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IN-VITRO ASSESSMENT, ISOLATION AND SPECTRAL ANALYSIS OF THE PETROLEUM ETHER EXTRACT OF PUMPKIN SEED

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Keywords:

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ABSTRACT: *Cucurbita Maxima*, commonly known as pumpkin, which belongs to the family Cucurbitaceae, have numerous bio-active compounds such as polyphenols, dietary fibers, carotenoids, and minerals. This seed possesses anti-inflammatory and anti-oxidant properties and can be used in the neuro regenerative effects. In the present paper, an attempt has been made to reveal the phytochemical screening, anti-oxidant, anti-inflammatory activity and GC-MS spectral analysis of the isolated fatty acids compound of the plant *Cucurbita maxima*. From the anti-oxidant and anti-inflammatory activity of the petroleum ether extract showed good activity and proceeded for isolation; six fatty acid compounds were isolated from the seed *Cucurbita maxima* and analyzed using GC-MS and NMR. From the analysis, 10 compounds were identified by comparing their retention time and peak area with that of literature and interpretation of MS. The majority of phytoconstituents were 9-octadecenoic acid (Z), Oleic acid, 2-oxo-Cycloheptanevaleric acid and 14-Methyl-13-oxa-15-aza-dispirob [5.0.5.3] pentadec-14-ene. Hence this study helps in the screening of many bioactive components for the treatment of many diseases.

INTRODUCTION: As the synthetic drugs possess many toxic activities, much research attention is needed towards natural sources to fight against many diseases [1]. Medicinal plants have been identified for many years and are considered as a potential source for the pharmaceutical agent. The beneficial effects for plant material are due to the presence of their secondary metabolites [2]. In traditional medicine, the plant has a great potential for producing new drugs to treat Chronic and infectious diseases.

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The phytomedicines present in the plant called secondary metabolites more important in the treatment of various diseases [3]. So the use of plants is attractive and more essential for their anti-inflammatory properties but also their capacity to avoid cellular damage induced by free radicals [4]. Pumpkin seed is a horticultural crop that has numerous cultivated varieties. Pumpkin seed possesses different nutrients and phytochemicals such as carbohydrates, organic acids, vitamins, polyphenols, dietary fibers, and carotenoids, *etc*., which plays an important role in nutritive and pharmaceuticals [5].

The living organism produces its energy through the oxidation process. If membrane lipid oxidizes free radicals produced as intermediates during the regular pathway of aerobic metabolisms such as reactive oxygen species (ROS) and reactive

nitrogen species (RNS) as a consequence, many cellular components like proteins, lipids, and DNA get damaged. The free radical produces cell damage that leads to aging, cardiovascular disease, cancer, and brain damage [6]. Free radicals are the major intermediaries that enhance the inflammatory process, and as a result, their neutralization by radical scavengers can attenuate inflammation [7]. The plants can decrease the production of free radicals and minimize the disease caused by oxidative stress and inflammation [8].

Cucurbita maxima are a large climbing herb, annual or perennial. Its aerial part consists of a flexible succulent stem with trifoliate leaves [9]. *Cucurbita maxima* seeds are coupled with a range of biological activities, including antioxidant effects, and their effective radicals scavengers were found due to the fatty acid groups, namely Oleic and linoleic acids [10]. It is used as a vegetable and also in the traditional system as antidiabetic, antitumor, antihypertensive, anti-inflammatory immuno-modulatory and antibacterial [11]. The plant has been used traditionally as medicine in many countries such as China, India, Yugoslavia, Brazil, and America [12]. Based on conventional knowledge of the medicinal system, the present study was carried out to estimate the total phenol and flavonoid content of different solvents. *In-vitro* methods of assessment were used to determine the anti-oxidant and anti-inflammatory activity of the extract of the plant *Cucurbita maxima.* The petroleum ether extracts were subjected to isolation and analyzed for the GC-MS spectral studies. This work will accelerate in identifying the therapeutic importance of the plant.

MATERIALS AND METHODS:

Collection and Authentication of the Plant: The seeds of *Cucurbita maxima* were collected from the botanical garden of Acharya N. G. Ranga Agricultural University, Hyderabad, Telangana. The plant was identified and authenticated with specimen no: PLS - 2986.

Extraction Procedure: The selected plant part was shade dried and powdered. The 1 kg each of the coarse powder of the plant on dry weight basis was subjected for cold maceration using petroleum ether for 72 h. The extract was filtered, and the filtrate was concentrated under reduced pressure in a vacuum at 30 °C using the rotary evaporator. The concentrated extract was further dried in a desiccator to obtain the dry extract. The percentage yield of the dry extract was calculated and used for the qualitative phytochemical analysis. The dried extract was subjected to *in-vitro* anti-oxidant and anti-inflammatory studies. After *in-vitro* studies, the extract was subjected to isolation.

Separation of Fatty Acid Methyl Ester (FAME): The petroleum ether extract was refluxed for 4 h with 200 ml of methanol and acetyl chloride (95:5). After dilution with water, the resultant mixture was extracted with n-hexane containing 0.01% butylated hydroxyl toluene (3 × 100 ml). The combined organic layer was concentrated to obtain methyl ester of fatty acid, which was a mixture of saturated and unsaturated methyl esters of fatty acids. The FAME obtained was then subjected to FTIR analysis.

Urea Complexation [13]: Fatty acid mixtures are generally separated into fractions rich in saturated fatty acids and unsaturated acids by the urea complexation method. The FAME (6 g) obtained was treated with methanol and urea (1 g) and gently heated to 60 °C with stirring till a homo-genous mixture was formed. The urea-FAME adduct was subjected to crystallization at room temperature. The crystals, removed by filtration, were urea complexed fraction (UCF) and the remaining solution was the non-urea complexed fraction (NUCF). The filtrate was then diluted with 30 ml of n- hexane with stirring, and the hexane fraction was separated for the extraction of unsaturated fatty acids. The hexane fraction was evaporated to get a waxy white colored crystal and named as compound I. The compound 1 isolated was subjected to NMR & Mass spectral analysis.

***In-vitro* Anti-oxidant Activity:**

Hydrogen Peroxide Radical Scavenging Assay:

Principle: This assay is based on the *in-vivo* generation of hydrogen peroxide by several oxidizing enzymes, which is scavenged, either directly or indirectly, *via* the hydroxyl radical (OH), the reduction product. Here, the incubation of a scavenger in the presence of hydrogen peroxide caused the loss or decay of hydrogen peroxide, which is measured at 230 nm[14].

Procedure: The H_2O_2 scavenging power of the extracts was evaluated as per the standard method. 40 mM Hydrogen peroxide solution was prepared in PBS (pH 7.4). The extracts (1000 - 18.125 µg/ml), constituted in distilled water, were added to the solution of hydrogen peroxide (0.6 ml). The blank solution comprised of PBS without H_2O_2, and the absorbance at 230 nm was recorded. Taking ascorbic acid as the standard, the results were tabulated in the percentage of H_2O_2 scavenging. The study was performed in triplicate.

Superoxide Radical Scavenging Assay:

Principle: The extent of superoxide radical scavenging was determined by the alkaline DMSO method. The capability of the superoxide anion (generated by adding sodium hydroxide to DMSO) to reduce the nitro blue tetrazolium (NBT) to formazan dye is analyzed, which is measured at 560 nm [15].

Procedure: To 0.3 ml of varying concentration of the extracts (1000 - 18.125 µg/ml) obtained by serial dilution technique, 1 ml alkaline DMSO and 20 mM NBT (0.2 ml, prepared using 50 mg in 10 ml PBS, pH 7.4) was added. The absorbance was recorded at 560 nm.

Lipid Peroxidation Assay:

Principle: ROS, such as hydroxyl radicals extract a proton from lipids to form a conjugated lipid radical, thereby initiating the process of lipid peroxidation. The conjugated lipid radical reacts rapidly with polar oxygen moiety to form hydroperoxy lipid radicals, which are sources for the formation of free radicals. These sequentially attack polyunsaturated fatty acids present in the cell membrane leading to a cascade of chemical reactions. The lipid peroxidation complexes thus formed may oxidize biomolecules such as DNA, proteins, and lipids, thereby resulting in cellular damage [16].

Procedure: Lipid peroxidation inhibitory activity of the extracts was analyzed as per the standard protocol. A liposome mixture was prepared using egg lecithin (3 mg/ml, phosphate buffer, pH 7.4), followed by sonication. The test samples constituted of the liposome mixture (1 ml) with varying concentrations (1000 - 18.125 µg/ml) of the extracts. The liposome mixture without the test sample was assigned as control. Lipid peroxidation was commenced when ferric chloride solution (10 µl) and 200 mM L-ascorbic acid (10 µl) was added. After incubation at 37 °C for 1 h, the process was quenched by supplementing a solution of 0.25 N HCl with 0.375% thiobarbituric acid (10 µl) and 15% trichloroacetic acid. The resultant samples were then boiled for 15 min, followed by cooling. After centrifuging, the supernatant was collected for recording the absorbance at 532 nm.

***In-vitro* Anti-Inflammatory Activity:**

HRBC Membrane Stabilization Method:

Principle: The anti-Inflammatory activity was performed by the Human Red Blood Cell (HRBC) membrane stabilization method. As the HRBC membrane is analogous to the components of lysosomal membrane, the capability to prevent the hypotonicity-induced lysis of HRBC membrane is taken as a measure of membrane stabilizing effect of the extracts. Diclofenac is taken as standard [17].

Procedure: Equal volume of fresh human blood and sterilized Alsever solution was constituted. Alsever solution was prepared using 0.05% citric acid, 0.42% sodium chloride, 0.8% sodium citrate and 2% dextrose in water. After centrifuging the sample at 3000 rpm for 10 min, the packed cells were washed thrice using 0.85% isosaline (pH 7.2). The volume of the blood was noted for preparing a 10% v/v suspension with isosaline (RBC suspension).The test sample consisted of extracts of varied concentrations (18.25 - 1000 µg/ml) with RBC suspension (0.5 ml) and a hypotonic solution (5 ml, 50 mM NaCl) in 10 mM sodium PBS (pH 7.4). RBC suspension (0.5 ml) with hypotonic solution alone was used as control. After incubation for 10 min at room temperature, the resultant solutions were centrifuged at 3000 rpm. The collected supernatant was used to record the absorbance (560 nm). The percentage inhibition of membrane stabilization or hemolysis was calculated by

Percentage inhibition of hemolysis = Optical density of test sample / Optical density of control × 100

Protein Denaturation:

Procedure: The test samples (0.5 ml) comprised of egg albumin (5% aqueous solution, 0.45 ml) and extracts (0.05 ml) at varied concentrations (1000 - 18.125 µg/ml). After incubation for 30 min at 37

^{0}C and subsequent cooling, PBS (pH 6.3, 2.5 ml) was added to each sample. The absorbance was recorded at 660 nm. Distilled water (0.05 ml) without bovine serum albumin was used as control. Using Ibuprofen as standard, the percentage inhibition of protein denaturation was determined by;

Percentage inhibition of protein denaturation = At / Ac × 100

Where, At = absorbance of a test sample, Ac = absorbance of control. The concentration required for 50% inhibition (IC_{50}) was tabulated from the plot of percentage inhibition with respect to control versus treatment concentration.

GC-MS Analysis [18]: The phytoconstituents present in the petroleum ether extract analyzed on Shimadzu QP- 2010 GC-MS. The following conditions were used: ZB-5MS column Phenomenex Zebron (30 m × 0.25 mm × 0.25 mm); Helium (99.999%) carrier gas at a constant flow of 1ml/min; 1µl injection; injector split ratio of 1:40; injector temperature 240 °C; electron impact mode at 70eV; ion source temperature 280 °C.

The oven temperature was automatic from 100 °C (isothermal for 5 min), with an increase of 10 ºC/min to 250 °C (isothermal for 5 min) and 10 °C/min to 280 °C (isothermal for 15 min). The individual phytoconstituents were identified by comparing their MS with the spectra of known compounds stored in the spectral database, NBS; WILEY and NEST attached to the GC-MS instrument and reported.

NMR Analysis: Proton NMR spectra were recorded on a Varian 400 spectrometer. Proton chemical shifts are reported in ppm (d) relative to internal tetramethylsilane (TMS, d 0.0 ppm), or with the solvent reference relative to TMS employed as an internal standard (CDCl3, d 7.26 ppm). Data are reported as follows: chemical shift (multiplicity [singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m)], coupling constants [Hz], integration). Carbon NMR spectra were recorded on a Varian 400 (100 MHz) or 500 (125 MHz) spectrometers with complete proton decoupling. Carbon chemical shifts are reported in ppm (d) relative to TMS with the respective solvent resonance as the internal standard (CDCl3, d 77.0 ppm).

Statistical Analysis: The statistical data expressed as Mean ± SD were evaluated by one way analysis of variance (one way ANOVA), followed by Turkey Posts test. Values of $p < 0.001$ were considered statistically significant. Graph Pad Prism Version 5.0 was used for graphical and statistical evaluations**.**

RESULTS AND DISCUSSION: Since prehistoric time, mankind is using herbs to treat acute and chronic diseases. Natural anti-oxidants are the most important source present [6] to in this plant for the treatment of diseases like cancer, cardiovascular and neurodegenerative diseases by scavenging the free radical, which is the main reason for the pathogenesis of these diseases. Qualitative Phytochemical analysis revealed the presence of Protein, Phenolics, fixed oils and fats.

TABLE 1: PHYTOCHEMICAL SCREENING OF THE EXTRACTS OF *CUCURBITA MAXIMA* SEEDS

S. no.	Chemical Test	PE
1	Carbohydrates	-
2	Proteins	+
3	Alkaloids	-
4	Glycosides	-
5	Phenolics	+
6	Flavonoids	-
7	Fixed oils & Fats	+

Cucurbita maxima seeds would be considered as traditional medicine for treating many chronic and acute diseases of different etiology. Phenolics, Proteins, Fixed oils and Fats possess good anti-oxidant and anti-inflammatory properties [9].

***In-vitro* Anti-oxidant Activity:**

Hydrogen Peroxide Radical Scavenging Activity: The petroleum ether extract of *Cucurbita maxima* seed was capable of scavenging hydrogen peroxide in a concentration-dependent manner. The IC_{50} value of the petroleum ether extract is 17.23 µg/ml at a concentration range of 200 µg/ml, which is having a similar effect to that of ascorbic acid 40.1 µg/ml at 200 µg/ml. Scavenging of H_2O_2 by the plant extract might be recognized by phenolics. The results show that the radical scavenging activity of petroleum ether extract is significant to the standard.

Superoxide Radical Scavenging Activity: The petroleum ether extract of the plant showed maximum superoxide anion scavenging activity. The IC_{50} of the petroleum ether extract of

Cucurbita maxima was 90.10 µg/ml, which is significant to that of the standard. These show that the extract has significantly better activity.

Lipid Peroxidation Scavenging Activity: The lipid peroxidation inhibition activity of the extract was compared with standard ascorbic acid.

From the result, the petroleum ether extract of *Cucurbita maxima* seed was giving significant activity with that of the standard (26.65 µg/ml) exhibited inhibition at 250 µg/ml, respectively. The inhibitory effect of lipid peroxidation could be due to the presence of Phytoconstituents in the extract.

TABLE 2: *IN-VITRO* ANTIOXIDANT ACTIVITY OF THE EXTRACTS OF *CUCURBITA MAXIMA* SEED

S. no.	Extracts	H_2O_2 IC_{50} Values (µg/ml)	SO IC_{50} Values (µg/ml)	LPO IC_{50} Values (µg/ml)
1	Petroleum ether	17.23 ± 0.90	90.10 ± 1.17	26.65 ± 1.01
2	Ascorbic acid	22.15 ± 0.351	102.25 ± 0.73	35.23 ± 0.54

Each value is expressed as Mean ± SD, n=3. H_2O_2: Hydrogen peroxide; $SO^{\cdot}$: Superoxide radical; LPO: Lipid peroxidation, AA: Ascorbic acid.

***In-vitro* Anti-Inflammatory Activity:**

Protein Denaturation Method: The finding has clearly shown that all the extracts of the three plants *Cucurbita maxima* seeds, was shown a concentration-dependent inhibition of protein (albumin) denaturation. The IC_{50} value of the petroleum ether extract of CM found to be 82.5 ± 0.763 µg/ml was found significant to that of standard.

HRBC Membrane Stabilization Method: The results have shown that the petroleum ether extract of *Cucurbita maxima* prevented hypotonicity induced HRBC membrane lysis to the extent of 187.5 ± 1.040 µg/ml at the concentration of 500 µg/ml, which was significant to that of standard 188.75 ± 1.289. The results are tabulated.

TABLE 3: PROTEIN DENATURATION AND MEM-BRANE STABILIZATION EFFECT OF *CUCURBITA MAXIMA*

S. no.	Extracts	Protein Denaturation IC_{50} Values	Membrane Stabilization IC_{50} Value
1	Petroleum ether	82.5 ± 0.763	187.5 ± 1.040

Values are Mean ± SD, ***P<0.0001: compared to standard, IBU: Ibuprofen

GC-MS Spectrum for the Petroleum Ether Extract:

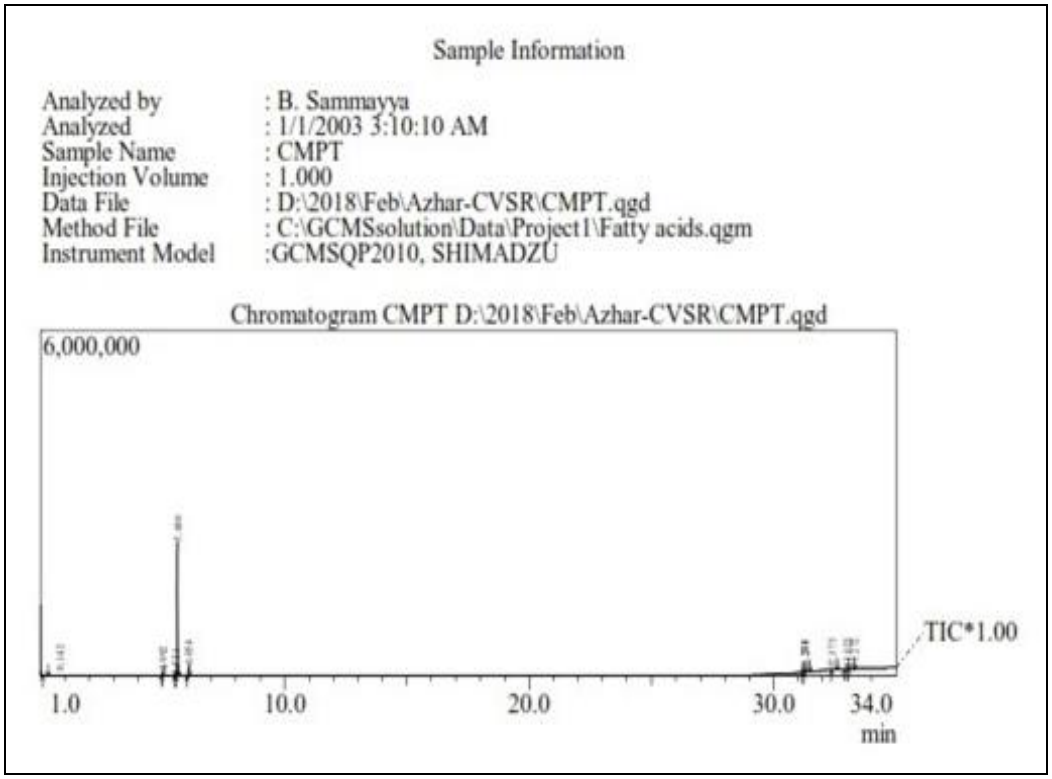

FIG. 1: GC-MS RESULTS OF FAME OF PETROLEUM ETHER EXTRACT OF *CUCURBITA MAXIMA* SEEDS

TABLE 4: RESULTS OF GC-MS

Peak no.	R. Time	Area	Area %	Height	Base m/z	Name
1	0.143	728116	2.33	78454	55.05	9-Octadecenoic acid (Z), Oleic acid.
2	4.992	97621	1.71	71635	41.05	β-Myrcene, 2-methyl-6-methylene -2,7-octadiene
3	5.531	36994	0.65	21894	119.10	p-Cymene, 1-Methyl-4-isopropylbenzene
4	5.606	4947721	86.57	2341045	68.05	L-limonene
5	6.054	297639	5.21	181617	93.10	gamma-Terpinene
6	31.244	27648	0.48	6157	109.10	3,3-Difluoro-4,8-dimethyl-1,7-nonadien-4-ol
7	31.275	63890	1.12	5574	109.15	3,3,3-Dichloro-1-propene, 3,3,3-Trichloro-1-propene
8	32.375	46596	0.82	3523	123.15	14-Methyl-13-oxa-15-aza-dispiro[5.0.5.3]pentadec-14-ene
9	33.023	29951	0.52	6337	99.10	9-Decen-2-ol
10	33.272	33911	0.59	3544	133.05	2-oxo-Cycloheptanevaleric acid

NMR Spectral Analysis: The presence of four highly deshielded proton signals δ_H6.27 (dd, *J* = 14.9, 11.1 Hz, 1H), 5.91 (q, *J* = 10.1 Hz, 1H), 5.63 (q, *J* = 11.2 Hz, 1H) and 5.26 (dd, *J* = 18.4, 7.6 Hz, 1H) are attributed to the presence of four ethylenic protons.

The singlet signal at δ_H11.97 (s, 1H) and signal at δ_H2.51 (t, *J* = 7.8 Hz, 2H) were attributed to hydroxyl proton and methylene protons adjacent to a carboxylic acid. Signals at δ_H2.20 – 2.01 (m, 6H) reveal the presence of methylene protons adjacent to conjugated double bonds.

The remaining downfield signals at δ_H1.53 - 1.43 (m, 2H), δ_H1.38 –- 1.22 (m, 14H) and at δ_H 0.86 (t, *J* = 5.9 Hz, 3H) has indicated the presence of β methylene protons, terminal methyl protons, and other methylene protons. The C^{13}-NMR spectra revealed the presence of carbonyl carbon at δ_C 174.32. Two signals at δ_C 128.63 and 125.51, which are in the range of δ_C 110-150, are implied to four unsaturated carbon atoms. The spectral data of the compound displays structural features analogous to that of Oleic acid. Thus, it is deduced that the isolated compound is Oleic acid.

H^1NMR Spectrum:

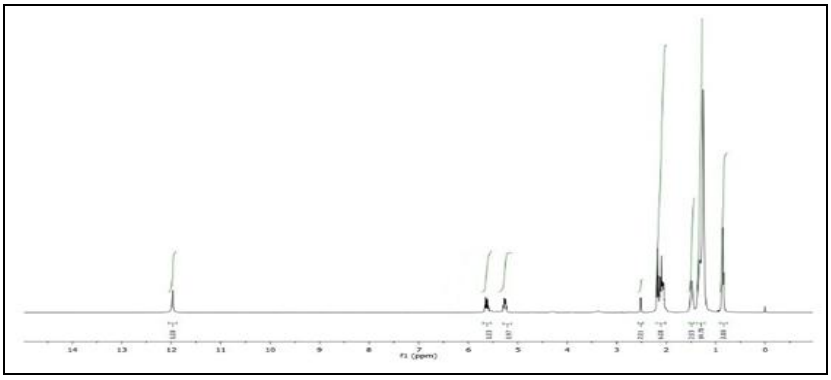

FIG. 2: H^1-NMR SPECTRUM OF FAME SEPARATION FROM PETROLEUM ETHER EXTRACT OF *CUCURBITA MAXIMA* SEEDS

C^{13}-NMR:

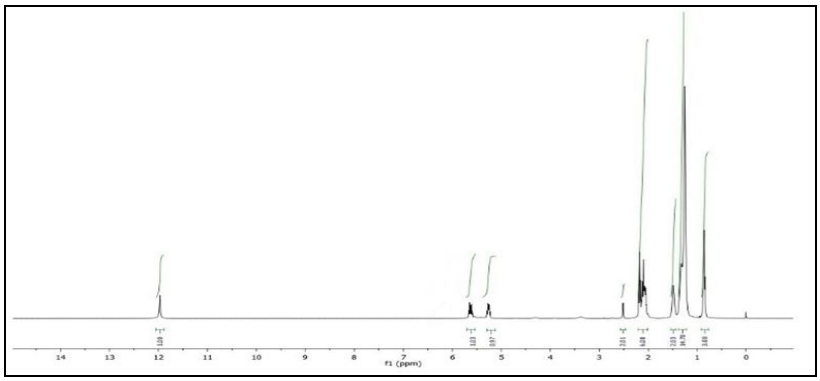

FIG. 3: C^{13} NMR SPECTRUM OF FAME SEPARATION FROM PETROLEUM ETHER EXTRACT OF *CUCURBITA MAXIMA* SEEDS

CONCLUSION: From the result of this study, the *Cucurbita maxima* seed has been revealed to contain anti-oxidant and anti-inflammatory activity which is responsible for many diseases; the existence of bioactive compounds in *Cucurbita maxima* determines the pharmaceutical importance. By isolating and identifying these phyto-constituents, novel drugs can be formulated to treat different diseases. Further studies will be required to find out the molecular level.

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CONFLICTS OF INTEREST: None

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