ChemSearch Journal 5(1): 27 – 38, June, 2014
Publication of Chemical Society of Nigeria, Kano Chapter

Received: 22/11/2013 Accepted: 07/01/2014

Bioassay Guided Chromatographic Isolation Of Lactation Inducing Agent From *Hippocratea Obtusifolia*

[1]Datti, Y. and [2]Idris, M. M.
[1]Department of Chemistry, Northwest University, Kano.
[2]Department of Pure and Applied Chemistry, Bayero University, Kano.
Email: whydee@yahoo.co.uk

ABSTRACT

The powdered leaves of *Hippocratea obtusifolia* (commonly known as *gwadayi* in Hausa) was percolated with ethanol and then sequentially extracted with petroleum ether, chloroform, ethyl acetate and methanol. The extracts were respectively labeled HO1, HO2, HO3, and HO4, with the ethanolic extract labeled HO. The extracts were subjected to bioactivity against lactation inducement, and the chloroform extract labeled HO2 was found to be the most active. The HO2 extract was then subjected to column and thin layer chromatography with similar fractions pooled together. The pooled fractions were then subjected to bioactivity testing against lactation inducement, and the fraction labeled HO2-13 was found to be the most active, this was subsequently subjected to column and thin layer chromatography with similar fractions pooled together. The pooled fractions were again subjected to bioactivity testing against lactation inducement, and the fraction labeled HO2-13-111 was found to be the most active. The fraction HO2-13-111 was subjected spectroscopic analysis, using Infrared, Mass spectroscopy, and both ^{1}H and ^{13}C NMR data obtained. The structure of the compound present in fraction HO2-13-111 was then elucidated based on the obtained data.

Keywords: Force-Feeding, Lactation, Progesterone, Thin-Layer Column Chromatography.

INTRODUCTION

One outstanding feature that distinguishes the class mammalia from the rest of the members of the kingdom animalia, is that their young ones are fed from the milk secreted by the mothers mammary glands (Neville and Peaker 1984). The provision of a specialized maternal body fluid for neonatal nutrition allows birth to occur at an early stage of development and provide time of intense maternal infant interaction during early behavioral development. In addition, the nutritional reserves of the mothers can sustain the infant through a period of famine, as it contains all the nutrients required for the development of the body (Collier *et al*, 1977). These nutrients include carbohydrates, proteins, lipids and minerals (Hurley, 1998).

Lactogenesis is the term meaning the initiation of lactation. Lactation is the process of functional differentiation which mammary tissue undergoes when changing from a non-lactating to a lactating stage. This process is normally associated with the end of pregnancy and around the time of parturition (Nagasawa *et al.,* 1969). Because lactogenesis is particularly dependent upon specific set of hormones (called lactogenic complex of hormones). Mammary tissue from most state of non-lactating mammary gland also can be made to undergo some degree of lactogenesis by administration of high amount of such hormones even in non-pregnant animals (Davis *et al.,* 1983). The production of breast milk is controlled by an interplay of various hormones, with prolactin being the predominant hormone involved. The maturation of breast tissue, resulting in milk production, is controlled by many other factors besides prolactin, including estrogen, progesterone, insulin, growth hormone, cortisol, thyroxine, and human placental lactogen (Michael, 2008).

Throughout human history, there have been infants who have been nursed by surrogate mothers. Non-maternal lactation may have occurred as a results of maternal death or illness or because the birth mother gave over, or shared the care of her baby with another mother **(**Akers *et al.,* 1981). Sometimes the surrogate mother was already breastfeeding another baby and milk supply simply increased due to additional demand, to meet the growth needs for two or more babies. In the event that no already-nursing mother was available, reports from several continents describe effort by non-lactating woman to induce lactation by putting the baby to breast (Turner *et al.,* 1956).

The inducement of lactation without the needs of a proceeding pregnancy could offer several practical and economical advantages (Bauman *et al.,* 1977). Lactation has been induced in ruminants using steroids (progesterone and estrogen) as a short-term treatment followed by a

wide variety of drugs aimed in increasing prolactin secretion. Those treatment protocols have resulted in lactation with milk production ranging between 25% and 82% of physiological post-partum lactation (Palmer *et al.*, 2002).

Inducement of lactation has also been achieved both locally and traditionally, especially among the Hausa communities. Factors necessitating the need to have the lactation induced, include among others, the insufficiency of the milk from the birth mother, and the need to induce lactation on a birth mother, (who could not produce the milk). The method employed is mainly dependent on the necessitating factor: As for the insufficiency of the milk from the birth mother, the use of a local porridge called *kunun kanwa* (literally potash gruel) is employed. By immediately taking this porridge, hot and steaming, it is widely believed and accepted that the milk content will be increased to nearly maximum. Even though, there has not been any scientific proof to this, this tradition alongside the role played by the famous *wankan jego* (the hot water birth) in solving the problem of milk insufficiency, is widely accepted within the Hausa culture and tradition.

However, when it comes to the situation where there is no milk at all, in many cases use is made of some plants that are believed to be useful for that purpose. In India lactation inducing herbal remedies are commonly used by nursing mothers to meet the nutritional requirement of their children (Nudrat *et al*., 2007). Notable plants used by the Warlis tribe in India to induce lactation in nursing mothers include *Carica papaya* Linn, *Asparagus racemosa* Wt, *Trigonella foenum graecum* Linn and *Madhuca indica* Gmellin (Nudrat *et al.,* 2007). Other notable plants believed to be useful for the purpose of inducement of lactation, are *Kigelia aethiopica*, known as *hantsar giwa* in Hausa, as well as *Hippocratea obtusofolia* or *gwadayi* in Hausa.

Hippocratea obtusofolia is a species of flowering plant in the family *Hippocrateacea* which occur in some part of West African countries like Nigeria, Niger, Senegal and Cameroon. *Hippocratea obtusifolia*, a shrub with a green leaves and stalk growing up to 1m tall, is an evergreen where rainfall occurs throughout the year, but deciduous where there is long dry season, it produces sticky white fluid of detached (Huxley, 1992).

AIMS AND OBJECTIVES

This research is aimed at evaluating, isolating and structure elucidation of the bioactive lactating inducing agent from *Hippocratea obtusifolia*.

MATERIALS AND METHOD

The Plant Materials

The leaves and stalk of *Hippocratea obtusofolia* was collected at Dawaki, in Dawakin Tofa Local Government Area of Kano state, and were identified and authenticated at the Department of Biological Science, Bayero University, Kano.

Extraction Of The Plant

Five hundred grams of the dried and ground form of the plant *Hippocratea obtusofolia* was put in 2.5dm^3 brown capacity brown bottle, and 1.5dm^3 of ethanol added. The set up was left for two weeks with constant shaking, after which the extract was filtered and then concentrated using rotavapor (Vishnoi, 1979) to get the crude extract labeled HO. Part of the crude residue HO was then dissolved using aqueous methanol, and then partitioned with petroleum ether using separating funnel to get the petroleum ether fraction. The petroleum ether fraction was concentrated, dried, weighed and labeled HO1. The aqueous methanol portion was further partitioned with chloroform to get the chloroform fraction. The chloroform fraction was concentrated, dried, weighed and labeled as HO2. The aqueous methanol portion was then partitioned with ethyl acetate to get the ethyl acetate fraction. The ethyl acetate fraction was concentrated, dried, weighed and labeled as HO3. The final aqueous methanol portion was then concentrated, dried, weighed and labeled as HO4. Each of the five extracts HO, HO1, HO2, HO3 and HO4 was tested for lactation inducing activity.

Activity Testing

All the five fractions HO, HO1, HO2, HO3 and HO4 were subjected to bio-activity test. Twenty four female guinea pigs of average weight of 200g were randomly divided into 6 groups of four each. The first five groups A, B, C, D and E were each force-fed with the labeled portion HO, HO1, HO2, HO3 and HO4 of *Hippocratea obtusofolia* respectively for a period of two weeks. Each guinea pig received 2g/Kg body weight of the extract dissolved in 2cm^3 of distilled water. The force-feeding was carried out using a 2cm^3 syringe and endogastric tube. The last group F served as the control and was given distilled water free from the sample extract (Salim, *et al.,* 2005). After 2 weeks each group was quantitatively examined for the presence or any sign of lactation (Palmer *et al.,* 2002). At the end the chloroform extract HO2 was found to be the most active.

Purification Of The Chloroform Fraction (HO2)

Having found that the chloroform fraction HO2 is the most active, it was then subjected to column chromatography. The column was eluted using the solvents in the following order: petroleum ether (1dm^3), petroleum ether:dichloromethane (7:3, 2dm^3), petroleum ether:dichloromethane (3:2,

1.5dm^3), petroleum ether:dichloromethane (1:1, 1dm^3), petroleum ether:dichloromethane (2:3, 1.2dm^3), petroleum ether:dichloromethane (3:7, 1dm^3), dichloromethane (1.5dm^3), dichloromethane:ethylacetate (4:1, 1dm^3), dichloromethane:ethylacetate (1:1, 1dm^3), dichloromethane:ethylacetate (1:4, 1dm^3), ethylacetate (1dm^3). The eluents were collected in 100cm^3 volumes. All the fractions were spotted on a TLC plate coated with silica gel, and fractions that are similar were combined and labeled as pooled fractions HO2-13, HO2-40, HO2-60, HO2-79, HO2-90 and HO2-110.

Bioactive Evaluation Of The Pooled Fractions

Three of the five pooled fractions, HO2-13, HO2-60 and HO2-90 that were the most abundant, were subjected to bio-activity test. Eight female guinea pigs of average weight of 200g were randomly divided into 4 groups of 2 guinea pigs each (Salim, *et al.*, 2005). The first three groups G, H and I were each force-fed with the three pooled fractions HO2-13, HO2-60 and HO2-90, for a period of two weeks. Each guinea pig received 1g/kg body weight of the extract dissolved in 2cm^3 of distilled water and force-feeding was carried out using a 2cm^3 syringe and endogastric tube. The last group J served as the control and was given distilled water free from the sample extract (Salim, *et al.,* 2005). After 2 weeks each group was quantitatively examined for the presence or any sign of lactation (Palmer *et al.,* 2002). At the end the chloroform extract HO2-13 was found to be the most active.

Purification Of Fraction HO2-13 (Active Fraction)

Having found that fraction HO2-13 showed appreciable activity, it was then subjected to micro-column chromatography. The order of elution was petroleum ether (500mls), petroleum ether:dichloromethane (9:1, 500mls), petroleum ether:dichloromethane (4:1, 500mls), petroleum ether:dichloromethane (7:3, 500mls), petroleum ether:dichloromethane (3:2, 500mls), petroleum ether:dichloromethane (1:1, 500mls), petroleum ether:dichloromethane (2:3, 500mls), petroleum ether:dichloromethane (3:7, 500mls), petroleum ether:dichloromethane (1:4, 500mls), petroleum ether:dichloromethane (1:9, 500mls), dichloromethane (500mls). The eluents were collected in 20cm^3 volumes. All the fractions were spotted on a TLC plate coated with silica gel, and fractions that are similar were combined and labeled as pooled fractions HO2-13-15, HO2-13-111 and HO2-13-135.

.

Bioactivity Evaluation Of The Pooled Fractions (II)

The pooled fractions, HO2-13-111 and HO2-13-149 were the subjected to bio-activity test. Six female guinea pigs of average weight of 200g were randomly divided into 3, with the first two groups K and L each force-fed with the two pooled fractions HO2-13-111 and HO2-13-149, for a period of two weeks. Each guinea pig received 0.5g/kg body weight of the extract dissolved in 2cm^3 of distilled water and force-feeding was carried out using a 2cm^3 syringe and endogastric tube. The last group M served as the control and was given distilled water free from the sample extract (Salim *et al.,* 2005). After 2 weeks each group was quantitatively examined for the presence or any sign of lactation (Palmer *et al.,* 2002). At the end the chloroform extract HO2-13-111 was found to be pure and the most active. The purity was confirmed by subjecting the isolated HO2-13-111 to TLC using varying solvent ratios, and in each case a single spot was obtained.

Spectroscopic Analysis

The active fraction HO2-13-111 was then subjected to spectroscopic analysis, and the parameters obtained are Infrared, mass spectroscopy and both ^{1}H and ^{13}C-nmr.

RESULTS AND DISCUSSION

After subjecting all the five extracts to bioassay, the result obtained is as tabulated in Table 1.

Table 1: Result of Bioactivity Test 1

EXTRACT	HO	HO1	HO2	HO3	HO4	CONTROL
ACTIVITY	+	-	+	-	-	-

Key: + = Extract is active
- = Extract is not active

After subjecting the HO2 fraction to chromatography and TLC, as well as pooling the fractions that were found to be similar, the following weights were obtained (Table 2).

Table 2: Weight and Code of the Pooled Fractions 1

FRACTION	R_F-VALUES	POOLED INTO	FRACTION CODE	WEIGHT (g)
6-34	0.72	13	HO2-13	2.23
39-41	0.36	40	HO2-40	1.05
44-70	0.29	60	HO2-60	1.32
73-80	0.55	79	HO2-79	0.98
85-99	0.42	90	HO2-90	1.97
103-116	0.98	111	HO2-110	0.67

Table 3: Result of Bioactivity Test 2

EXTRACT	HO2-13	HO2-40	HO2-60	HO2-79	HO2-90	HO2-110
ACTIVITY	+	-	-	-	-	-

Key: + = Extract is active
- = Extract is not active

After subjecting the HO2-13 fraction to chromatography and TLC, as well as pooling the fractions that were found to be similar, the following weights were obtained (Table 4).

Table 4: Weight and Code of the Pooled Fractions 2

FRACTION	R_F-VALUES	POOLED INTO	FRACTION CODE	WEIGHT (mg)
17-50	0.87	15	HO2-13-15	57.23
60-123	0.56	111	HO2-13-111	98.32
129-146	0.31	135	HO2-13-135	47.70

After subjecting the pure isolate HO2-13-111 to spectroscopic analysis using Infrared, mass spectroscopy, ^{1}H-nmr and ^{13}C-nmr, and the results obtained are as shown in Figures 1- 6 on the attached spectral data sheets.

The IR (Fig.1) of HO2-13-111 shows a peak at 1727.4 cm^{-1} representing a carbonyl functional group, a peak at 2936.7cm^{-1} representing a C-H stretching, while the band at 1661.2cm^{-1} can be attributed to =C-H bending of alkenes, and a peak at 1246.1cm^{-1} representing a carbon-oxygen single bond stretching, while the peak seen at 1450.6cm^{-1} may be attributed to to C-H bending of alkanes (Williams and Fleming 2004). The absence of an OH signal (3300-2500cm^{-1}) in the IR indicates that the carbonyl groups are not from carboxylic acids, and this left us with the possibility of having either a ketonic carbonyl group, or an ester, or both. However, the fact that we do not have an IR signal at 1750-1735 cm^{-1}, which is characteristic of esters, rather we have a peak 1727.4cm^{-1}, which is characteristic of ketone, means that the carbonyl group is from a ketone.

The ^{1}Hnmr (Fig. 2) reveals a cluster of peaks from 0-2ppm, which according to many literature consulted (Gonzalez *et al.,* 1981; Molnar *et al.,* 2006; Badgujar and Jain, 2009; Anand *et al.,* 2011) can be attributed to triterpenes. Other peaks from the Hmnr include a sharp singlet at 2.00ppm, a singlet at 4.70ppm, a doublet at 2.25ppm, a quartet at 4.10ppm, a doublet of triplet at 4.38ppm, a triplet at 4.60ppm, a doublet of quartet at 2.31ppm, and a singlet at 3.7ppm.

The ^{13}Cnmr data (Fig. 3) of HO2-13-111 showed the presence of resonances signifying the presence of 32 carbons. The results obtained from the dept spectrum (Fig. 4) of this compound reveal that 25 out of the 32 carbons are protonated, while the remaining 7 are unprotonated. Deduction from the dept (Table 5) shows that there are 9 methyl carbons, 11 methylene carbons, 5 methine carbons, and consequently 7 quaternary carbons. Notable signals from the ^{13}Cnmr (Fig. 3) include a peak at ∂14.7ppm which may be associated with carbon 23, a peak at ∂171.8ppm which can be attributed to carbon 3, a peak at ∂142.5ppm which can be attributed to carbon 24, a peak at ∂129.8ppm which can be attributed to carbon 25, a peak at ∂81.5ppm which can be due to carbon 15, a peak at ∂55.6ppm which can be due to carbon 2, and a peak at ∂43.4 which can be due to carbon 13. The fact that the proton on carbon 15 appeared as a doublet of triplet instead of a triplet, is an indication that there is a shift of a methyl group from carbon 14 to carbon 8 (Venkata and Chaturvedula 2013).

Table 5: (13C - Adept of HO2 – 13 – 111)

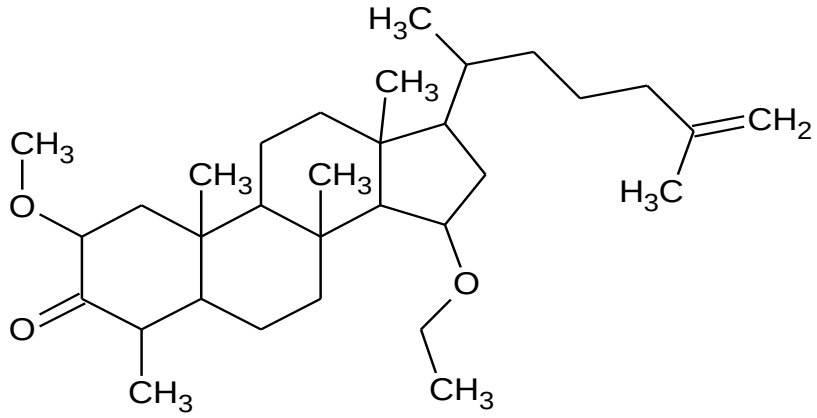
ADEPT SPECTRUM ANALYSIS

index		frequency	ppm	intensity
1	D	6530.1	129.859	35.080
2	D	4096.3	81.459	50.885
3	D	2800.0	55.682	37.741
4	D	2576.1	51.229	35.589
5	T	1945.9	38.696	33.347
6	D	1935.6	38.491	35.447
7	T	1900.5	37.794	34.954
8	T	1883.7	37.460	33.459
9	T	1740.7	34.615	33.842
10	T	1680.4	33.416	34.166
11	Q	1579.7	31.413	44.341
12	Q	1469.8	29.229	44.597
13	Q	1407.2	27.985	54.820
14	T	1388.6	27.613	32.783
15	T	1321.8	26.285	34.450
16	Q	1273.0	25.314	46.078
17	T	1194.8	23.759	45.803
18	T	1188.3	23.630	25.486
19	Q	1073.8	21.354	79.335
20	T	1068.1	21.240	30.582
21	T	917.4	18.244	35.972
22	Q	847.2	16.848	60.810
23	Q	833.9	16.582	55.942
24	Q	812.5	16.158	49.973
25	Q	736.2	14.640	48.337

Number of protonated carbons: 25

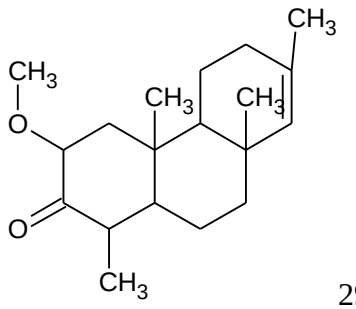
CH : 5
CH2: 11
CH3: 9

Combining the information above, we can come up with a structure having the framework of a triterpene with one ketonic group at carbon 3, a double bond at carbon 24, and two C-O-C bonds at carbons 2 and 15. Having a clear quatet Hnmr at 4.10ppm is an indication of the presence of a highly shielded ethyl group, with the methylene group splitted into a quatet by the methyl group. This means that one of the two C-O-C bonds is ethoxy, while the remaining three may be methoxy, and preferably the C-O-C on carbon 15 is chosen to be the one having the ethoxy group. The protons on the double bond split into a singlet at 4.7ppm, while the protons on the methyl group attached to the double split into a sharp singlet peak at 2.00ppm, the proton on carbon 3 will split into a doublet of quatet at 2.31ppm, and the methoxy proton will split into a singlet at 3.7ppm. Consequently, the structure of the isolated compound HO2-13-111 is proposed as;

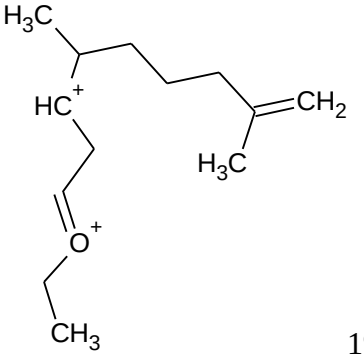


The proposed structure whose formula weight is 486, with molecular formula $C_{32}H_{54}O_2$ has the following fragmentation pattern, as evidently shown by the mass spectroscopy (Fig. 5).

Heterolytic cleavage of structure 486 by shifting the bond between C14 and 15 to the bond between C15 and O (creating a double bond) will create a positive charge on C14, which will cause the bond between C13 and 17 to shift to the bond between C13 and 14 (creating a double bond). This splits the structure into two structures with formula weights 290 and 196 (base peak).



290



196

When the proposed structure heterolytic cleaves by losing a propylene group, a structure with formula weight 445 results, which can itself heterolytically loose a methoxy group to yield another structure with formula weight 414. Structure 414 can heterolytically cleave to generate another structure with formula weight 318, which itself can lose a methyl group to yield a structure with formula weight 304.

CONCLUSION

The chloroform fraction HO2 of *Hippocratea obtusofolia* was found to be the most active as far as inducement of lactation is concerned. Activity guided fractionation and chromatography of HO2 led to tne isolation of a fraction HO2-13 which retained the observed activity. This was further chromatographed to obtain fractions HO2-13-111 and HO2-13-149, with the latter being the most active.

REFERENCES

Akers, R. M., Bauman, D. E., Cauco, A V., Goodman, G. T., and Tucker, H. A. (1981) *Endocrinology* 109:23.

Anand, SP., Jeyachandran, R., and Nandagopalan, N. (2011) NMR Spectral Analysis on Root Extract of *Zehneria scabra* - A Vital Medicinal Climber *J. Pharm. Sci. & Res.* 3(1):1015-1018.

Badgujar VB., and Jain PS. (2009) A Phytochemical Analysis of the Medicinal Plant: *Helicteres isora International Journal of Pharmtech Research* 1(4):1376-1377.

Bauman, D. E., Collier, R. J., and Tucker, A. (1977) *Proc. Soc. Exp. Boil. Med.* 155:189

Chaturvedula, V. S. P., Chen, S., Yu, O., and Mao, G. (2013) Isolation, NMR spectral analysis and hydrolysis studies of a hepta pyranosyl diterpene glycoside from *Stevia rebaudiana* Bertoni. *Biomolecules* 3:733-740.

Collier, R. J., Bauman, D. E., and Hays, R. L. (1977) Effect of Reserpine on Milk Production and Serum Prolactin on Cows Hormonally Induced into Lactation. *J. Dairy sci.* 60:896.

Davis, S. R., Welch R. A. S., Pearce, M. G., and Peterson, A. J. (1983) Induction of Lactation in Nonpregnant Cows by Estradiol-17B and Progesterone from an Intraviginal Sponge. *J. Dairy sci.* 66:450-457.

Gonzalez, AG., Fraga, BM., Gonzalez, P., Hernandez, MG., and RAVELO, AG. (1981).13CNMR spectra of olean-18-ene derivatives *Phytochemistry*, 20(8):1919-1921

Hurley, W. L. (1998) Human Milk and Lactation. *J. An Sci*. 47:308.

Huxley, A. (1992) *Hippocrateceae: The New Royal Horticulture Society Dictionary of Gardening*. Macmillan 2:735.

Michael, P. G. (2008) Galactogogues: Medications That Induce Lactation *J Hum Lact* . 18(3):274-279.

Molnar, J., Gyemant, N., Tanaka, M., Hohmann, J., Bergmann-Leitner, E., Molnar, P., Deli, J., Didiziapetris, R., Ferreira, MJU. (2006) Inhibition of multidrug resistance of cancer cells by natural diterpenes, triterpenes and carotenoids. *Curr. Pharm. Design* 12:287−311.

Nagasawa, H., Chen, CL., and Meites, J. (1969) *Proc. Soc. Exp. Biol. Med.* 132:859.

Nevelle, M. C. and Peaker, M. (1984) Mammary Gland Lactation and Lactogenesis. *Journal of physiology* 313: 561-570.

Nudrat, Z. S., Rich, D., and Usha, M. (2007) Herbal remedies used by Warlis of Dahanu to induce lactation in nursing mothers *Indian Journal of Traditional Knowledge* 6(4):602-605.

Palmer, C. P., Anold, G. and Ponter, D. C. (2002) Qualitative and Quantitative Assessment of Milk Production After Pharmaceutical Induction of Lactation in the Mare. *J. vet. int. med.* 27:145-147.

Salim, M. A., Esomonu, U. G., El-Talu, A. B., Anuka, J. A., and Atiku M. K. (2005) Effect of Ingestion of Ethanol Extract of *Garcinia kola* Seed on Erythrocytes in Winster Rats *Nigeria J. Physiological Sci.* 20(1-2):31-32.

Turner, C. W., Yammota H., and Ruppert, L. (1956) The Experimental Induction of Growth of the Cows Udder and the Initiation of Milk Secretion. *J. Dairy sci.* 39: 1717.

Venkata, S. P. and Chaturvedula, R. H. (2013) Isolation and NMR Spectral Studies of Dihydromyricetin *J. of Pharmacognosy and Phytochemistry* 2(4): 113-115.

Vishnoi, N. K. (1979) *Advanced practical organic chemistry*. Vicas publishing house PVT Ltd. Ghaziabad, India.

Williams, D. H. and Fleming, I. (2004) *Spectroscopic methods in organic chemistry*. Tata McGraw-Hill Publishing Co. Ltd., New Delhi.

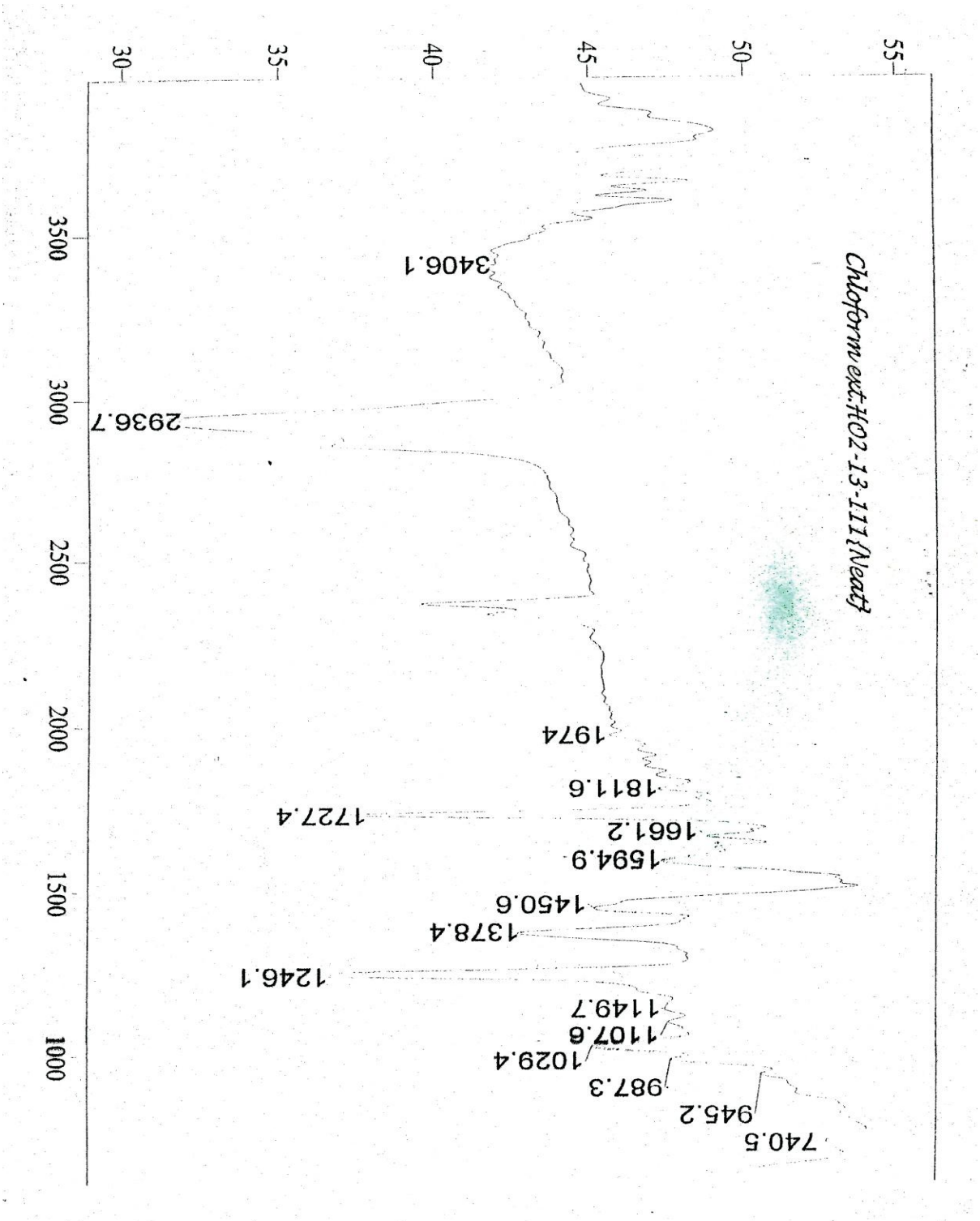


Fig. 1: IR of HO2-13-111

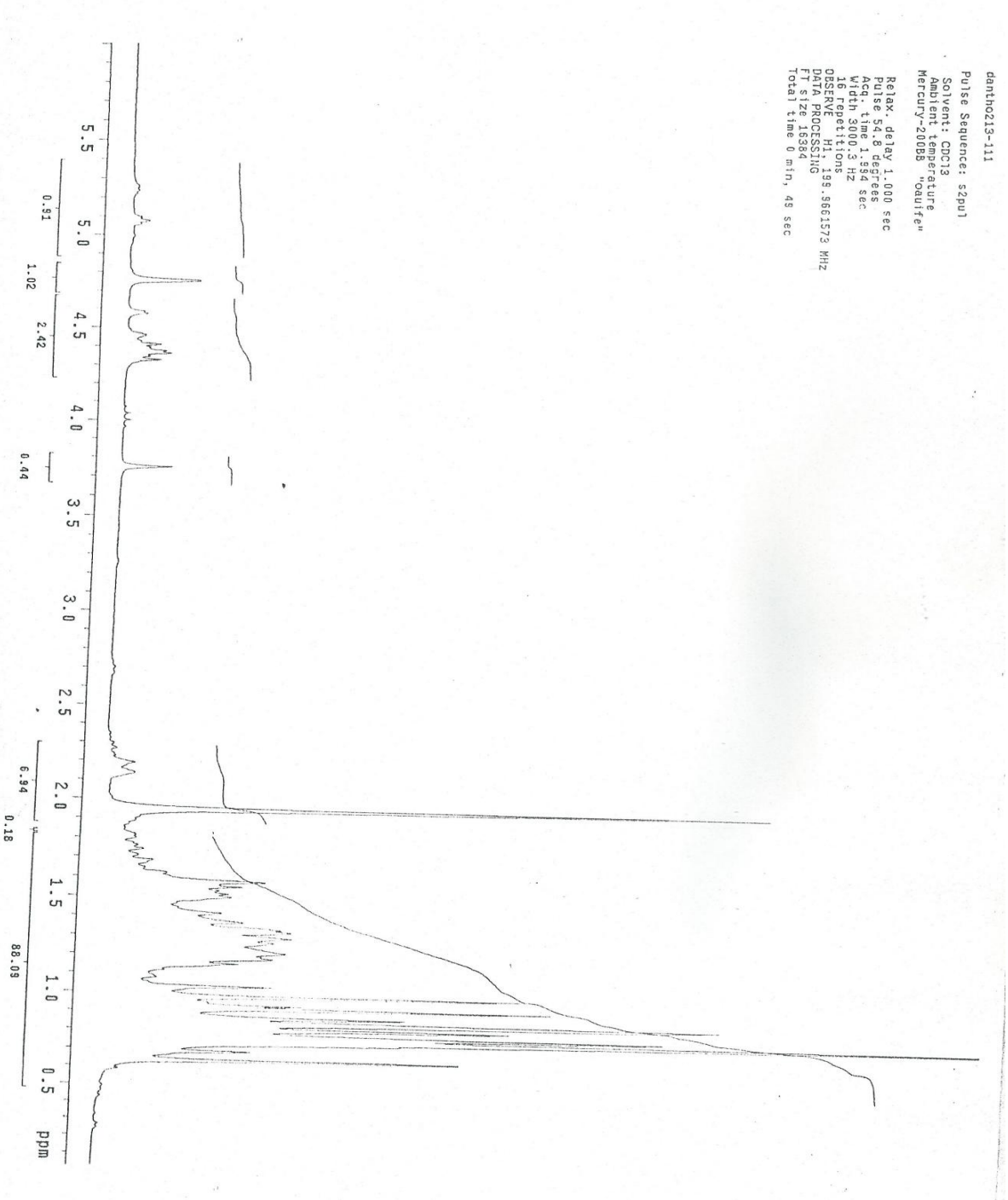


Fig. 2: H-nmr of HO2-13-111

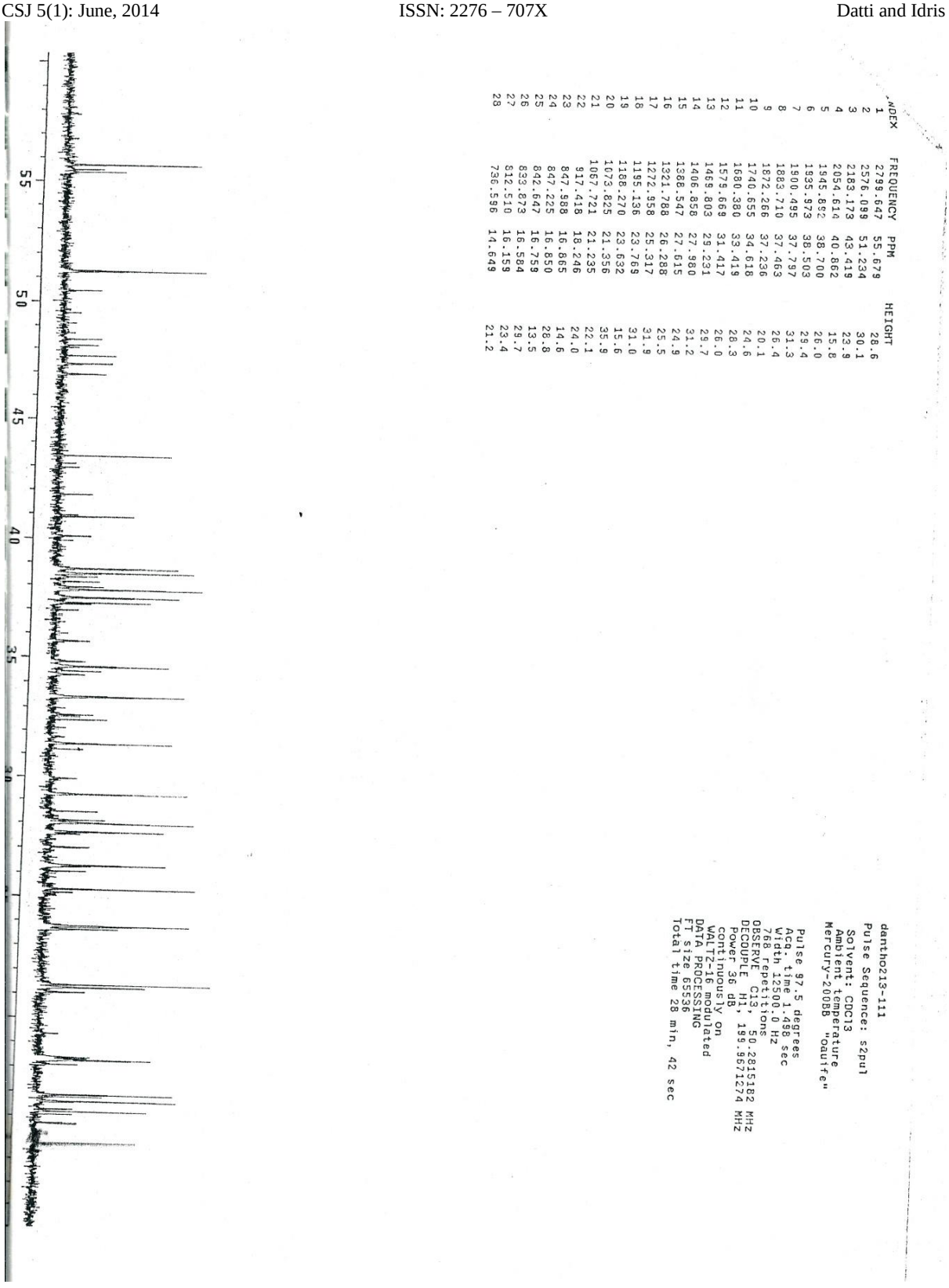


Fig. 3: C-nmr of HO2-13-111

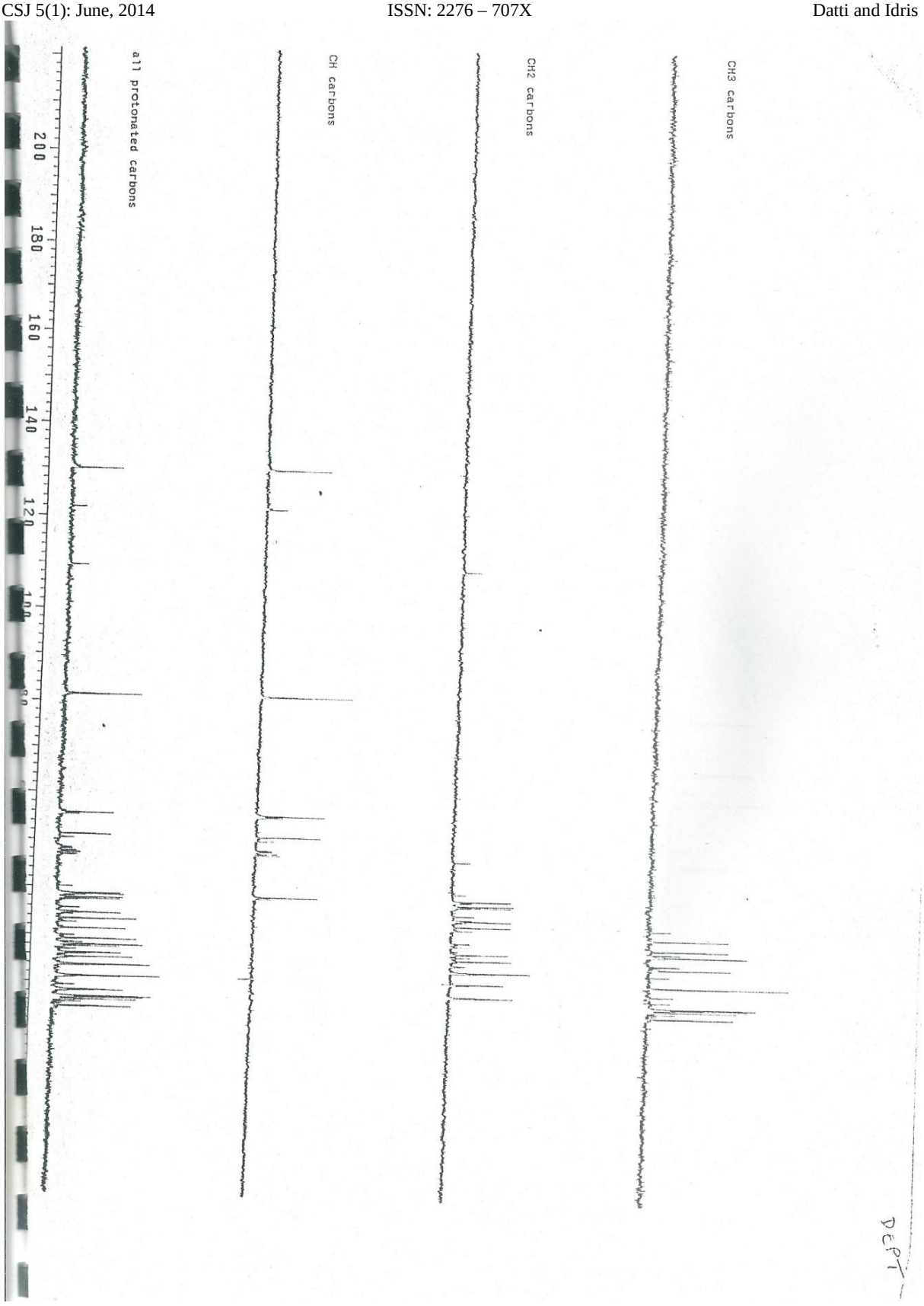


Fig. 4 (C-Dept of HO2-13-111)

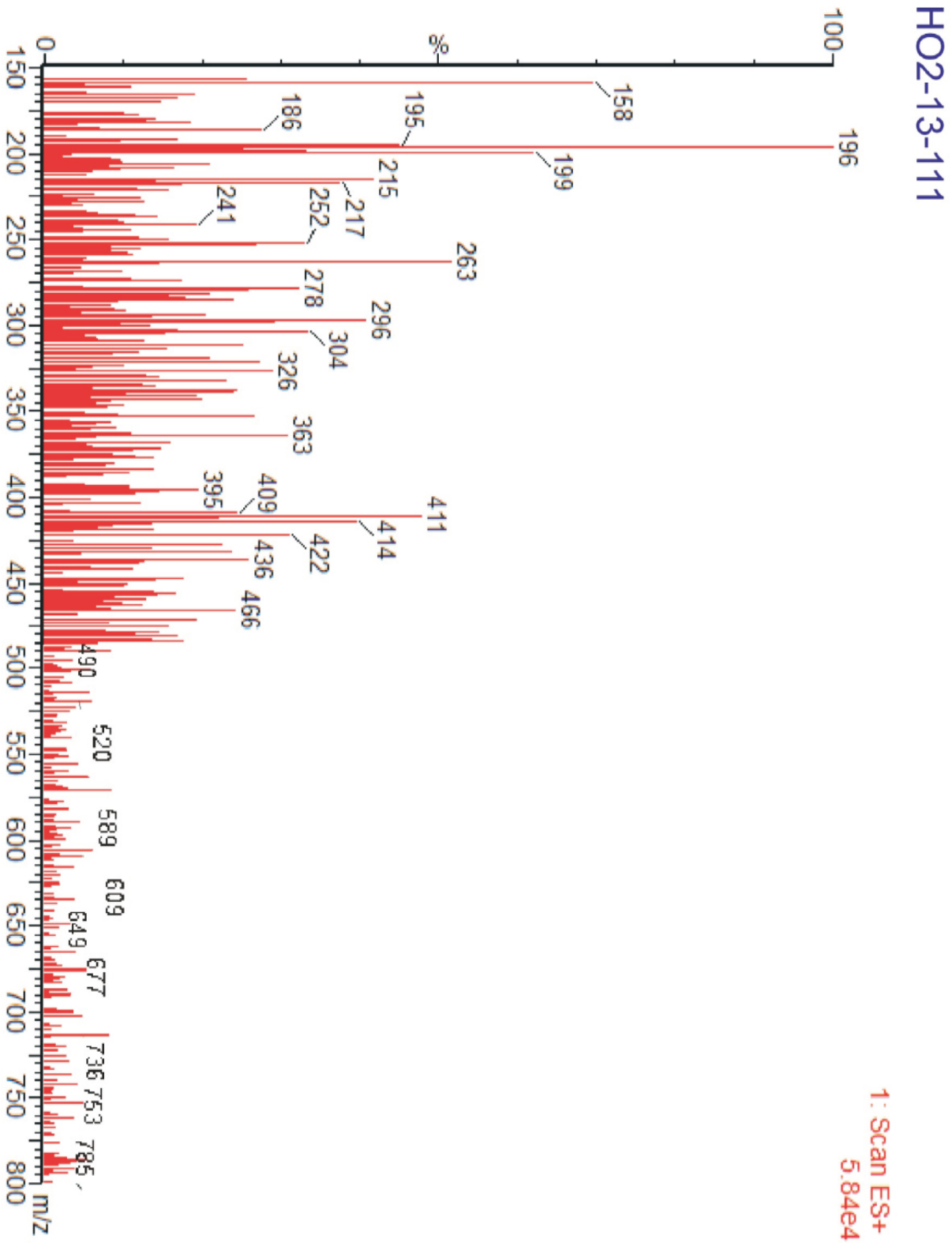


Fig. 5: Mass Spect. of HO2-13-111