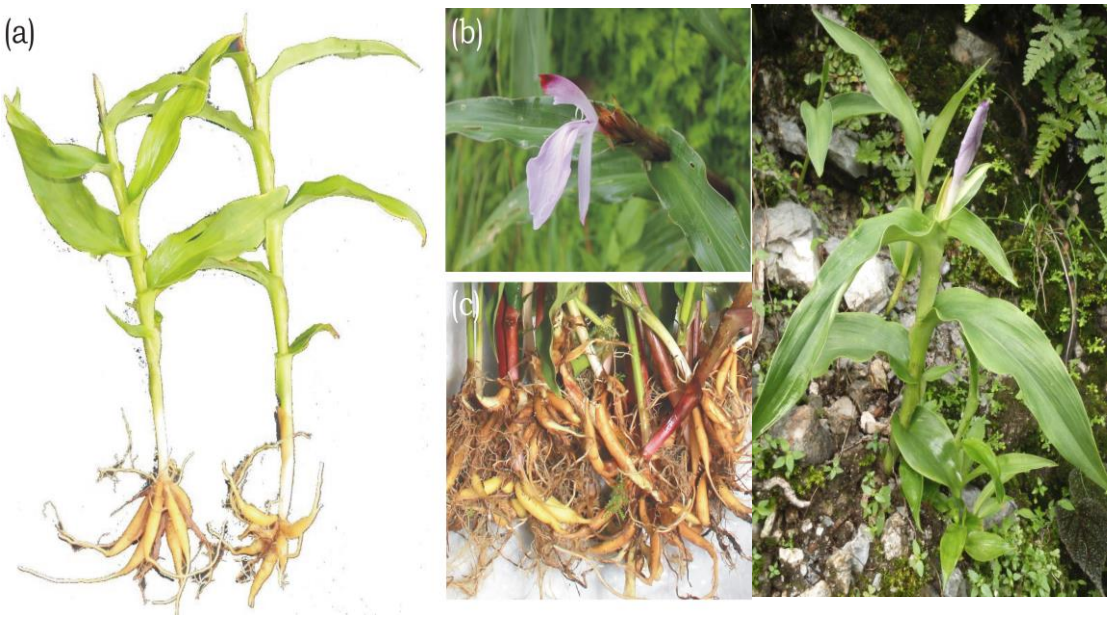


Figure 2: Different part of kakoli (a) whole plant (b) flower (c) root

Figure 3: Kakoli plants in Himalaya rigion

2.5.1 Extraction and isolation

The leaves of Kakoli were shade dried and finely powdered using a grinder. Powdered leaves of Kakoli (16 g) were suspended in ethyl acetate (250 mL) and stirred magnetically at room temperature for 24 h. The mixture was then filtered (filter paper) and the volatile components were removed under reduced pressure to afford a brownish solid material (0.636 g). The crude extract was then purified by successive column chromatography (thrice, Si-gel, 100–200 mesh) using 10-20 % ethyl acetate/ petroleum ether as the eluant. The purified product appeared as a white crystalline solid (0.038 g, 0.23% yield). MP = 162 – 165°C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.38 (1H, t, J = 4.7 Hz), 5.20 (1H, dd, J = 8.4,15.1 Hz), 5.04 (1H, dd J = 8.4,15.1 Hz) 3.55 (1H, m), 2.036 (1H, m), 1.03 (d, 3H), 1.01 (s, 3H) 0.84 (d, 3H), 0.83 (d, 3H), 0.81 (d, 3H), 0.72 (s, 3H), 2.31-1.01 (25H: phytosterol proton, m)

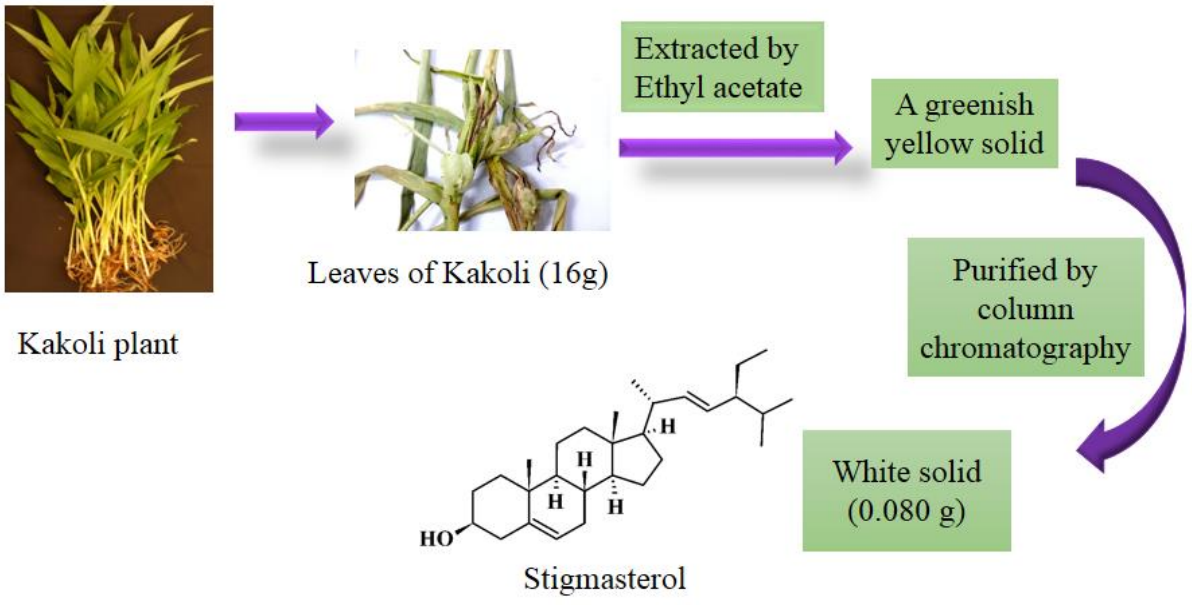


Figure 4: Schematic representation of the extraction of stigmasterol from kakoli leaves

ppm. ^{13}C NMR ($CDCl_3$, 100 MHz): δ 140.76, 138.30, 129.29, 121.70, 71.81, 56.88,

Figure 5: Image of different part of kakoli plant

55.97, 51.23, 50.18, 42.21, 42.22, 40.47, 39.7, 37.26, 36.52, 36.7, 31.97, 31.67, 29.2, 28.4, 25.6, 24.5, 21.4, 21.3, 22.3, 19.93, 19.15, 12.23, 12.05.

2.5.2 Results and discussion

2.5.2.1 Extraction, Purification & Isolation of Stigmasterol

Compound **1** was isolated from the powdered leaves of *Roscoea purpurea* by solvent extraction technique and purified by column chromatography (see experimental section). The compound **1** was obtained as a white crystalline solid and its structure was established as follows: High resolution mass spectrometry of the sample showed a molecular ion peak (M+) at 412.3721 corresponding to a molecular formula $C_{29}H_{48}O$ (calc. 412.3705). The stretching frequency at 3350 cm^{-1} in FTIR spectrum supported the presence of the -OH group in the molecule. The

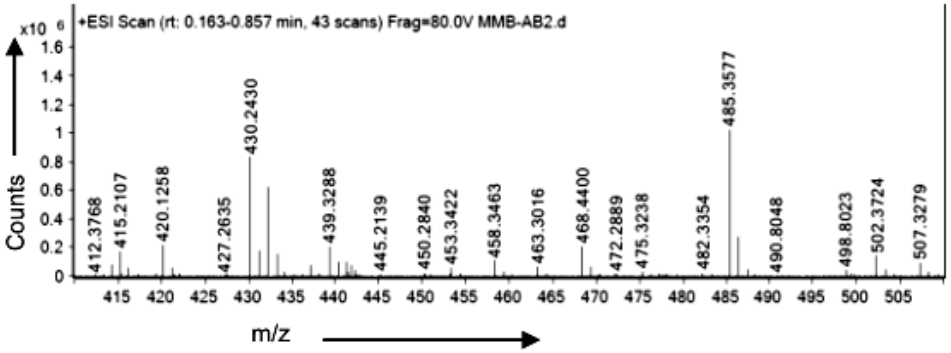
1688 cm^{-1} peak in FTIR supported the presence of C=C bond(s).

Formula calculator elements limits

Element	Min	Max
C	0	29
H	0	48
O	0	1

Formula calculator result

Formula	Best	Mass	Tgt mass	Diff (ppm)
$C_{29}H_{48}O$	True	412.3721	412.3705	-3.96



The ^{13}C NMR spectrum showed 29 carbon signals supporting the molecular formula $C_{29}H_{48}O$. The peaks at 140.8, 138.3, 129.3 and 121.7 indicated the presence of four olefinic C's or two C=C bonds. The 71.8 ppm peak indicated the presence of secondary C(OH). Additionally, the presence of the 1H NMR spectrum exhibited two methyl singlets (δ 1.01 and 0.71 ppm), and two-proton double doublet at *δ* 5.20 (*J* = 8.4, 15.1 Hz), 5.04 (*J* = 8.4, 15.1 Hz)) for one double bond system, one-proton multiplate at δ 3.546 an R-proton geminal to a hydroxyl group. The multiplicity of each carbon was achieved by the DEPT experiment, which confirmed the presence of one secondary hydroxyl group and five methyl groups. The NMR data obtained for the compound are compatible to those reported compound. Stigmasterol **1** is a tetracyclic plant based phytosterol, obtainable from the leaves of Indian medicinal

plant *Roscoea purpurea*, commonly known as Kakoli found in Himalayan area of India. The compound was extracted from several plants ware reported previously but to our knowledge stigmasterol is the first compound isolated from the Indian medicinal plant Roscoea purpurea. The molecule constitute a rigid tetracyclic backbone (6-6-6-5) with one secondary hydroxyl group in one end and one long hydrocarbon chain in the other end of the molecule. Energy minimized structure revealed that the molecule is 1.73 nm long.

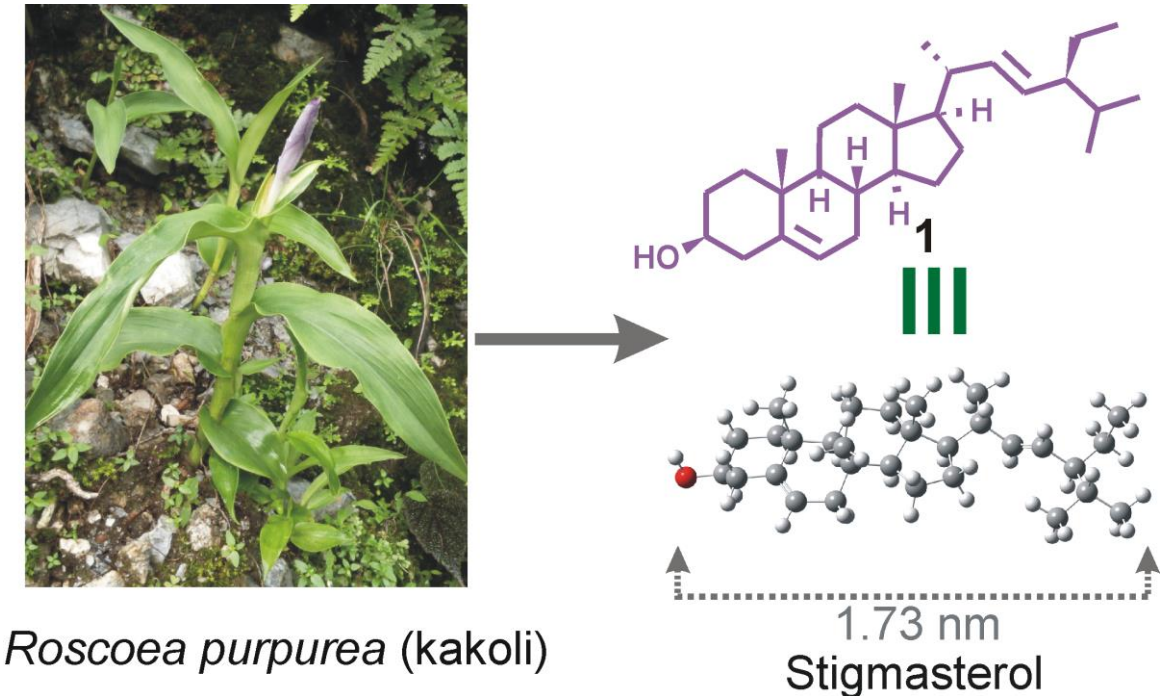
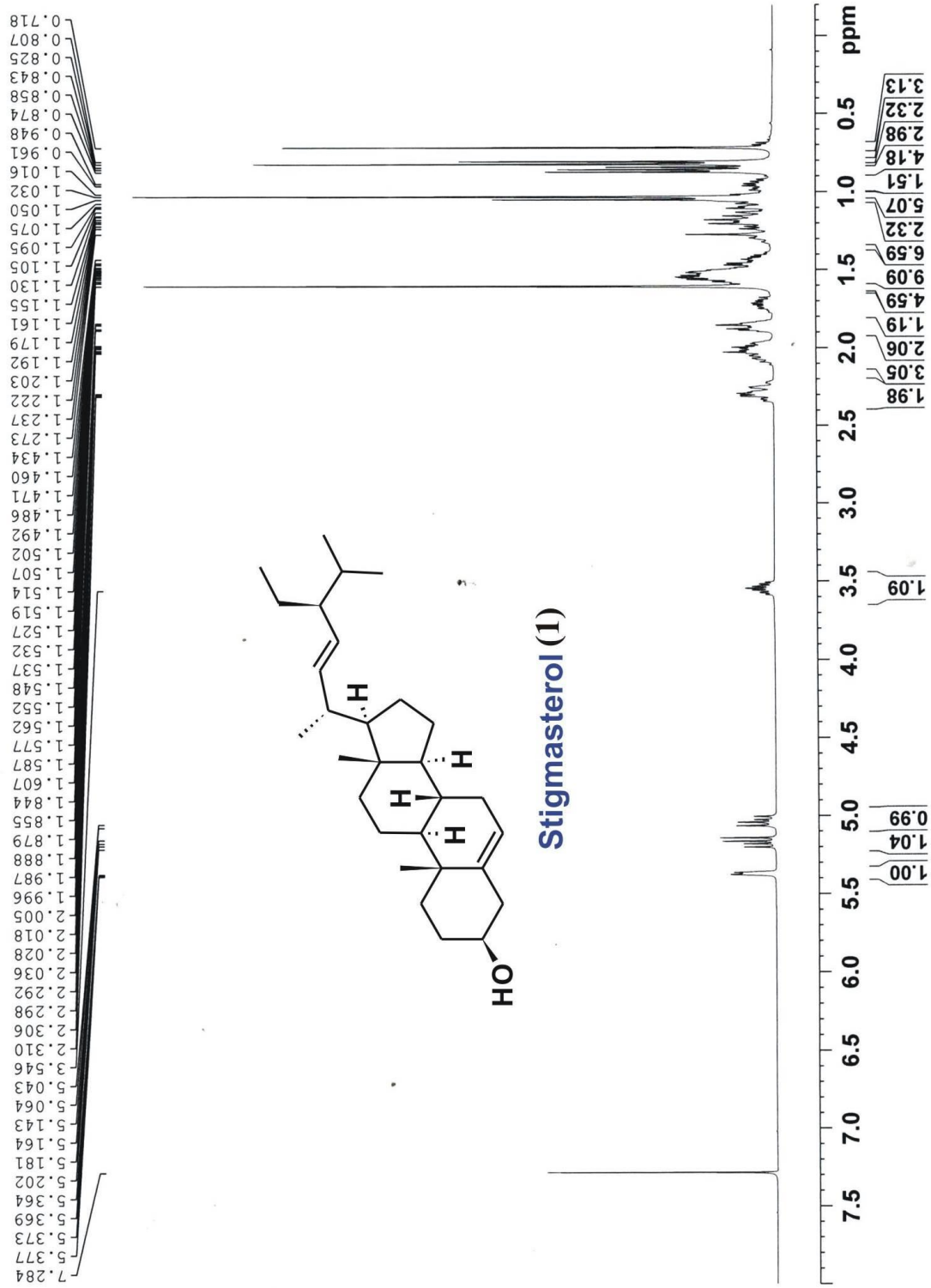
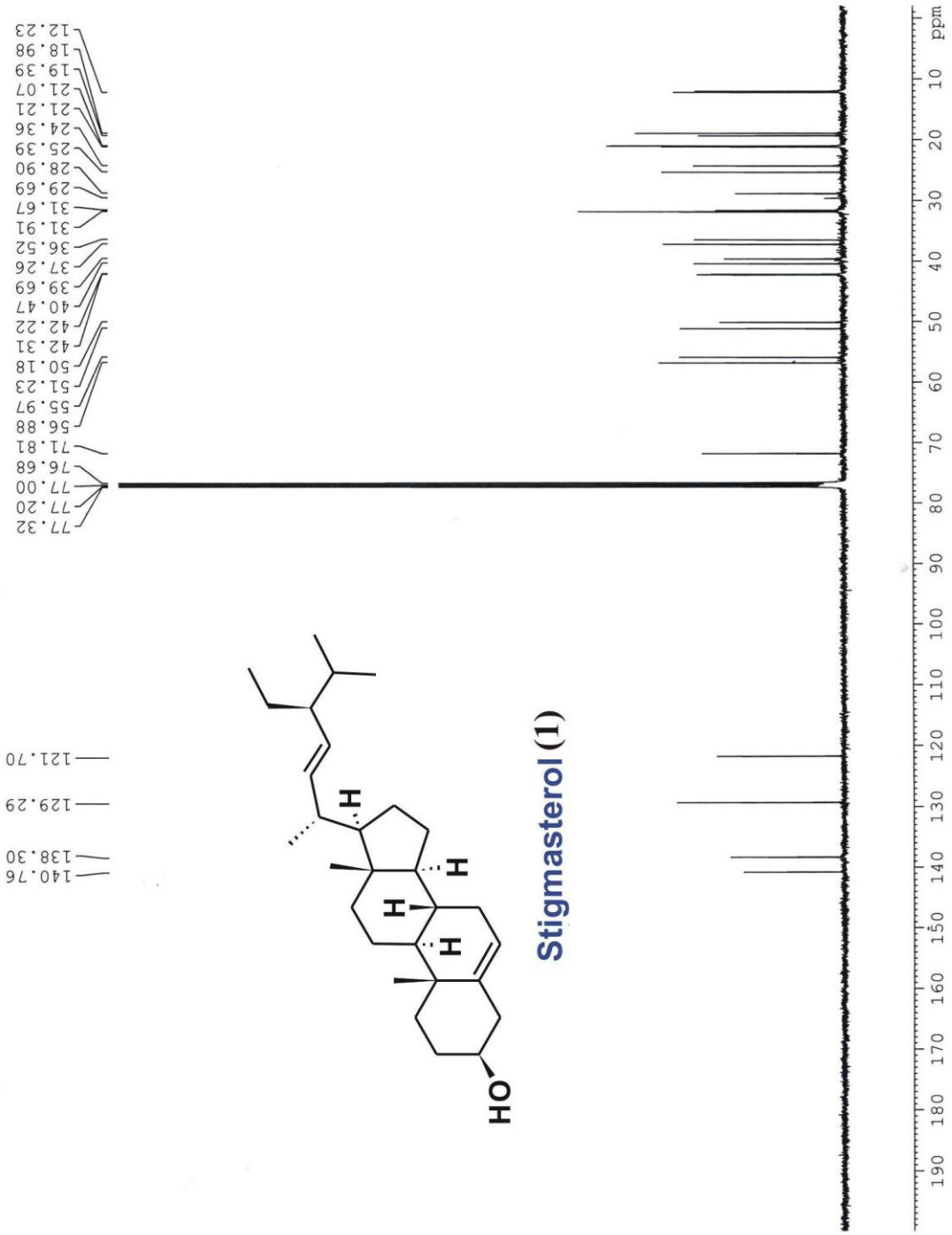


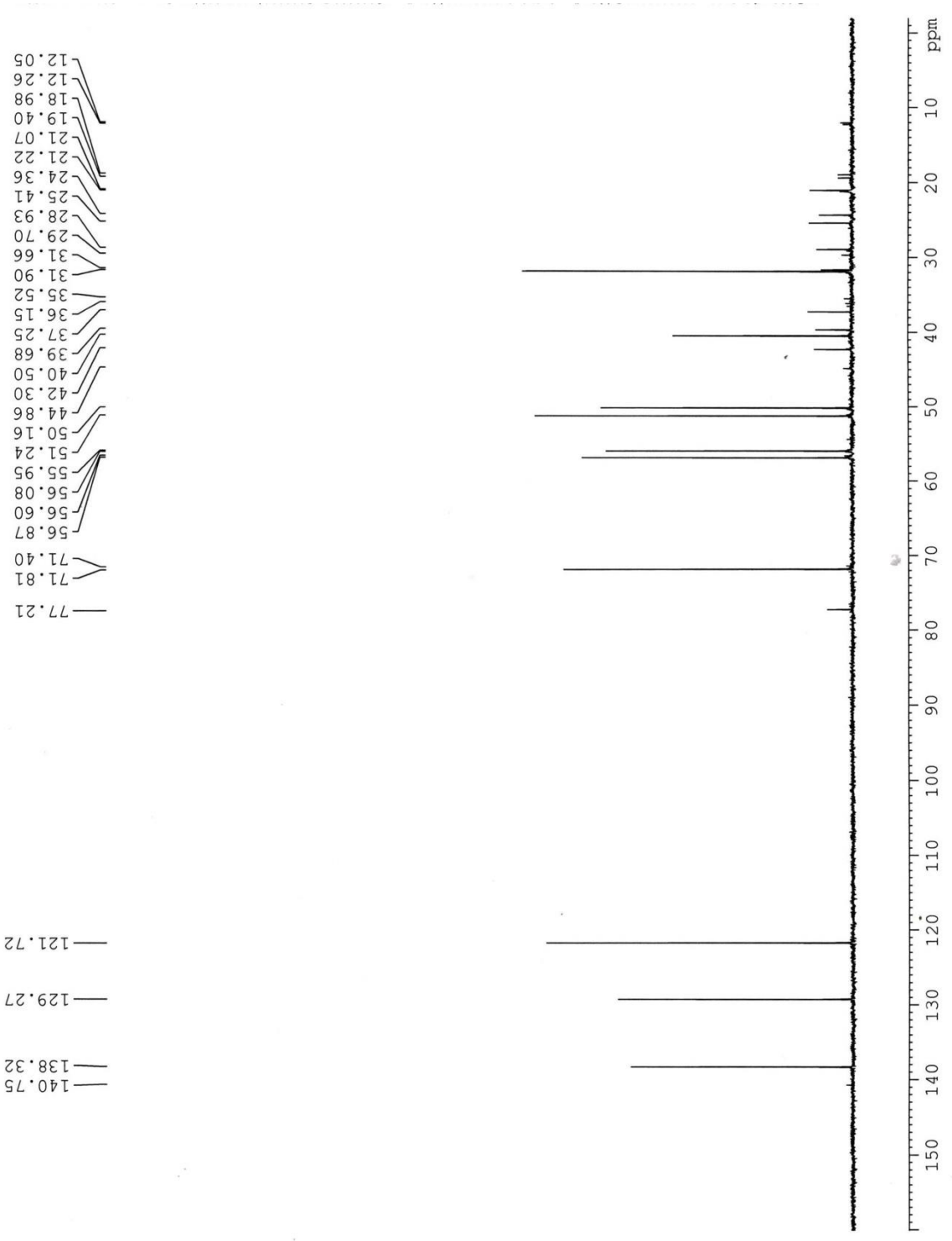
Figure 6: Chemical structure and Energy minimized structure of stigmasterol



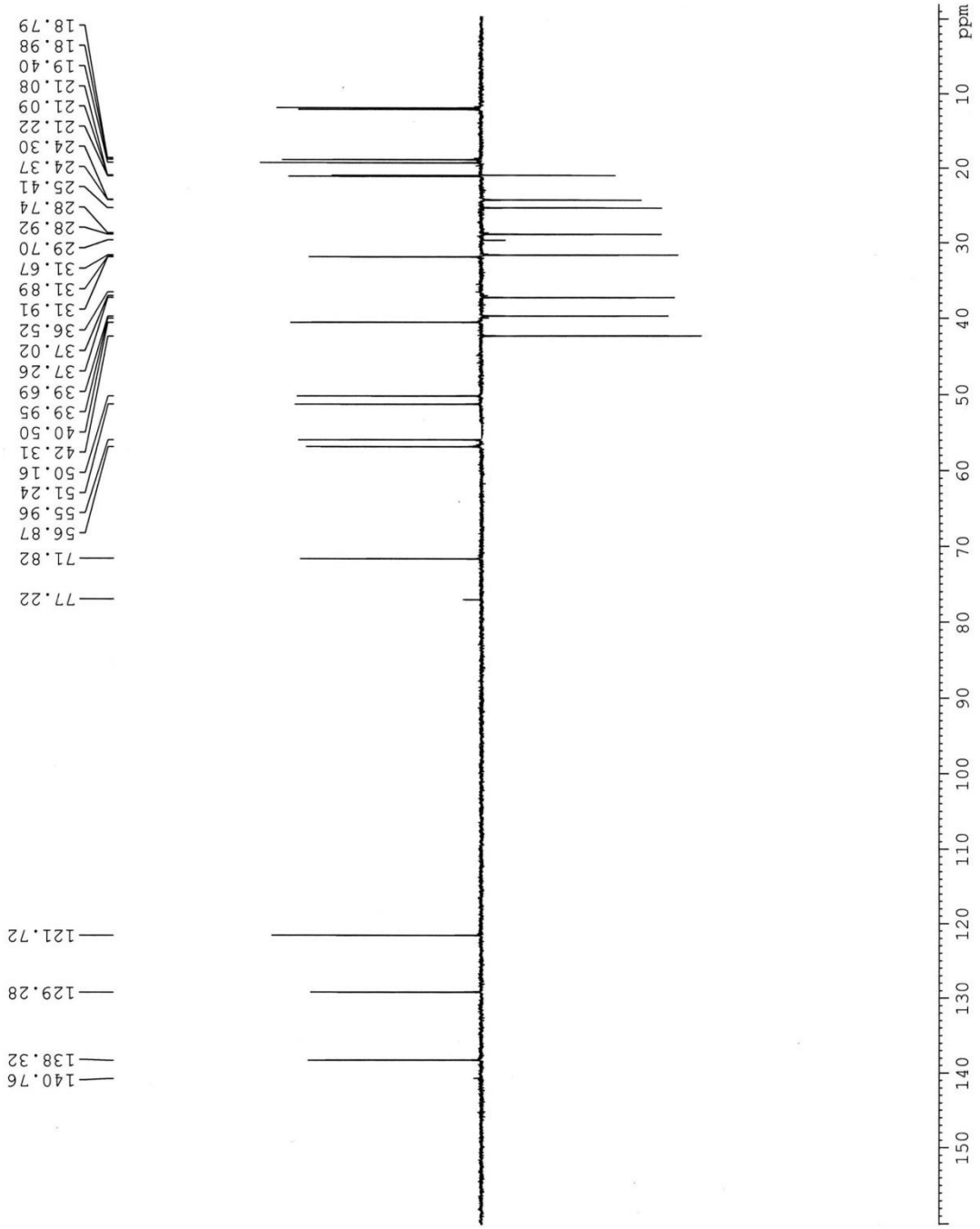
[1]HNMR of Stigmasterol



^{13}C NMR of Stigmasterol


12.05
12.26
18.98
19.40
21.07
21.22
24.36
25.41
28.93
29.70
31.66
31.90
35.52
36.15
37.25
39.68
40.50
42.30
44.86
50.16
51.24
55.95
56.08
56.60
56.87
71.40
71.81
77.21
121.72
129.27
138.32
140.75
ppm
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150

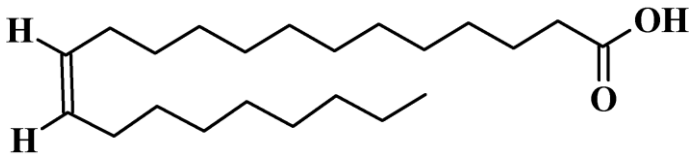
DEPT 90


18.79
18.98
19.40
21.08
21.09
21.22
24.30
24.37
25.41
28.74
28.92
29.70
31.67
31.89
31.91
36.52
37.02
37.26
39.69
39.95
40.50
42.31
50.16
51.24
55.96
56.87
71.82
77.22
121.72
129.28
138.32
140.76
ppm
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150

DEPT 135

2.6 Isolation of Erucic acid from Jeevak

Erucic acid **2** is a plant based fatty acid, obtainable from the pseudobulb of Indian medicinal plant *Crepidium acuminatum*, commonly known as Jeevak found in Himalayan area of India. The compound was extracted from several plants was



Erucic acid (**2**)

reported previously but to our knowledge Erucic acid is the first compound isolated from the indian medicinal plant *Crepidium acuminatum*. The structure of this compound (**2**) was determined by NMR techniques including ^{1}H, ^{13}C NMR, DEPT. Erucic acid (**2**) was obtained from ethyl acetate crude extract from the pseudobulb of *Crepidium acuminatum* by Si-gel column chromatography, eluting with petroleum ether/ethyl acetate. The molecular formula of 2 was determined by HRMS.

Figure 7: Jeevak plant and pseudobulb of jeevak

2.6.1 Experimental procedure

Powdered pseudo bulbs of *Crepidium acuminatum* (10 g) were extracted with ethyl acetate (250 mL) by using an extraction apparatus (capacity 500 mL) during 24 h at room temperature. The volatiles were removed under reduced pressure to afford a brownish solid material (0.636 g). The crude extract was purified by successive column chromatography (thrice, Si-gel, 100–200 mesh) using 10-20 % ethyl acetate/ petroleum ether as the eluent. *m/z* calculated for $C_{22}H_{42}O_2NH_4$ $[M^+ NH_4]^+$ 356.3527; found 356.3513.

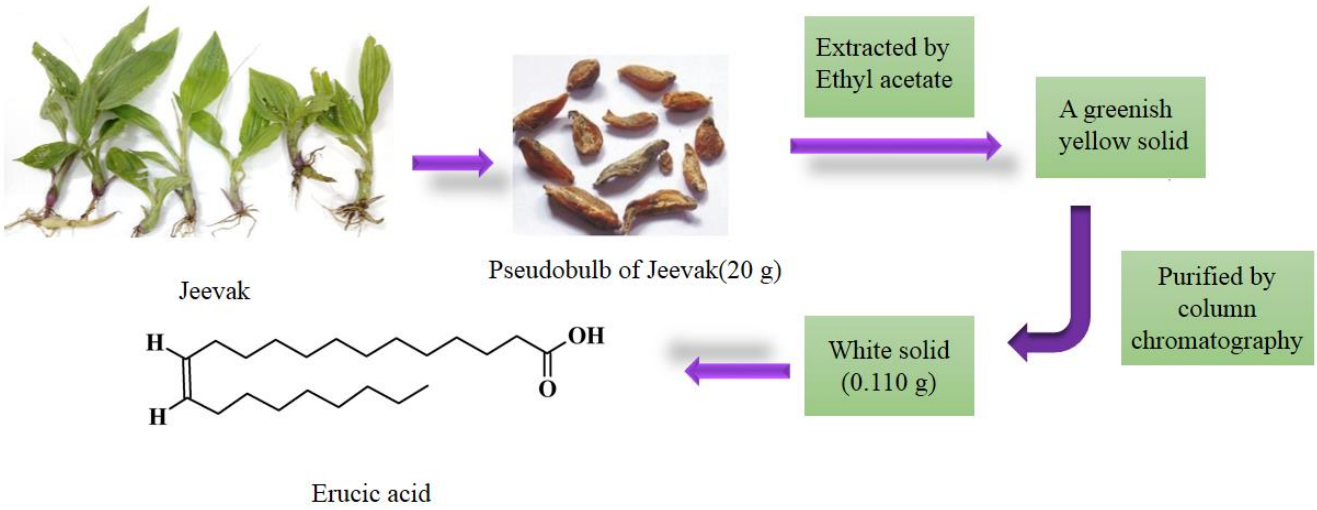
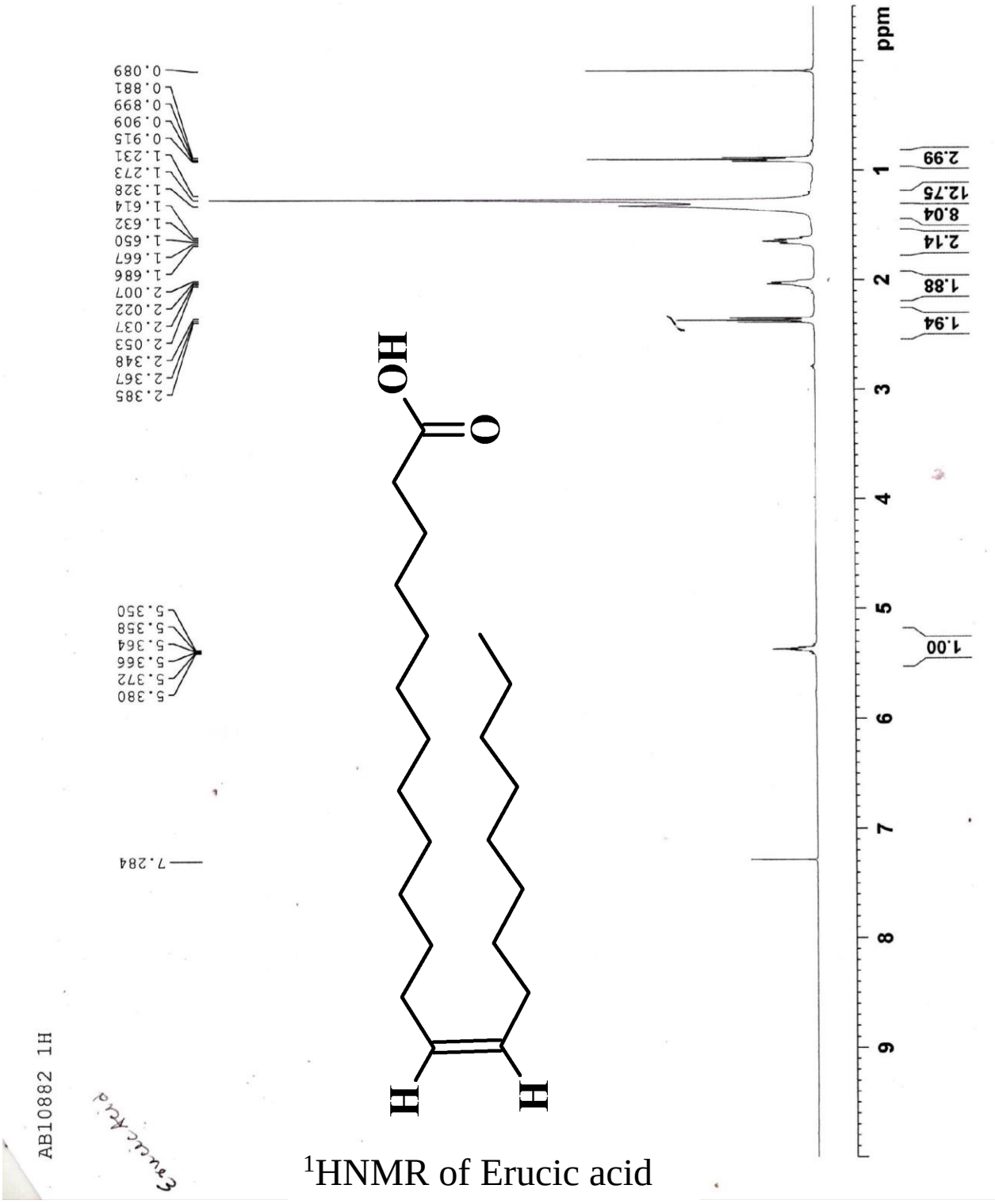
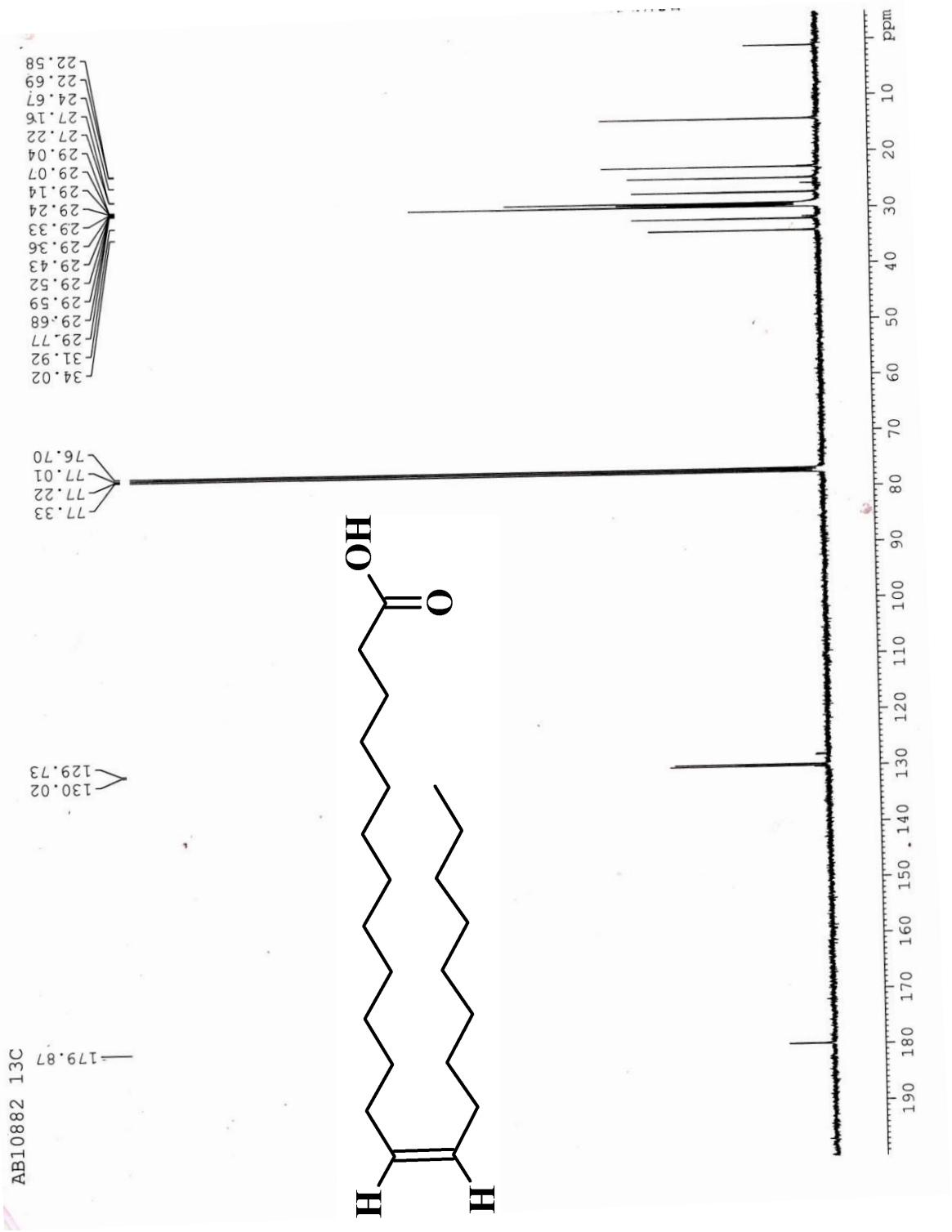


Figure 8: Schematic representation of the extraction of erucic acid from pseudobulb of jeevak

Figure 9: Image of jeevak



[1]HNMR of Erucic acid



^{13}C NMR of Erucic acid

HRMS spectra of Erucic acid

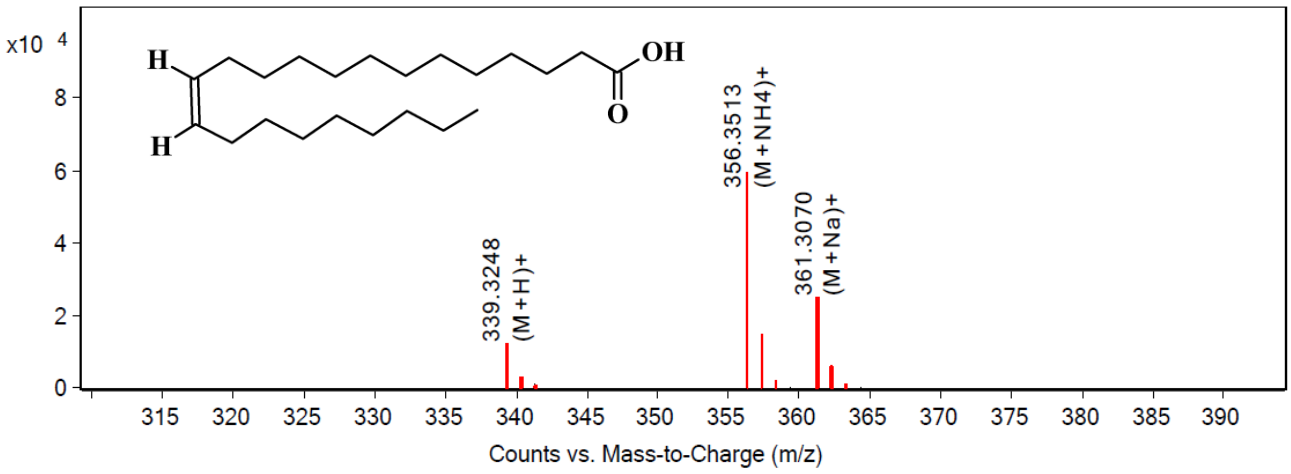


Table 3: Medicinal uses and formulations of Astavarga plants

Astavarga species	Therapeutic uses	Mode of use
Kakoli	Asthma, tuberculosis	Powder boiled with orange peel and taken with water
Kshirakakoli	Pain	Powder of Withania sominifera, L. polyphyllum, and Sida acuta taken with milk
Jeevak	Bronchitis	1 g powder mixed with M. muscifera, L. polyphyllum, F. roylei and Asparagus racemosus taken early morning with water
Rishbhak	Tonic	Dried powder taken with boiled milk
Meda	Suboleative	Powder of both Polygonatum species, M. musifera and Desmodium gangeticum taken with milk
Mahameda	Tonic, promote body heat	1gm dried powder is taken with milk
Riddhi	Galactagogue	Add tuber powder to Astavarga and swarnabhashma (from gold calcinations)
Vriddhi	Energy promoter	Boiled as vegetable

2.7 Antioxidant property of Astavarga plants

The plants of Astavarga group are well known for vitality strengthening properties and anti-ageing properties. However, systematic investigations with respect to these properties are not known. Generally, vitality strengthening and anti-ageing activities are controlled by antioxidant properties present in the different group of chemicals i.e., phenol, flavonoid, isoflavone, flavones, anthocyanin, etc. As such, phytochemical investigation in some species of Astavarga like *Habenaria edgeworthii*, *Habenaria intermedia* and *Roscoea proceraand*, *Polygonatum verticillatum* with regards to phenol, flavonoid, thiamine, riboflavin, mineral contents, etc. has been carried out[21,22,2324,25]. Therefore we investigate the anti-

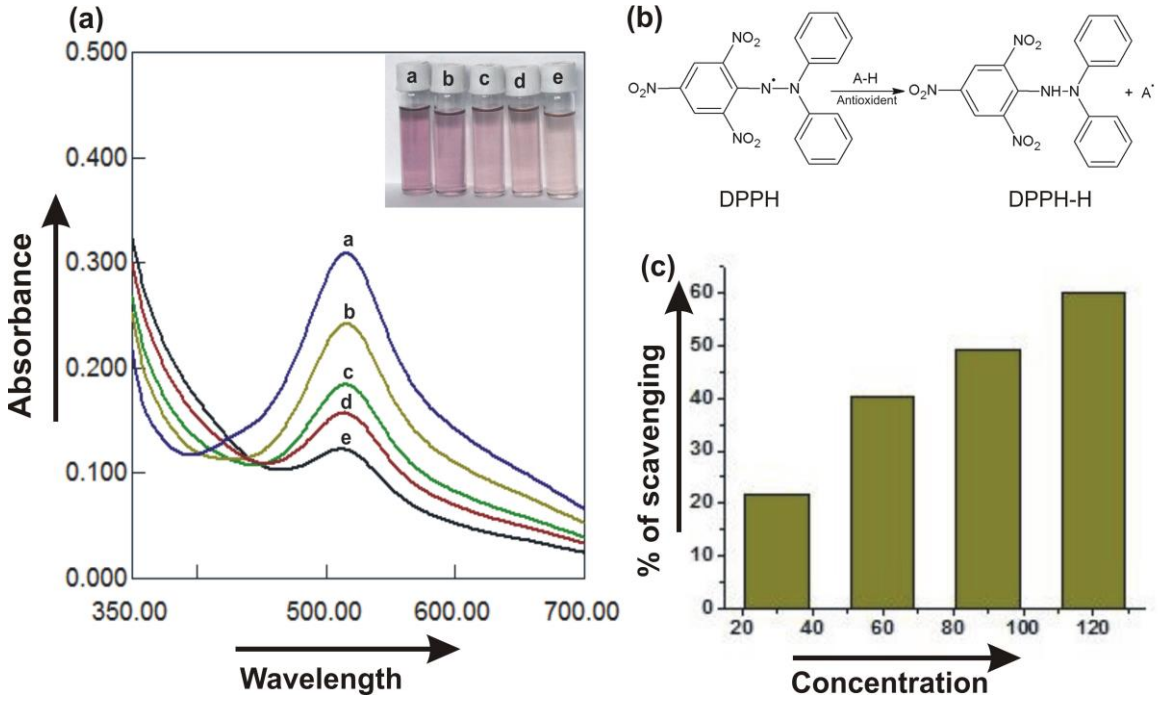


Figure 10: Antioxidant activity studies of the tuber extract of *Vrddhi*: (i) UV-visible spectra of (a) DPPH, (b -e) DPPH + with ethanol extract of tuber 30, 60, 90, 120 μgmL^{-1}., Inset: photographs of the vials containing the respective solutions; (ii) reaction scheme showing quenching of DPPH radical by the antioxidant (A-H); (iii) plot of % DPPH radical scavenging by the ethanol extract of tuber.

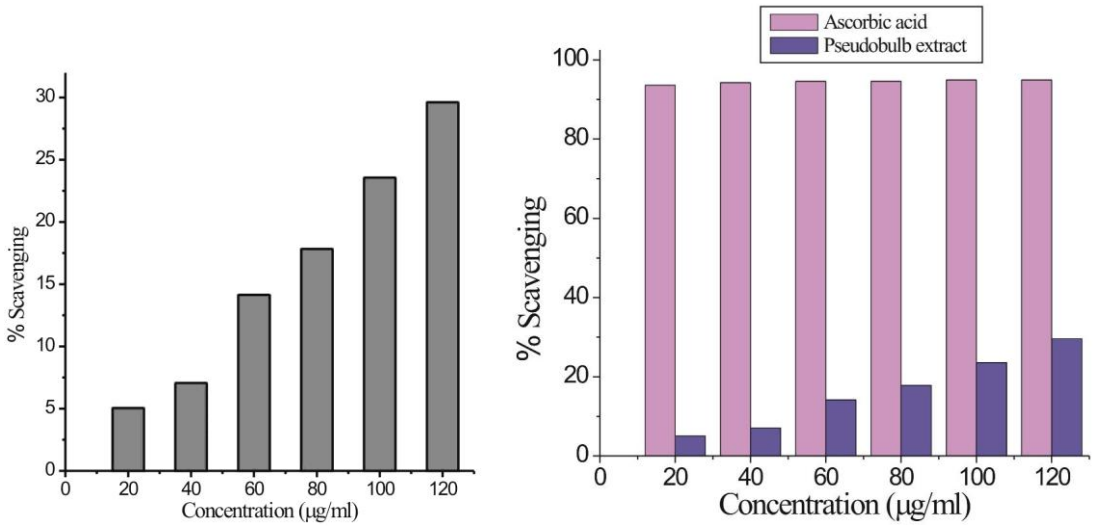


Figure 11: (a) Scavenging property of jeevak(b)Comparison of radical scavenging activity of pseudobulb extract with naturally occuring Ascorbic acid standard

conditions. *Vrddhi*, a terrestrial tuberous orchid, growing upto 75 cm in height, is found across the Himalayas at an altitude 2500 – 3000 m above sea level (Figure 1). Tuberous roots are small, ellipsoid, usually 1-2.5 cm long, 0.5 - 1 cm in diameter, white inside and fleshy (Figure 1b). It is used for the treatment of asthma, fever, skin diseases, leprosy, blood disorders, burning sensation, general debility, etc.

Mass spectral studies of the methanol extract of the tubers carried out in our laboratory showed the presence of several polyphenolic compounds including flavanoids along with steroids and other plant secondary metabolites (Figure 1c). Evidence for the presence of phenolic compounds was also obtained from a positive ferric chloride test. As the phenolic compounds have antioxidant properties, we tested the antioxidant activity of the ethanol extract of the dried tubers against a long lived 2,2-diphenylpicrylhydrazyl (DPPH) radical at room temperature.[26]

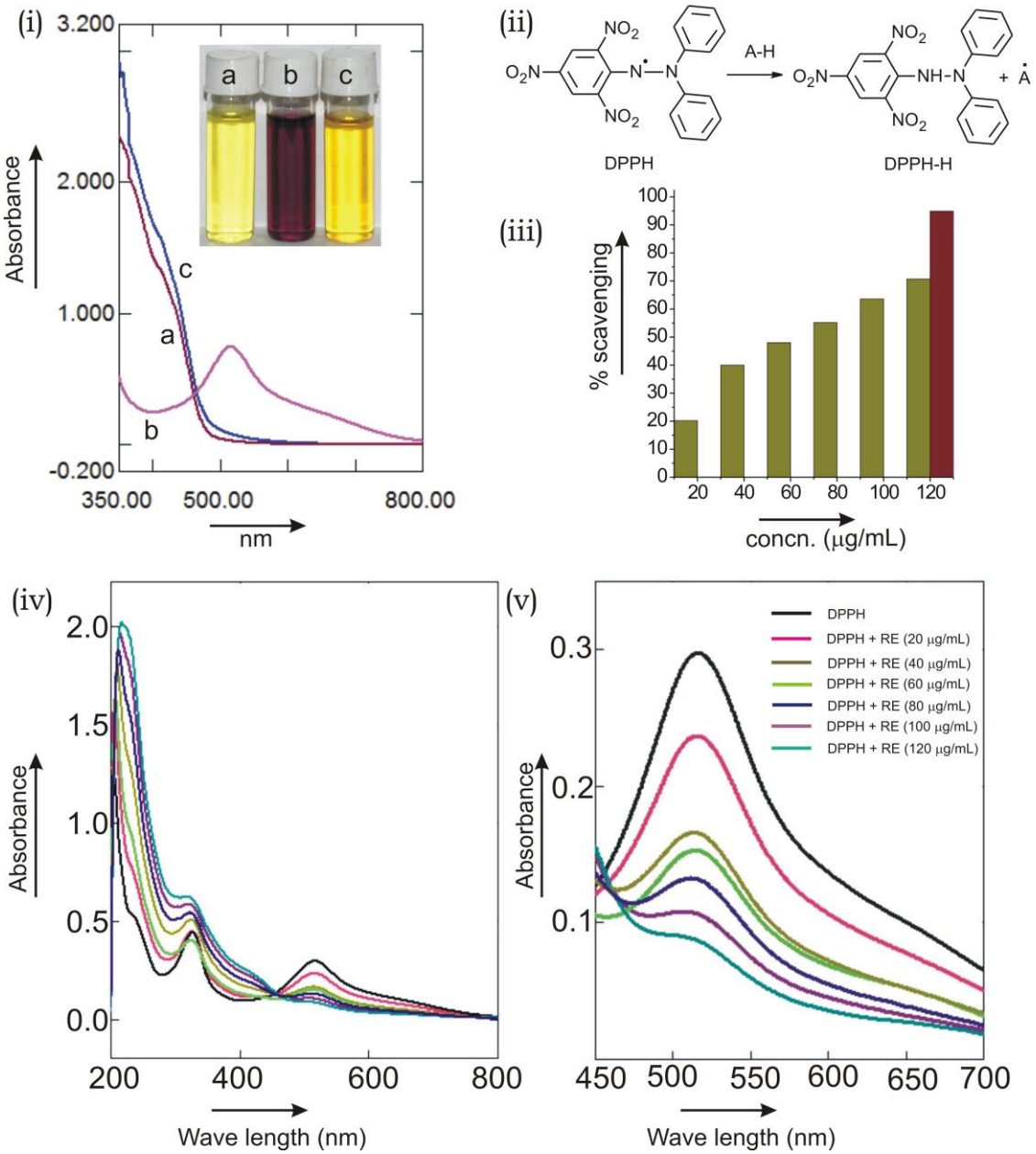


Figure 12: (A) Antioxidant activity studies of the rhizome extract of Kakoli: (i) UV-visible spectra of (a) Extract, (b) DPPH, (c) DPPH + ethanol extract, Inset: photographs of the vials containing the respective solutions; (ii) reaction scheme showing quenching of DPPH by the antioxidant (A-H); (iii) plot of % DPPH readical scavenging by the methanol extract of rhizome (20 - 120 µg/mL) and its comparison with ascorbic acid (120 µg/mL, brown); (iv) and (v) UV-visible spectrum of DPPH with increasing concentration of the methanol extract of rhizome (RE).

2.7.1.1 Determination of Antioxidant activity by DPPH Assay:

The 2,2-diphenylpicrylhydrazyl (DPPH) assay is widely used in plant biochemistry to evaluate the properties of plant constituents for scavenging free radicals. The free radical scavenging activity of the methanol and ethanol extracts of tubers of *Vrddhi* was tested against DPPH.[27] Antioxidants with active hydrogen react with DPPH radical and convert it to 1-1-diphenyl-2-picryl hydrazine. The radical scavenging potential of the extract is indicated by the degree of discoloration. Evaluation of the reducing ability of antioxidants present in the extract towards DPPH radical can be carried out by monitoring the decrease in the absorbance intensity at 517 nm in the UV-visible spectroscopy (Figure 10). The decrease in the absorption intensity of DPPH takes place because of the reaction between antioxidant (A-H) present in the root extract and DPPH radical (Figure 10b). % radical scavenging activity was calculated to be 60.19 when concentration of the tuber extract is 120 μg/mL. The anti-oxidant activity of pseudobulb of jeevak and rhizome extract of kakoli was studied same procedure as above (Figure 11 and figure 12)

2.8 Conclusion

Investigations of chemical constituents of medicinal plants will aid in understanding the chemical basis of biological and medicinal activities. With an aim to investigate the chemical constituents of various parts of the medicinal plants Kakoli (*Roscoea purpurea*) and Jeevak (*Crepidium acuminatum*), we have isolated stigmasterol from the leaves of *Roscoea purpurea* and erucic acid from the pseudo-bulb of jeevak and characterized by spectroscopic methods. To our knowledge, this is the first report of the isolation of stigmasterol and erucic acid in Kakol and Jeevak respectively.

Molecular modeling studies have revealed that stigmasterol can act as a functional nano-entity with tremendous potential application in supramolecular chemistry and nano-science. Similarly molecular modeling studies of erucic acid have revealed that this is a typical amphiphiles with functional nano entity.

2.9 References

1 R.R. Rao, Ethnobotany, **1994**, *8*, 14–24.

2 V.K. Joshi, Evolution of the concept of Astavarga. Indianv J Hist Sci. **1983**, *18*, 9–14.

3 A. Dhyani Lilium polyphyllum – rarest of rare lilies. Lilies and related plants. London (England): **2009**, Royal Horticulture Society. p. 85–91.

4 L. Giri, T. Belwal, A. Bahukhandi, R. Suyal, I. D Bhatt, R.S Rawal, S. K Nandi, *Ind. Crops and Prod,* **2017**, *102*, 173–179.

5 D. R. Mathur **2003**. Yogtarangini. Varanasi (India): Chaukhamba Vidhyabhawan.

6 D, Pandey, **2005**. Sarangadharasanhita. Varanasi (India): Chaukhamba Amarabharati Prakashan.

7 L. Giri, P. Dhyani, S. Rawat, , I.D. Bhatt, S.K. Nandi, R.S. Rawal, V. Pande, *Ind. Crops Prod.* **2012**, *39*, 1–6.

8 P. Pushpangadan Ethnobiology in India: A Status Report, Government of India, New Delhi, India, **1995**.

9 C. P. Kala, P. P. Dhyani, B. S.Sajwan Journal of Ethnobiology and Ethnomedicine. **2006**, *2*, 32-37.

10 S. Pant, S. S. Samant Ethnobotanical observation in the Mornaula Reserve Forest of Kumoun, West Himalaya, India, Ethnobotanical Leaflets, **2010**, *14*, 193-217.

11 S. K. Uniyal, A. Awasthi, G. S. Rawat, *Current Science.* **2002**, *82*, 1246-1252

12 D. K. Singh, P. K. Hajra, British Council, New Delhi, **1996**, 23-38.

13 D. P. Semwal, C. P. Kala, A. B. Bhatt, India, *Medicinal Plants*, **2010**, *2*, 51-57.

14 M. Sundriyal, R. C. *Current Science*. **2003**, *85*, 731-736.

15 C. P. Kala *Journal of Sustainable Development and World Ecology*, **2004**, *11*, 205–210.

16 G. Singh, G. S. Rawat, *Indian Journal of Fundamental and Applied Life Sciences*. **2011**, *1*, 35-46.

17 R. Ingalhalli, H. Rathod, H. Desai, *International Journal of Ayurveda and Pharma Research*, 2322 - 0910.

18 B. G. Bag, S. S. Dash, A. Roy. *IJRCE*, **2014**, *4*, 174-180.

19 A. Balakrishna, A. Srivastava, R.K. Mishra, S. P. Patel, R. K. Vashistha, A. Singh, V. Jadon, P. Saxena. *International Journal of Medicinal and Aromatic Plants*. **2012**, *2*, 661-676

20 A. Dhyania, B.P. Nautiyalb, M.C. Nautiyala, *International Journal of Biodiversity Science, Ecosystem Services & Management*, **2010**, *6*, 13–19

21 L. Giri, A. Jugran, S. Rawat, , P. Dhyani, H. Andola, , I.D. Bhatt, R.S. Rawal, U. Dhar, *Acta Physiol. Plant*, **2012**, *34*,869–875.

22 L. Giri, A. K. Jugran, A. Bahukhandi, P. Dhyani, I.D. Bhatt, R.S. Rawal, S.K. Nandi, U. Dhar, Appl. *Biochem. Biotechnol*. **2016**, *181*, 267–282.

23 H. Khan, M. Saeed, N. Muhammad, S. Perviz, *Toxicol. Ind. Health*, **2016**, *32*, 841–847.

24 H. Khan, M. Saeed, N. Muhammad, R. Ghaffar, S.A. Khan, S. Hassan, *Pak J. Pharm Sci.* **2012**, *25*, 463–467.

25 H. Khan, M. Saeed, N. Muhammad, S. Perviz, *Toxicol. Ind. Health*, **2016**, *32*, 841–847.

26. S.S. Gowri, S. Pavitha, K. Vasantha, *Int J Pharm Pharm Sci*, **2011,** *3*, 160.

27. R .Subramanian, P. Subbramaniyan, V. Raj, *Springer plus*, **2013,** *28*, 1.