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Phytochemical Investigation and Characterization on the Leaf Extract of *Prunus africana*

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Authors' contributions

This work was carried out in collaboration among all authors. Author Teshale Ayano Begeno designed the study, collected the data, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors AET and Temesgen Abera Bafa managed the analyses of the study, interpretation of the data and critical revisions of the manuscript. Author WBN managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Prunus africana (*P. africana*) is a geographically wide spread tree to forest habitats of the African continent. *P. africana* is one of the most popular plant in traditional medicine for threatening various ailments. It is mainly is used to treat benign prostate hyperplasia (BHP). The study aimed at phytochemical investigation and characterization on the leaves extract of *P. africana*. The air dried and powdered plant material (155 g) was first soaked with 500 ml n-hexane for 72 hours and yielded 1.3 g of n-hexane extract. Residue was soaked with 400 ml of ethyl acetate for 72 hours and afforded 1.8 g of ethyl acetate extract. Finally, residue was soaked with 400 ml of methanol and yielded 12.6 g of methanol extract. The ethyl acetate extract of the leaves of *P. africana* afforded a compound coded as CM whose structural determination was accomplished by means of spectroscopic techniques. The compound, CM was isolated and characterized from the leaves of *P. africana*. It was shown that spot on TLC only up on spraying 1% vanillin sulphuric acid and after

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heating for a few minutes. Generally, chromatographic techniques like PTLC and HPLC are required to isolate more compounds from leaves of *P. africana*. Also MS and 2D NMR spectroscopic techniques are needed to fully characterize the isolated compound.

Keywords: P. africana; phytochemical; characterization; spectroscopic techniques.

1. INTRODUCTION

Infectious diseases are the leading causes of death in tropical countries accounting for approximately one half of all deaths and underlying causes of significant problem, i.e., 8% deaths occurring in developed nations such as USA [1]. Plants have a long history of being used for a wide variety of purposes where therapeutic uses are inclusive. Majority of developing countries (70-95%) in Asia, Africa, Latin America and the Middle East use traditional medicine for the management of primary health care [2]. Phytochemical study of plants is of the great importance in developing drugs. Natural products have been used as a major source of drugs for centuries, with more than 25% of the pharmaceuticals in use today derived from natural products [3]. The World Health Organization (WHO) has also recognized that the importance of traditional medicine and has created strategies, guidelines and standards for botanical medicines [4].

1.1 *Prunus africana (P. africana)*

Many well-known and beloved species of Rosaceae family have great economic importance. Hence, they are known as "edible temperate zone fruits". The Rosaceae is the 19th largest family of plant which includes more than 100 genera and 2830-3100 species among which *P. africana* has well claimed medicinal value. *P. africana* is a geographically wide spread tree to forest habitats of the African continent. It is an evergreen hardwood tree; over 30 m-60 m in height and up to 1.5 m diameter. The leaves are simple and alternately arranged. The flowers are small, androgynous and insect-pollinated. It is commonly known as African cherry or *Pygeum africanum*. It is widely distributed in Angola, Mozambique, Zambia, Zimbabwe, Burundi, Congo, Kenya, Rwanda, Nigeria, Sao Tome, and Ethiopia (Northwest and Southeast highlands, Harerge, Illubabor, Kefa, Arsi and Wolega) [5,6].

Many African traditional healers have used the plant to treat various health ailments like diarrhoea, dysmenorrhoea, epilepsy, impotency, infertility, irregular menstruation, kidney disease, mental illness, eye disorders, obesity, arthritis, haemorrhage, hemorrhoids, hypertension, anti-gonorrheic, anti-inflammatory, anti-malarial, chest pain, anti-parasitic, and anti-rheumatic [7,8]. The pharmacological efficacy is believed to be due to various known and unknown compounds. Among the known compounds, three groups are greatly important: (A) Phytosterols especially β-sitosterol, have anti-inflammatory properties that inhibit the swelling of the prostate gland, (B) Pentacyclic triterpenoids that provide anti-edematous activity, and (C) Ferulic acid esters, which have a powerful hypocholestero-lernic activity in the prostate, as well as anti-tumor activity [9]. Leaves are used as an inhalant for fever or are drunk as an infusion to improve appetite, remedy for stomach pain and insanity [10,11]. In Ethiopia, the leaves of the species were used to dress wounds. Leaf infusions were used for relieving fever and in increasing appetite [12]. Bark infusions were used in the treatment of chest pains and as a purgative for cattle [13]. In Africa, 500 tonnes of bark were harvested for use in traditional medicine annually [14]. The use of the species in pharmaceutical industry commercially began in 1970s. In 1980, only 200 tonnes of *P. africana* bark were harvested [15]. The demand for the bark in pharmaceutical industry increased from then and by 1997 the demand had risen to 3500 tonnes [16]. This involved processing and marketing of bark and bark extracts for the treatment of benign prostate hyperplasia [17]. Benign prostatic hyperplasia (BPH) is a condition common in most men and manifests itself as increased frequency in urination, pain in passing urine, inability to empty the bladder and post urinary dribbling [18]. Phytosterols eliminate vassal congestion and excess blood hence reduces the size of prostate adenomas. The pentacyclic triterpenoids block enzymatic activity thus inhibits inflammation in the prostate [19,20]. Thus, the study was aimed at phytochemical investigation and characterization, on the leaf extract of *P. africana*.

2. MATERIALS AND METHODS

2.1 Plant Material

The leaves of *P. africana* were collected from Shero kebele, Misha Woreda, Hadiya Administrative Zone, Southern Nations

Nationalities and People Regional State (SNNPR). It was authenticated by a botanist Mr. Wege Abebe and specimen was stored at the National Herbarium of Addis Ababa University (Voucher no.TA001), Addis Ababa, Ethiopia.

2.2 Instruments, Apparatus and Chemicals

^{1}H and ^{13}C NMR spectra were recorded on a Bruker Advance 400 MHz spectrometer (Germany) in $CDCl_3$ with TMS as internal standard. The ultra-violet and visible (UV-Vis) spectra were taken on GENESY'S 2PC UV-Vis scanning spectrometer (USA) (200-800 nm). IR spectra were obtained on Perkin-Elmer BX infrared spectrometer (USA) (400-4000 cm^{-1}) using KBr. Melting point was recorded using digital melting point apparatus; RV-10-basic Rotavapor (Germany) was used for separation of solvents. Analytical thin layer chromatograms were run on 0.2 mm thick layer of silica gel coated on aluminium foil. Column chromatography was performed using silica gel (70-230 mesh). In this study, all the chemicals were provided by Hi-Media Co. including methanol, acetone, chloroform, ethyl acetate, n-hexane, and others. Some of the apparatus were used: funnels, round bottom flasks, vials, glass wares, refrigerator, Whatman No.1 filter papers, grinder, drying oven, measuring cylinders, TLC Chamber and others.

2.3 Extraction and Isolation

The air dried and powdered plant material (155 g) was first soaked with 500 ml n-hexane for 72 hours and the extract was collected by filtering and concentrated under reduced pressure using the RV-10 basic Rotavapor. The solvent free residue was then soaked with 400 ml of ethyl acetate for 72 hours and the extract was collected. This filtrate was evaporated under reduced pressure using the

Fig. 1. *P. africana* (Picture taken by Teshale Ayano during data collection)

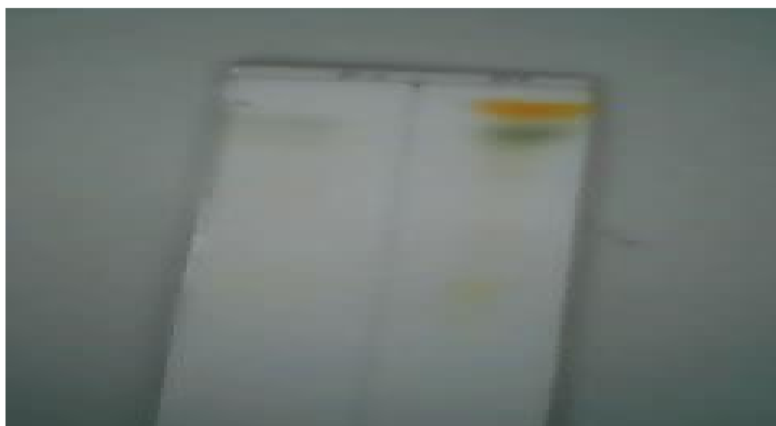
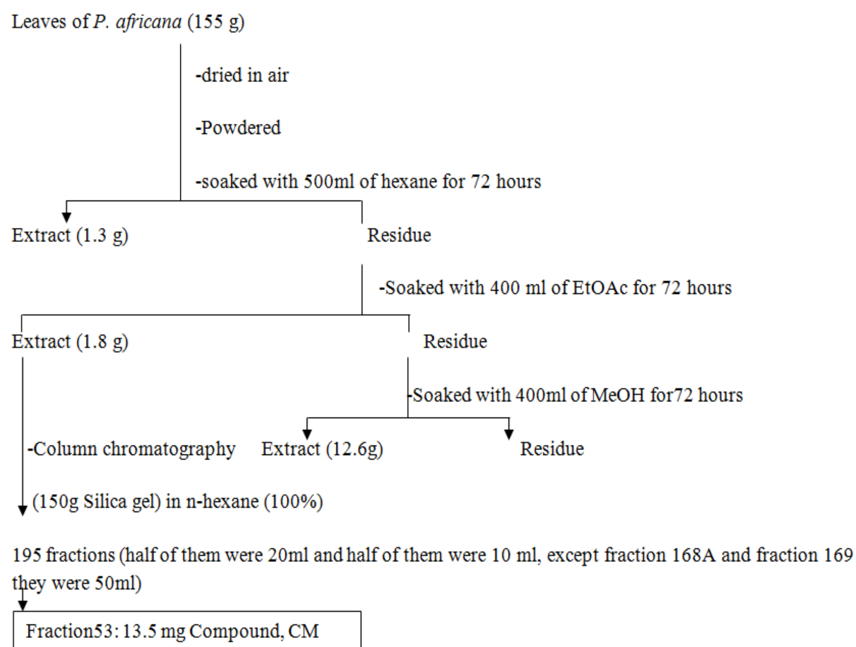


Fig. 2. TLC for Ethyl acetate extract (n-hexane: Ethyl acetate (7:3)) of *P. africana* leaves

Rotavapor. Finally, the solvent free residue was soaked with 400 ml of methanol, and then it was filtrated by using Whatman no.1 filter paper and concentrated under reduced pressure using the Rotavapor and its extract was afforded many spots on TLC. Compound on TLC was detected after spraying 1% vanillin sulphuric acid and after heating for a few minutes. There was no visible spot for n-hexane extract, but ethyl acetate extracts showed three colored spots by using solvent system of n-hexane: ethyl acetate (7:3) and crude extract was dissolved in itself with equivalent amount of silica gel, dried using Rotavapor and applied to a silica gel (150 g) column chromatography which was packed with n-hexane (100%).

The scheme of extraction is shown below:



Scheme 1. Method used to extract and isolate of leaves of *P. africana*

Table 1. Column chromatography of *P. africana* leaves extract of EtOAc by using solvent systems: n-hexane, ethyl acetate and methanol

S. no.	Solvent systems	Solvent ratio	Collected fractions
1	n-hexane: ethyl acetate	9.5:0.5	1-6
2	n-hexane: ethyl acetate	9:1	7-10
3	n-hexane: ethyl acetate	8:2	11-19
4	n-hexane: ethyl acetate	7:3	20-24
5	n-hexane: ethyl acetate	6:4	25-41
6	n-hexane: ethyl acetate	5:5	42-52
7	n-hexane: ethyl acetate	4:6	53-65
8	n-hexane: ethyl acetate	3:7	66-85
9	n-hexane: ethyl acetate	2:8	86-98
10	n-hexane: ethyl acetate	1:9	99-131
11	ethyl acetate	100%	132-150
12	ethyl acetate: methanol	9:1	151-158
13	ethyl acetate: methanol	8:2	159-163
14	ethyl acetate: methanol	7:3	164-168
15	ethyl acetate: methanol	6:4	168A-169
16	ethyl acetate: methanol	5:5	170-177
17	ethyl acetate: methanol	3:7	178-182
18	ethyl acetate: methanol	1:9	183-187
19	methanol	100%	188-194

Totally 195 fractions were collected. Out of 195 fractions which were collected using the solvent systems increased polarity, only those fractions from 50-80 showed the characteristic colored spots on TLC up on spraying 1% vanillin sulphuric acid and after heating for a few minutes. The remaining fractions did not show the characteristic colored spots on TLC up on spraying 1% vanillin sulphuric acid and after heating for a few minutes. Among fractions 50-80, fraction 53 showed single spot on TLC using the solvent system n-hexane: Ethyl acetate (7:3) upon spraying 1% vanillin sulphuric acid and after heating for a few minutes. Finally, the dried sample of this fraction was afforded 13.5 mg of the compound, CM.

2.4 Phytochemical Screening of Leaves Extract of *P. africana*

The phytochemical analysis of chemical constituents was done by using the following procedures.

1. **Tannins:** About 0.1 g of the extract put in a test tube and 20 ml of distilled water was added and heated to boiling. The mixture was then filtered and 0.1% of $FeCl_3$ was added to the filtrate and observations made. A brownish green colour or blue-black coloration indicated the presence of tannins.
2. **Saponins:** About 0.1 g of the extract was mixed with 5 ml of water and vigorously shaken. The formation of stable form indicated the presence of saponins.
3. **Flavonoids:** About 0.1 g of the extract was added in to a test tube. To the test tube 5 ml of dilute ammonia and 2 ml of concentrated sulphuric acid was added and heated for about 2 minutes. The appearance of a yellow colour indicated the presence of flavonoids.
4. **Terpenoids:** About 0.1 g of the extract was taken in a clean test tube; 2 ml of chloroform was added and vigorously shaken, then evaporated to dryness. To this, 2 ml of concentrated sulphuric acid was added and heated for about 2 minutes. A greyish colour indicated the presence of terpenoids.
5. **Glycosides:** About 0.1 g of the extract was mixed with 2 ml of chloroform and 2 ml of concentrated sulphuric acid was carefully added and shaken gently, then the observations were made. A red brown colour indicate the presence of steroidal ring (glycone portion of glycoside).
6. **Alkaloids:** About 0.1 g of the extract was mixed with 1% of HCl in a test tube. The test tube was then heated gently and filtered. To the filtrate a few drops of Wagner's reagents were added by the side of the test tube. A resulting precipitate confirmed the presence of alkaloids.
7. **Steroids:** About 0.1 g of the extract was put in a test tube and 10 ml of chloroform added and filtered. Then 2 ml of the filtrate was mixed with 2 ml of a mixture of acetic acid and concentrated sulphuric acid. Bluish green ring indicated the presence of steroids.
8. **Phenols**: About 0.1 g of the extract was put in a test tube and treated with a few drops of 2% of $FeCl_3$; blue green or black coloration indicated the presence of phenols.

3. RESULTS AND DISCUSSION

The air dried and powdered leaves of the *P. africana* (155 g) were extracted with solvents of n-hexane, ethyl acetate, and methanol and their yields 1.3 g, 1.8 g, and 12.6 g, respectively. These extracts when developed on TLC both the n-hexane and ethyl acetate extracts have shown three colored spots, but methanol extract was afforded many spots on TLC. The yellow organic extract of ethyl acetate (1.8 g) was subjected to column chromatography on silica gel and 195 fractions were collected.

Table 1. Results of phytochemical screening leaves extract of *P. africana*

Phytochemical constituents	Leaf extracts	
	Ethyl acetate	**Methanol**
Tannins	+	+
Saponins	+	+
Flavonoids	+	+
Terpenoids	+	+
Glycosides	–	+
Alkaloids	+	+
Steroids	+	+
Phenols	+	+

NB :(+) and (-) indicate the presence and absence of Phytochemical Constituents respectively

3.1 Characterization of Compound, CM

The compound, CM was obtained as a white solid that showed a characteristic color change to violet on TLC plate upon spraying 1% vanillin sulphuric acid and after heating for a few minutes.

It has retention factor, RF value 0.45 using hexane: ethyl acetate (7:3) as solvent system. In the IR spectrum of the compound, CM the absorption band at 3435 cm^{-1} showed the O-H stretching that indicated the presence of a hydroxyl group. The strong absorption band at 2925.5 cm^{-1} showed the presence of the C-H stretching of the olefinic group. The weak absorption band at 2856.5 cm^{-1} showed the presence of the C-H stretching for sp^3 groups. The strong absorption band at 1689 cm^{-1} showed the presence of the olefinic C=C stretching. The absorption band at 1257 cm^{-1} showed the presence of the C-O bond stretching.

The ^{1}H NMR spectrum showed multiplet peaks at δ 1.23, integrating for one proton, corresponded to the methine proton groups and assigned to C-1. Triplet peaks at δ 1.04, integrating for two protons, which were corresponded to the methylene proton groups and assigned to C-2. The triplet peaks at δ 3.4, integrating for one proton, corresponded to the methine proton group that assigned at C-3. The quartet peaks at δ 1.27 which integrating for one proton corresponded to the methine proton group and attached to C-5. Triplet peaks at δ 1.70, integrating for two protons corresponded to the methylene proton, which were assigned to C-6. Triplet peaks at δ 5.27, integrating for one proton, corresponded to the methine proton and assigned to C-7. Quartet peaks at δ 1.21, integrating for one proton corresponded to the methine proton which was attached to C-9. Triplet peaks at δ 1.80 which integrating for one proton corresponded to the methine proton and attached to C-10. Doublet peaks at δ 3.3 which integrating for four protons corresponded to the methylene proton and attached to C-11 and C-12. Singlet peak at δ 1.11 which integrating for six protons corresponded to the methyl protons and attached to C-13 and C-14.Singlet peak at δ 0.89 which integrating for three protons corresponded to the methyl protons and attached to C-15.

The ^{13}C NMR and DEPT spectrum of compound, CM showed well resolved resonance of 15C atoms of which 3, 4, 6, and 2 of them were methyl, methylene, methine, and quaternary carbon groups, respectively.

Finally, from the all above data, namely IR spectral data, ^{13}C NMR, DEPT, and ^{1}H NMR spectral data the proposed structure of compound, CM would be shown below:

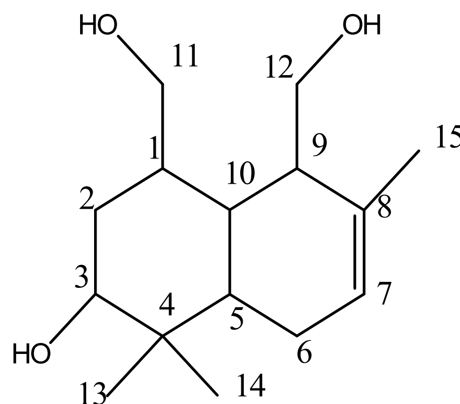


Fig. 3. The proposed structure of the compound, CM (1,2,3,4,4a,5,8,8a-octahydro-4,5-bis(hydroxymethyl)-1,1,6-trimethylnaphthalen-2-ol)

Table 3. IR spectral peak values and functional groups obtained from the leaves extract of *P. africana*

Extract prepared in	Peak values in cm^{-1}	Functional groups
Ethyl acetate	3435	-OH (hydroxyl group)
	2925.5	Sp^2 C-H stretching
	2856.5	Sp^3 C-H stretching
	1689	Olefinic group of C=C stretching
	1457.5	Methylene group bending
	1385	Methyl group bending
	1257	C-O bond stretching

Table 4. ^{1}H NMR spectral data of compound, CM

S. no.	Peaks (δ)	Peak multiplicities	Proton no.	Assigned carbon	Remark
1	1.23	Multiplet	One	C-1	Methine
2	1.04	Triplet	Two	C-2	Methylene
3	3.4	Triplet	One	C-3	Methine
4	1.27	Quartet	One	C-5	Methine
5	1.70	Triplet	Two	C-6	Methylene
6	5.27	Triplet	One	C-7	Methine
7	1.21	Quartet	One	C-9	Methine
8	1.80	Triplet	One	C-10	Methine
9	3.3	Doublet	four	C-11 and C-12	Methylene
10	1.11	Singlet	Six	C-13 and C-14	Methyl
11	0.89	Singlet	Three	C-15	Methyl

NB: CM is the "Code" given for the newly isolated compound

Table 5. ^{13}C NMR and DEPT (400 MHz, $CDCl_3$) spectral data of the compound, CM

Carbon no.	^{13}C NMR (in ppm)	DEPT (in ppm)	Remark
1	52.66	52.65	CH
2	38.61	38.62	CH_2
3	77.00	77.00	CH
4	37.00	-	C (Quaternary carbon)
5	47.55	47.55	CH
6	32.96	32.96	CH_2
7	125.87	125.87	CH
8	137.9	-	C (Quaternary carbon)
9	55.22	55.22	CH
10	39.05	39.06	CH
11	79.08	79.08	CH_2
12	77.21	77.22	CH_2
13	17.00	17.00	CH_3
14	17.00	17.00	CH_3
15	21.2	21.7	CH_3

4. CONCLUSIONS AND RECOMMENDATIONS

P. africana is a commercial by its stem bark which is most popular to treat benign prostate hyperplasia (BHP). In the this study, leaves of *p.africana* were showed the presence of phytochemical constituents such as alkaloids, flavonoids, terpenoids, saponins, tannins, steroids, glycosides, and phenols of methanol extracts of it and also confirms that the absence of glycosides in the ethyl acetate extracts of *P. africana* leaves. The leaves of the *P. africana* were extracted with solvents of n-hexane, ethyl acetate, and methanol and their yields 1.3 g, 1.8 g, and 12.6 g, respectively. The yellow organic extract of ethyl acetate (1.8 g) was subjected to column chromatography on silica gel and numbers of fractions were collected.

That is, from the IR spectrum, the absorption band at 3435 cm^{-1} shows the O-H stretching that confirms the presence of a hydroxyl group. Also the strong absorption band at 2925.5 cm^{-1} and 1689 cm^{-1} shows the presence of the C-H stretching and C=C stretching of the olefinic group respectively.

Finally, from this study, the compound CM was elucidated and characterised by incorporating all spectroscopic techniques such as IR spectral data, ^{13}C NMR, DEPT and ^{1}H NMR spectral data obtained.

Despite the traditional use of this plant for the treatments of various ailments, in many parts of the world there is no report on phytochemical analysis on the leaf of *P. africana*. This is confronted problem to compare and contrasts my work with the relative of other work. Thus, this study may serve as a baseline for researchers who are inspired and interested to conduct such type of research in the future.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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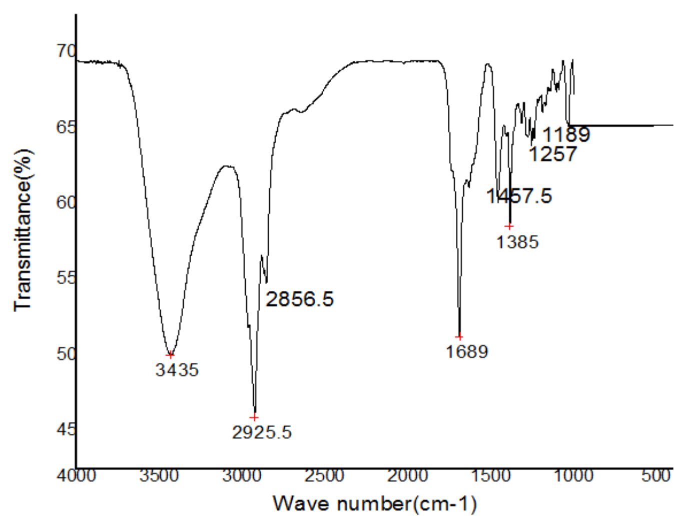
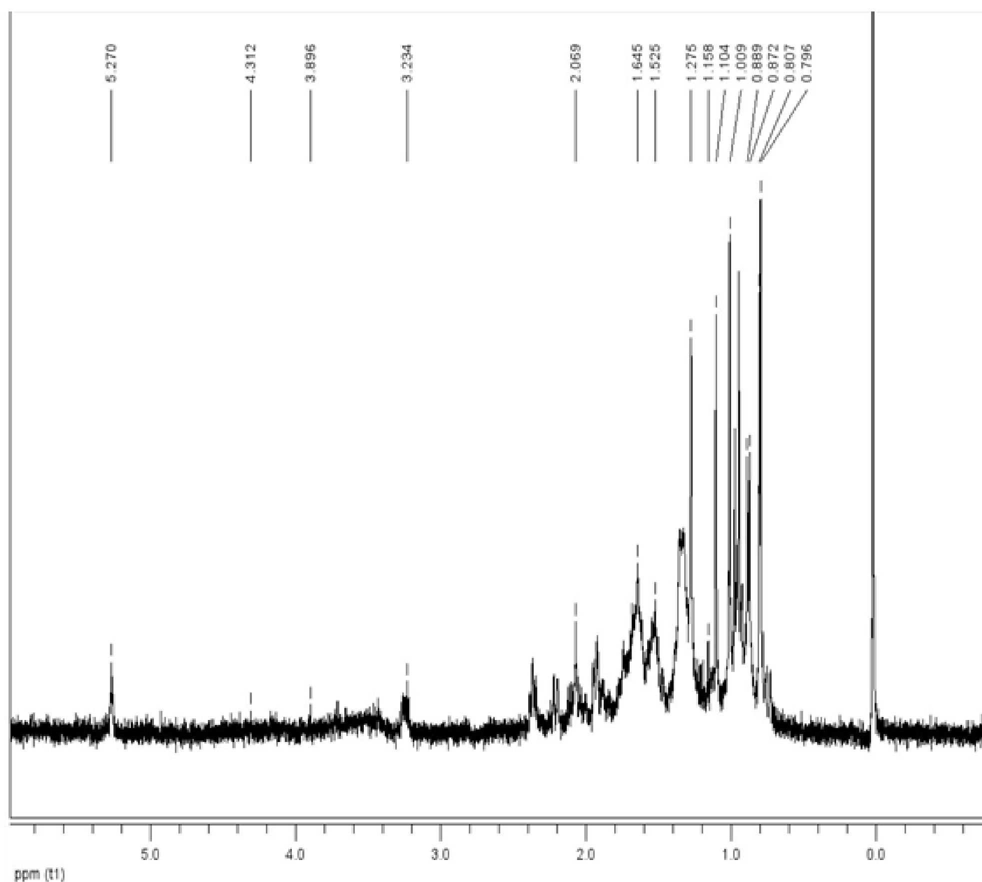
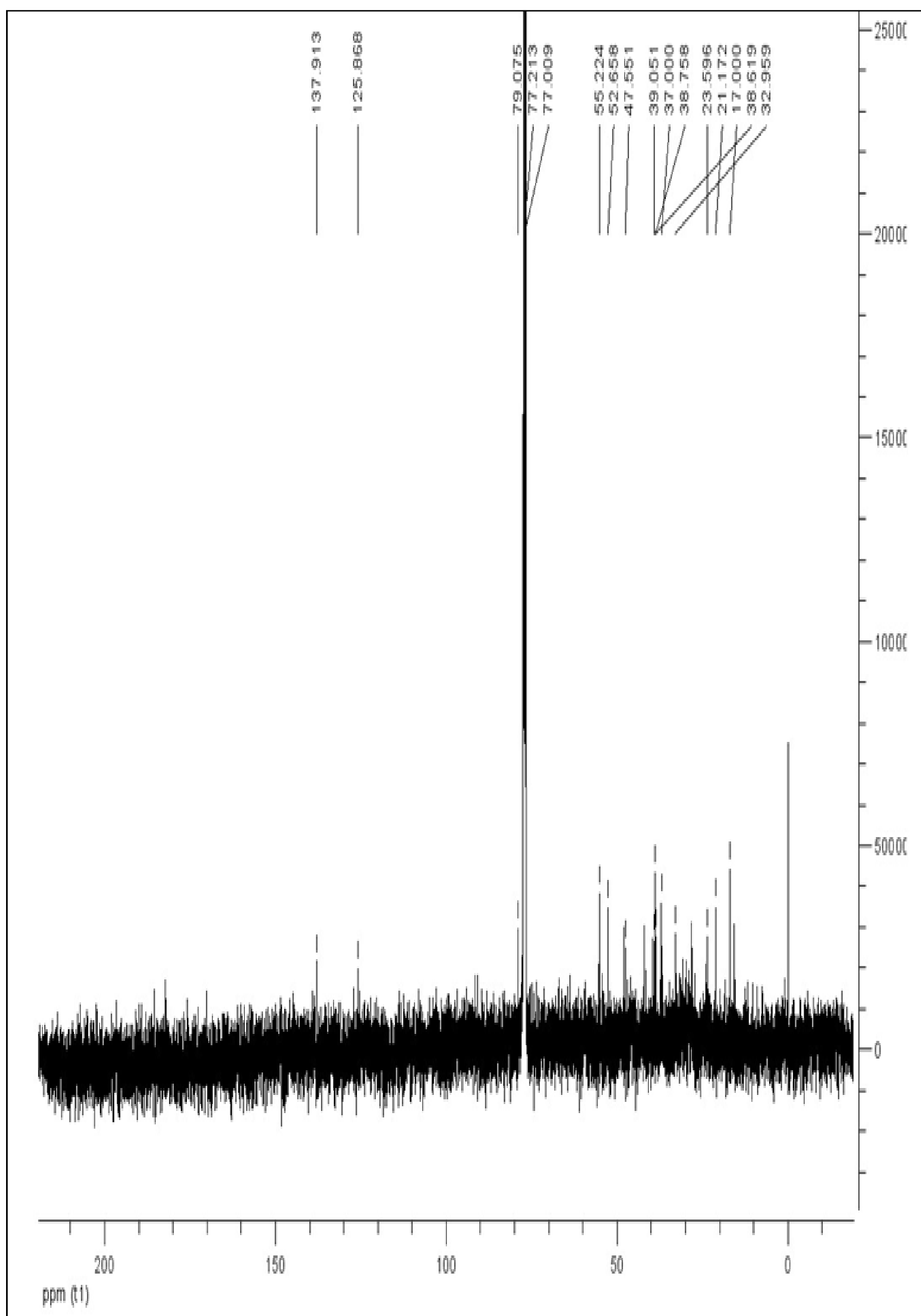
APPENDIXES



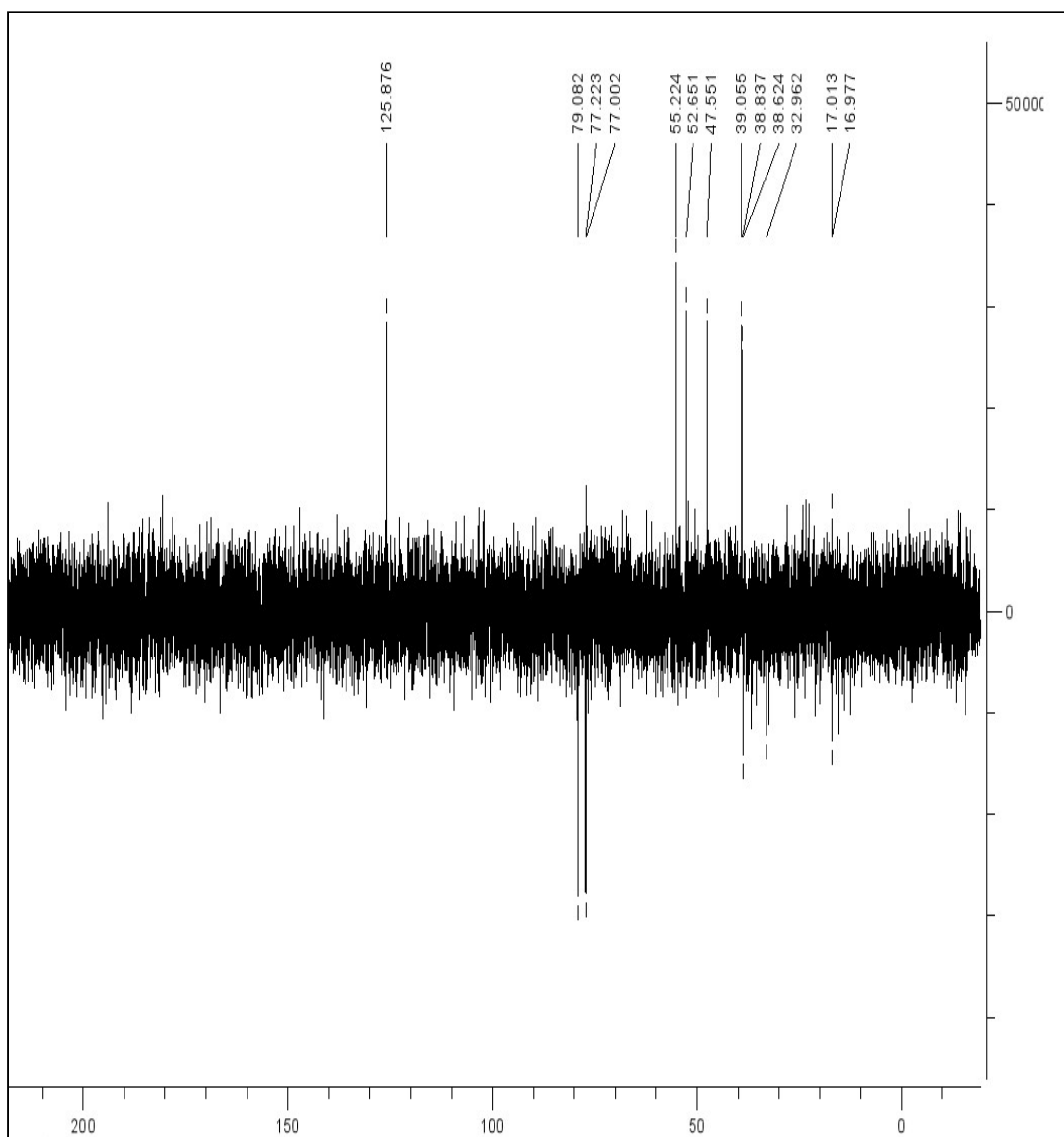
Appendix 1. IR spectrum of CM in KBr



Appendix 2. ^{1}H NMR Spectrum of CM in $CDCl_3$



Appendix 3. ^{13}C NMR spectrum of CM in $CDCl_3$



Appendix 4. DEPT spectrum of CM in $CDCl_3$

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