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Isolation , *In-Vitro* Evaluation, Spectral Analysis And Molecular Docking Studies of The Alcoholic Extract Of Dietry Crop *Cajanus Cajan*

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ABSTRACT

Cajanus Cajan (Fabaceae) is a multipurpose plant that is mostly used for human consumption. Various portions from the plants are being utilized as sedatives and are used in the treatment of cough, hepatitis, and diabetes in traditional medicine. In this study the alcoholic extracts of the leaves of *Cajanus cajan* were subjected to phytochemical analysis. Cetyl palmitate and betulinic acids were separated and characterized from the leaves of *Cajanus Cajan*. The characterization of phytoconstituents was done by FTIR, mass spectra, ^{1}H and ^{13}C NMR

analysis. The cytotoxicity of the extracted bioactive phytoconstituents was determined to be over 500 μg/ml using the MTT assay methodology in HepG2 and Huh7 cell lines. *In silico* docking and an *in vitro* investigation of betulinic acid against the target enzyme paraoxonase revealed that it has a higher affinity. As both isolated phytoconstituents having increased secretion for paraoxonase , will be potential candidates in control of atherosclerosis**.**

Key Words : *Cajanus Cajan* , cetyl palmitate , betulinic acid, paraoxonase , atherosclerosis.

1. INTRODUCTION

Cajanus cajan (L.) Millsp, a perennial herb in the Fabaceae families, is also named as pigeon pea, red gramme. It is among the second significant dietary legume crops present in tropical climates. India produces nearly 90 % of the global crop and ranks sixth in terms of both production and area when compared to other grain legumes [1]. Pigeon pea is a versatile plant that used it to generate a high-protein dhal. Silkworms are bred from the leaves, and the pods are consumed as a vegetable. The husks and green leaves have been utilized as feed and green manure [2]. Long ago, pigeon pea leaves were used to attack worms and pain killer.

Flavonoids and stilbenes were abundant in pigeon pea plants, and these compounds are assumed to be accountable for their health effects [4,5]. Boiling leaves are used as a laxative and to mitigate the symptoms of intoxication in eastern Rajasthan. The leaf paste is used to treat oral ulcer and inflammation [6]. Pigeon pea leaves, seeds, and young stems are being used to cure gingivitis and as a tooth brush in various parts of India and Tamil Nadu [7]. Saponins, a large quantity of tannins, and small amounts of resins, reducing sugars, and terpenoids can all be found [8]. Pinostrobin, cajaninstilbene acid were discovered to have significant antioxidant activity when isolated from ethanolic extracts of leaves [9]. Isoflavanoids extracted from ethanolic leaf extract also showed antimicrobial action [10]. Several protein fractions isolated from leaves were found to have hepatoprotective properties [11], the concentration of phenolics (flavanoids and tannins) provides anthelmintic properties [12]. Isolated flavonoids include genistein and genistin taken from the extracts of the leaves, iso flavonoids, (isorhamnetin, luteolin, apigenin, quercetin) from pigeon pea roots. *C Cajan* is the frequently utilized and grown crops since it is a significant source of protein and an important feed crop. It has also been utilized for therapeutic purposes numerous parts of the world, although its identify as a medicinal plant is unknown. Although several flavonoids, isoflavonoids, tannins, and protein fractions are separated from it and their medicinal benefits are identified but still phytochemists and pharmacologists have now neglected some bioactive components and pure chemicals. As a result, we decided to extract the novel bioactive phytoconstituent from *C cajan leaves*. The current study reports the isolation of cetyl palmitate and betulinic acid taken from alcoholic extract of the leaf of *C. cajan* , the characterization by FTIR, mass spectra, ^{1}H and ^{13}C NMR analysis and *in silico* docking and *invitro* study against the target enzyme paraoxonase. Paraoxonase is an enzymes that effectively hydrolyzes the oxidised phospholipids found in low-density lipoproteins, slowing their oxidation, reducing their pro-inflammatory effects, and preventing arteries from atherosclerosis

progression. The isolated phytoconstituents cetyl palmitate and betulinic acid, increases the secretion of enzyme paraoxonase and will have a key role in preventing atherosclerosis.

2. Materials and Methods

2.1 Plant material:

Pigeon pea plants being taken entire in Telangana, India. Leaves are divided, dried in shades, finely crushed, and preserved in airtight containers in sealed plastic bags.

2.2 Extraction of sample:

The continuous hot sequential soxhlet extraction methodology was used to extract phyto-compounds separate from leaves [13]. Hexane, ethyl acetate, and methanol, with polarities of 0.1, 4.4, and 5.1, respectively, were used in the extraction. Each sample was extracted for 8–10 hours with 500 mL of hexane, ethyl acetate, and finally methanol (40–60°C) in order of increasing polarity. The extract was then filtered with such a Whatman No. 1 filter at each step and concentrated with a water wash before being evaporated.

2.3 Isolation of compound 1 from methanol extract of Cajanus cajan

2.3.1 Fatty acid methyl ester separation (FAME)

Using 200 mL of methanol and acetyl chloride, the ethanolic extracts were refluxed for 4 hours (95:5). The resulting mixture is prepared with n-hexane containing 0.01 percent butylated hydroxyl toluene after dilution with water (3 x 100 ml). Concentrating the organic layers resulted in a fatty acid methyl ester that was a mix of unsaturated and saturated fatty acid methyl esters [14].

2.3.2 Urea complexation

The urea complexation technique is used to separate fatty acid mixtures into saturated and unsaturated fatty acid fractions. FAME (6 g) was combined with methanol and urea (1 g) before being gradually heated to 60 ^{0}C and agitated until a homogeneous mixture was achieved. The crystallisation of the urea-FAME adduct took place at room temperature. Screening was used to remove the urea complexed fraction (UCF), leaving only the non-urea complexed fraction (NUCF). To retrieve unsaturated fatty acids, the filtrate was diluted with 30 ml n-hexane while stirring, and the hexane fraction was separated. Compound 1 was made through evaporating the hexane fraction, which gave rise to a waxy white crystal [15].

2.4 Isolation of compounds 2 from ethanol extract of Cajanus cajan

Column chromatography was used for separating the ethanol extract (7 g) packed columns made of silica gel (60-120 mesh) with n-hexane as the packing solvent. To obtain multiple sub fractions, the column was eluted using growing polarity solvents (n-hexane, chloroform, ethyl acetate, and methanol). The eluents being collected as 5 ml fractions after the column was eluted

with n-hexane. To determine the presence of chemicals, each fraction was concentrated and subjected to TLC examination. Table 1 lists the mobile phases that were utilized.

Table1: The ethanol extract of *Cajanus cajan* was eluted using a column chromatography method.

Fraction No.	Eluent
1-47	N-hexane (100%)
48-92	N-hexane : $CHCl_3$ (3:1)
93-132	N-hexane : $CHCl_3$(1:1)
133-163	N-hexane : $CHCl_3$ (1:3)
164-199	$CHCl_3$ (100%)
200-240	$CHCl_3$: ethyl acetate (3:1)
241-274	$CHCl_3$: ethyl acetate (1:1)
275-312	$CHCl_3$: ethyl acetate (1:3)
313-354	Ethyl acetate (100%)
355-398	Ethyl acetate : MeOH(3:1)
399-430	Ethyl acetate : MeOH (1:1)
431-474	Ethyl acetate : MeOH (1:3)
475-500	MeOH

The ethanol extract of *Cajanus cajan* was eluted using a column chromatography method. TLC analysis revealed a single spot in the chloroform: ethyl acetate (1:3) fractions. The resulting yellowish white crystals were then exposed to IR , MS, and NMR characterisation.

2.5 In silico molecular docking studies

Glide version 6.7 of Schrodinger Suite 2015-2 was used to undertake a molecular docking research for chemicals extracted from plants with the targeted enzymes using Schrodinger Maestro version 9.9. The RCSB protein data library provided the X-ray crystal structure of the enzymes involved in atherosclerosis. The Protein Preparation Wizard programme was used to prepare the proteins (3.2). The energy optimised proteins were then used to create energy grids in a cubic box utilizing the default co-crystal ligand. Using the tool ACD Chemsketch, In the ".mol" format, the sequences to ligands (the pair of isolated molecules betulinic acid and cetyl palmitate) are produced. The LigPrep, version 3.4, was used to decrease the energy of these ligands utilizing the OPLS-2005 force field. For each of the generated proteins, individual docking investigations were carried out using the Schrodinger software Glide, version 6.7, in extra precision (XP) mode.

2.6 In vitro cytotoxicity studies

2.6.1 MTT assay for evaluating mitochondrial synthesis

Succinate dehydrogenase (a mitochondrial enzyme) is being studied for its ability to convert the tetrazolium salt 3-(4, 5-dimethylthiazol-2-yl)-2,5 diphenyl terazolium bromide (MTT) to formazan, a blue-colored product. If the cells are dead, the enzyme's reduction will not occur. The amount of formazan produced depends on the number of live cells are present [16].

2.6.1.1 Procedure:

On monolayer grown cells, trypsinization was conducted, and the cell count could be maintained to 1.0105 cells/ml utilizing the DMEM medium which possess 10 % FBS. Each well of a 96-well microtitre plate was filled with a diluted cell solution (100l, roughly 10,000 cells/well).The supernatant was discarded after the development of a partial monolayer (24 hours) and the resultant monolayer was rinsed with medium. At the microtitre plate, test samples (100l) in varying concentrations (made in maintenance media) were introduced to the wells having half monolayer. Microscopical observations were recorded every 24 hours after the incubation at 37 ^{0}C for 72 hours in a 5 % CO_2 atmosphere. The solution taken for sample were withdrawn after 72 hours and replaced with 20 l of MTT (2 mg/ml) in MEM (MEM without phenol red). After moderate shaking, the plate was incubated for 3 hours at 37 °C in a 5 % CO_2 environment. 50 l of isopropanol was added after the supernatant was separated. The plates were gently shook again to dissolve the formazan that had formed. A microplate reader was used to measure the absorbance at 540 nm. The percentage progress inhibition was calculated using the equation below.

$$\text{Cell viability} = \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100$$

The dosage responses for each cell line was used to calculate the concentration of drug/test samples required to suppress cell growth by 50 %.

2.7 In vitro paraoxonase activity (PON1)

2.7.1 Maintenance of cell lines:

HepG2 and Huh7 (human hepatoma cell lines) were cultured in RPMI-1640 (Roswell Park Memorial Institute medium) supplemented with 5% Fetal Bovine Serum, 100,000 U/L penicillin, 100 g/ml pyruvate, and 100 mg/L streptomycin at 37 °C in an incubator (95 % air, 5 % CO2) [17].

2.7.2 Conditioned medium (CM) collection:

Extracts was applied to HepG2 and Huh7 cells for 24 hours at 37 degrees Celsius. After washing the medium, it was supplemented with RPMI-1640 media containing 1 g of protein/ml

HDL generated from PON1 knockout mice's serum. The obtained medium was submitted to paraoxonase activitives assessment after 5 hours.

2.7.3 Paraoxonase activity on cells:

The amount of PON1 paraoxonase activitives secreted by cells were measured in the collected media. The activity of the enzyme paraoxonase was investigated (substrate). After the substrates was introduced, the absorbance was measured at 0 and 1 minutes.

The activity of enzyme can be calculated by:

$$1U \text{ of paraoxonase activity} = 1 \ \mu\text{mol of paraoxonase hydrolyzed/min.}$$

3. Results

3.1 Spectral analysis of compound 1

A carbonyl function absorbance band at 1733.69 cm^{-1} can be found in the FT-IR spectra of isolated chemical 1, along with C-O stretching at 1183.11 cm^{-1}. Aliphatic C-H symmetric and asymmetric stretching is discovered at 2917.77 $cm^{-1}$and 2848.35 cm^{-1}, corresponding.

In mass spectroscopy of the separated molecule, At 481.86, a molecular ion peak M+1 was identified.

Signals from ^{1}H NMR at H 4.256 and 4,106 ppm , respectively, represent Methylene protons near to oxygen. The ^{1}H NMR signal at H 2.877 ppm represents methylene protons near to the carbonyl group, whereas the ^{1}H NMR signal at H 2.877 ppm represents methylene protons adjacent to the carbonyl group. The presence of terminal methyl protons is indicated by a singlet signal at H 0.8-1.0 ppm. Remaining methylene protons are represented by multiplet signals in the H 1.5 to 1.8 ppm range. The combination of DMSO and moisture (DMSO-d6) causes the signals at H 2.49 and 3.35 ppm correspondingly.

Carbonyl carbon can be found at 169.45 ppm in the ^{13}C NMR spectra. Signals in the 10-30 ppm range indicate methyl and methylene carbons, with a value of C 52.66 ppm indicating the methylene carbon close to the oxygen atom.

Figure 1 - 4 depicts the spectrum. The structural properties of compound 1's spectral data are similar to those of cetyl palmitate. As a result, the isolated chemical 1 is determined to be cetyl palmitate.

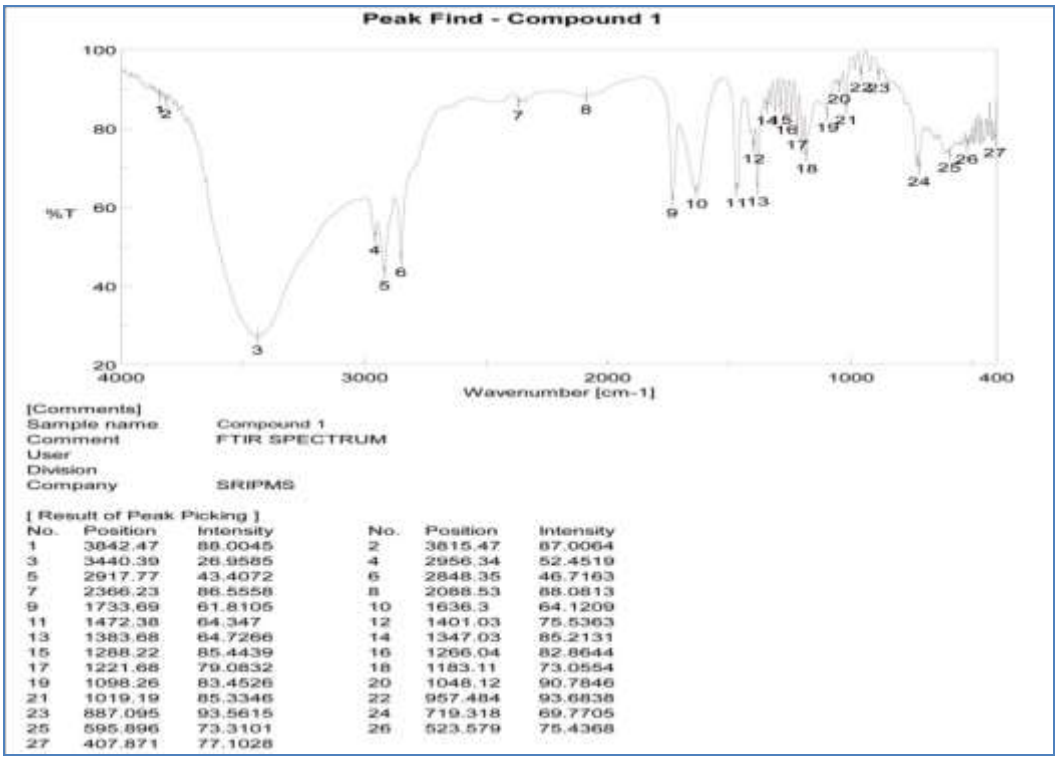

Figure 1: The isolated chemical 1's FT-IR spectrum

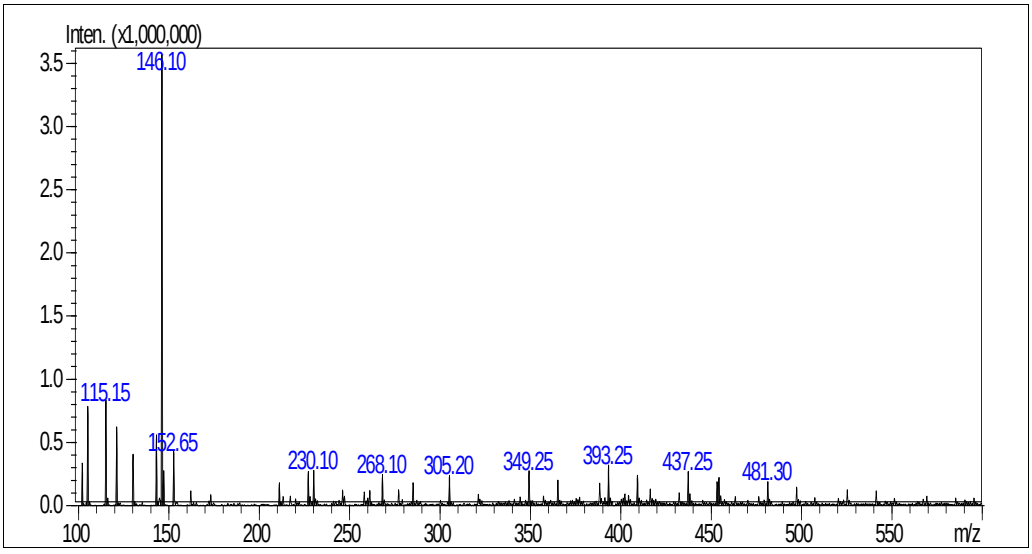

Figure 2 : The isolated compound's mass spectrum 1

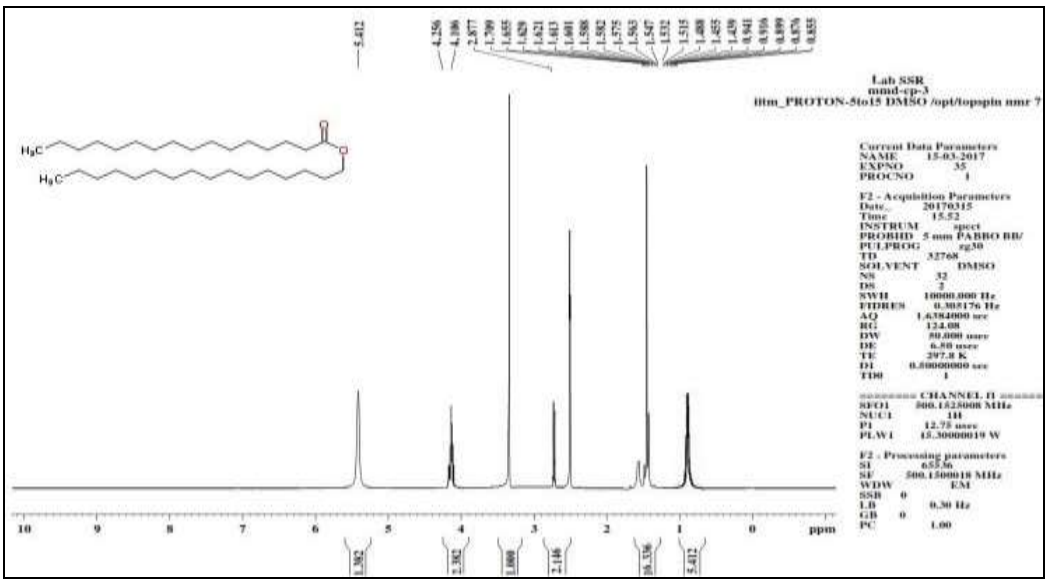

Figure3 :^{1}H NMR spectrum of the isolated compound 1

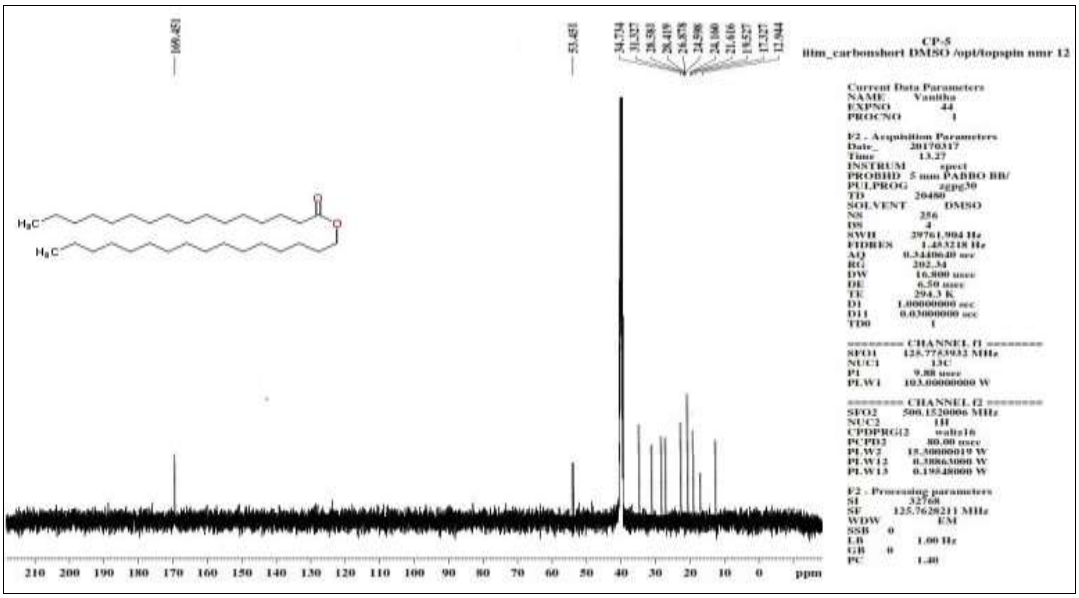

Figure 4: ^{13}C NMRspectrum of the isolated compound 1

3.2 Spectral analysis of compound 2

The presence of the –OH group can be seen in the FT-IR spectrum, The peak of the O-H stretching vibrations is 3432.67 cm^{-1}, while the peak of the vibrations of C-O stretching is 1020 cm^{-1}. The absorbance of asymmetric and symmetric C-O stretching vibrations is 1384.64 cm^{-1} and 1193 cm^{-1}, respectively. At 1639.2 cm^{-1}, carbonyl stretching vibrations were recorded.

A molecular ion peak M-1 peaks at 455.55 was discovered by mass spectroscopy of an isolated molecule.

The 5 tertiary methyl group signals at 0.98, 0.87, 0.8, 0.7, and 1.0 ppm in the ^{1}H NMR spectrum demonstrate the compound's triterpenoids nature. Isopropenyl function is indicated by the pair of substantially deshielded proton signals at 4.85 and 4.709 ppm, as well as an allylic methyl signal at 1.4 ppm. The protons linked to carbinolic carbon is shown by a signal at 3.14 ppm. (C3). Methane protons H19 and H18 are indicated by additional signals at 3.020 and 1.1 ppm , correspondingly. The existence of a carboxylic protons is confirmed by a weak signal at H 10.216 ppm.

A signal at 79.8 ppm, which is typical of carbinolic carbon, can be seen in ^{13}C-NMR data (C3). The presence of an isopropenyl side chain is indicated by couple of signals at C 152.2 ppm, as well as a methyl (C30) signal at 22.2 ppm. A signal at C 178.6 ppm confirms the presence of the carbonyl carbon (C28) group. At C 19.6, 19.7, 15.0, 16.2, 17.0 ppm, the spectrum also displays angular methyl carbons contains C23, C24, C25, C26, C27, as well as methlynic carbons C5, C9, C13, C18, C19, at C 49.7, 50.6, 52.1, 48.7, 48.7, 48.7, 48.7, 48.7, 48.7, 48.7, 48.7, 48.7, 48 ppm.

Figure 5 - 8 depicts the spectrum. The structural properties of compound 2's spectrum data are similar to those of betulinic acid. As a result, the isolated component 2 is determined to be betulinic acid.

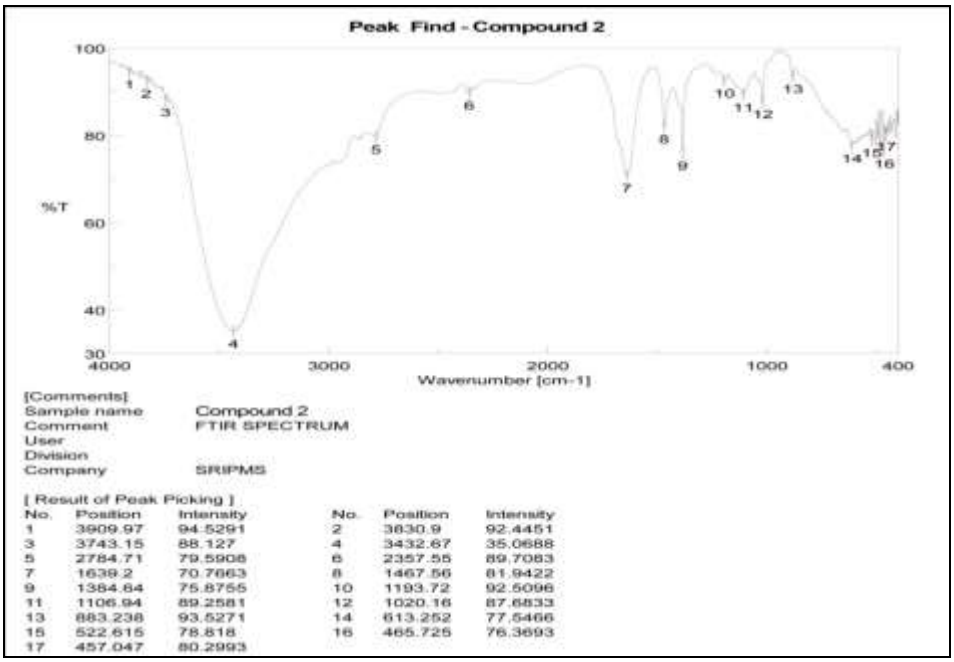

Figure 5: FT-IR spectrum

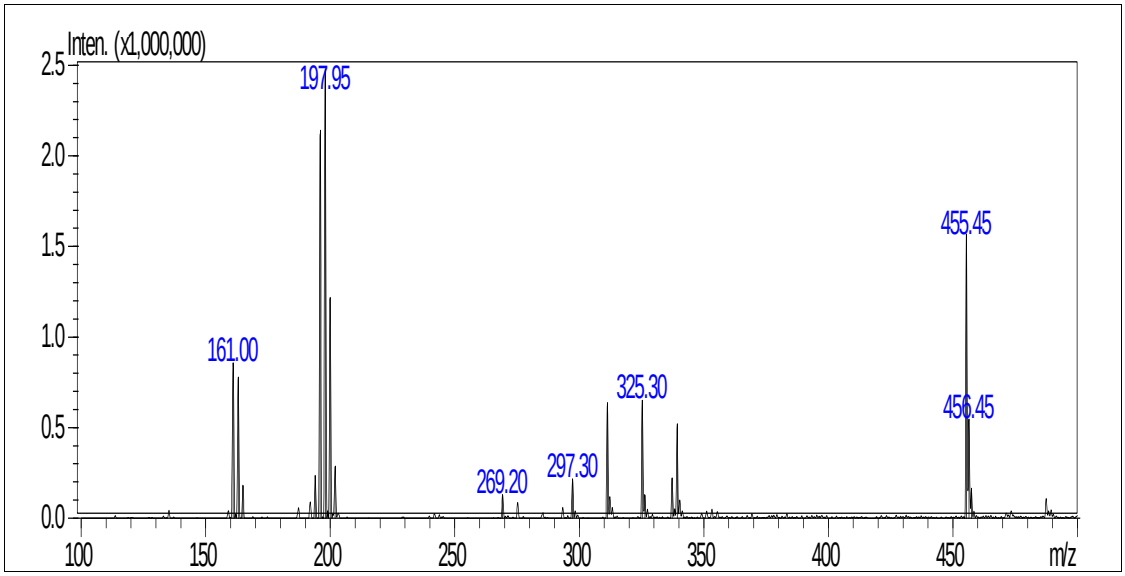

Figure 6: Mass spectrum of the isolated compound2

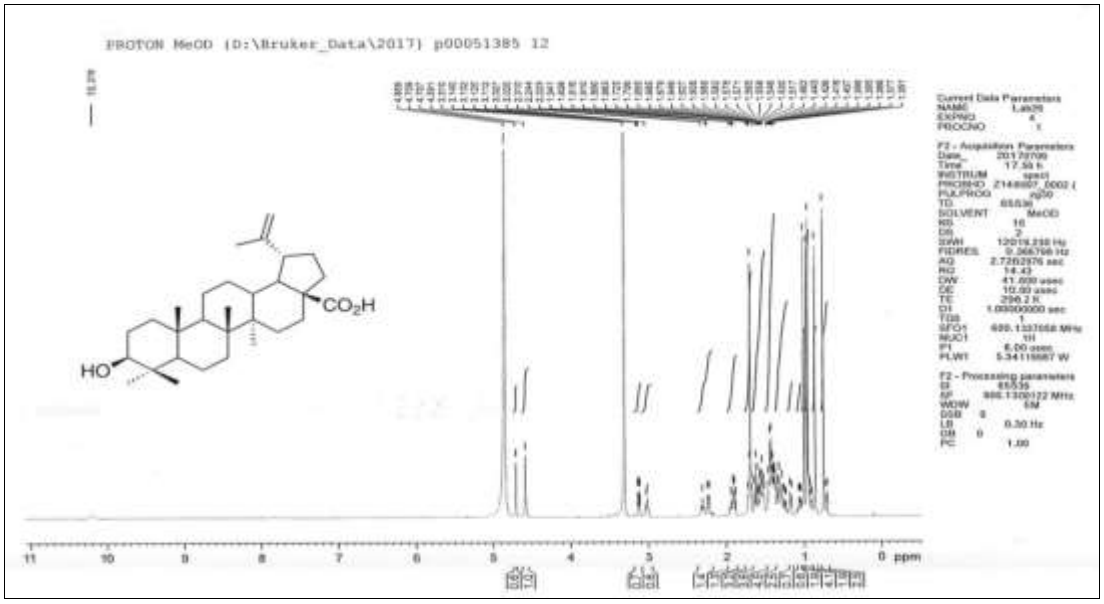

Figure7:^{1}H NMR spectrum of the isolated compound 2

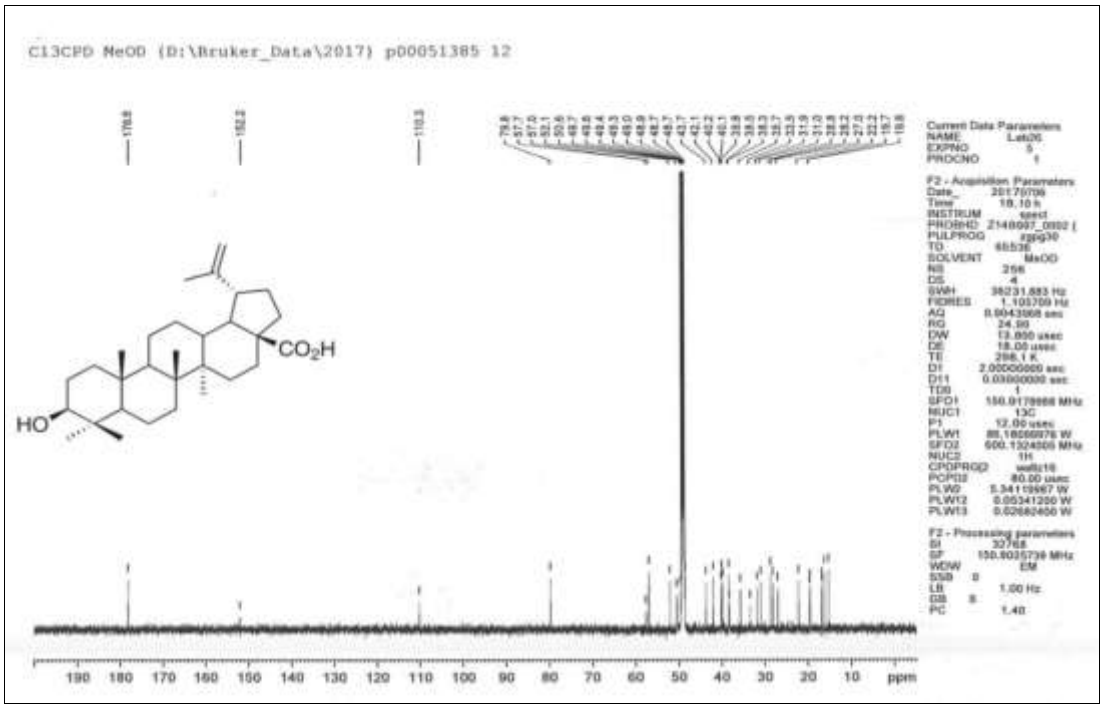

Figure 8: ^{13}C NMRspectrum of the isolated compound 2

3.3 In silico docking studies

PON1 has the highest binding affinity of all the enzymes tested in docking tests with the targeted enzymes implicated in atherosclerosis prevention. As a result, the PON1 enzyme has become the focus of the remaining research.

The *in silico* data clearly suggest that betulinic acid interacts with residues in hydrogen bond interactions that are comparable to those seen with atorvastatin (Lys192 and Asp269). Asn168 and Asn224 form hydrogen bond interactions with the ligand cetyl palmitate. Despite the fact that Asn168 is a required residue for PON1 activity, there is no evidence of interaction with the other important residues. This explains why cetyl palmitate has a lower docking score where Table 2 summarises the findings.

Table 2: Docking experiments with isolated molecules

Compound	Docking score (kcal/mol)	No. of interacting residues	Interacting residues	Type of interaction
Atorvastatin (Standard)	-7.661	5	Hip184, Lys192, Ile291, Phe292	H-bond, Hydrophobic interactions
Betulinic acid	-6.766	3	Hip184, Lys192, Asp269	Hydrophobic interactions
Cetyl palmitate	-6.543	2	Asn168, Asn224	H-bond

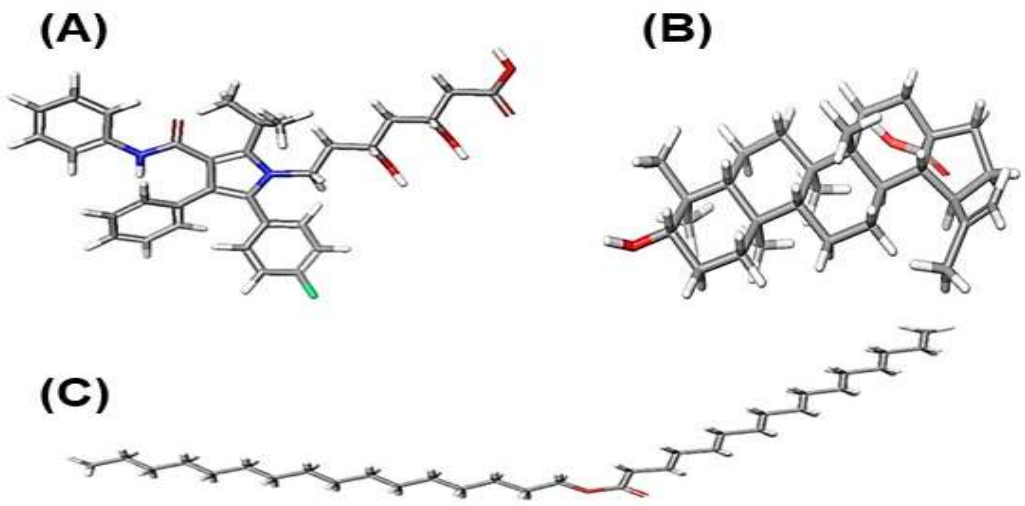

Figure 9: Chemical structures of (A) Atorvastatin, (B) Betulinic acid and (C) Cetyl palmitate

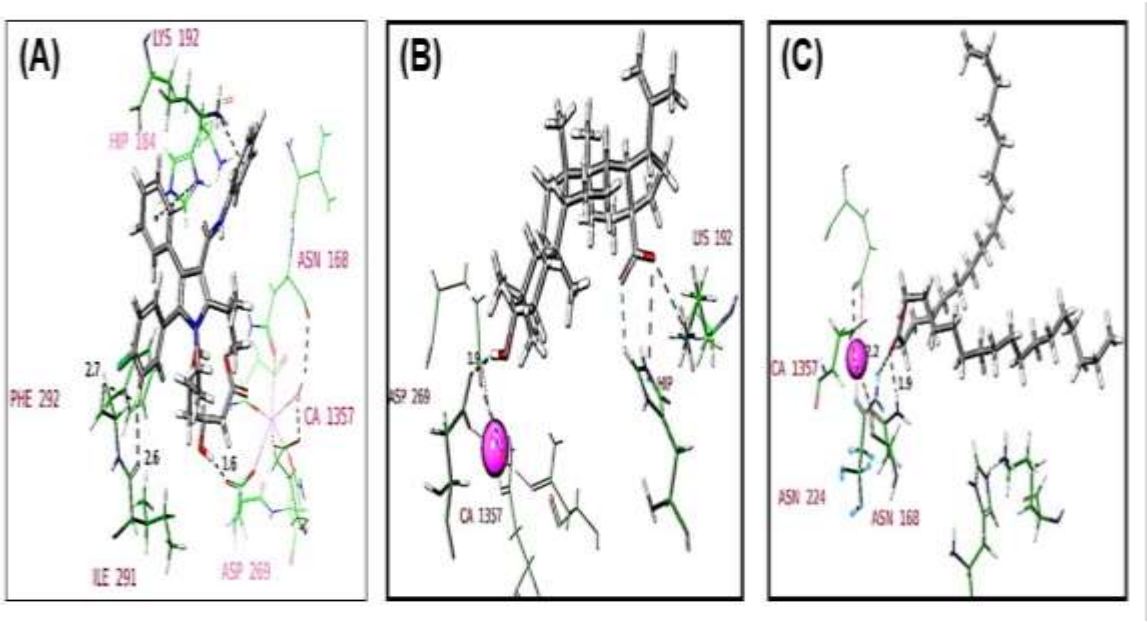

Figure10: Docked poses of (A) Atorvastatin, (B) Betulinic acid and (C) Cetyl palmitate with the paraoxonase enzyme

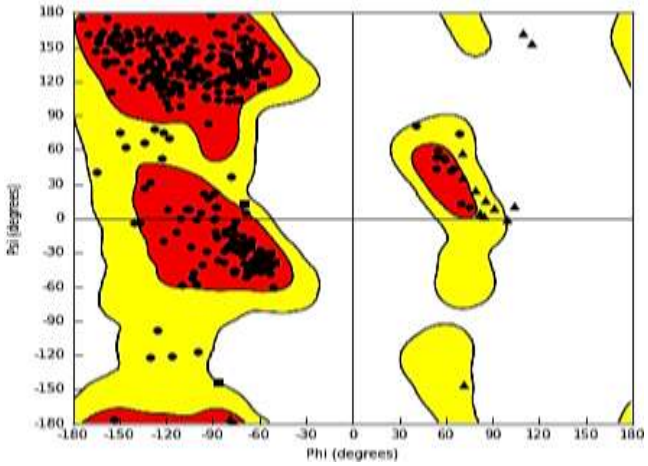

Figure11: Ramachandran plot for protein synthesis and validation

3.4 Cytotoxicity study of the isolated compounds

Using the MTT assay with dosages ranges from 500 to 62.5 g/ ml and the CTC50 value of each one extracted after 24 hours, 48 hours, and 72 hours of incubation, the extracted substance were examined for cytotoxicity on 3 dissimilar cell lines, Huh7,Vero, and HepG2.. Table 3 presents a summary of the findings.

Table 3: The extracted compounds were tested for cytotoxicity in Vero, HepG2, and Huh7 cell lines.

Compound	Cell line (CTC_{50} in µg/ml)*		
	Vero	HepG2	Huh7
Cetyl palmitate	141.5± 1.01	49.1± 2.11	42.7 ±1.17
Betulinic acid	164.5± 0.11	63.7 ± 0.11	48.4 ± 0.66

***Average of three determinations,Mean ± SD**

3.5 Paraoxonase activity of isolated substances produced by cells:

Using paraoxonase as a substrates to measure PON1 paraoxonase activity produced by cells in the collected media demonstrates that the separated chemicals significantly increases the cell secreted paraoxonase enzyme level compared to the control ($p < 0.001$). In HepG2 cell lines, betulinic acid had the highest activity has 1.39 fold compared to cetyl palmitate contains 1.47 fold and control has 1.47 fold. In Huh7 cell lines, betulinic acid has a greater activity than Cetyl palmitate, which possess 1.70 times and controls has 1.58 fold. The outcomes are in Table 4 and Figure 12.

Table 4: In HepG2 and Huh7 cell lines, isolated chemicals have an effect on cell secreted paraoxonase activity.

Compound	Cell lines (µmol / min / ml)*	
	HepG2	Huh7
Control	1.32±0.68	1.15 ± 0.68
Cetyl palmitate	1.84±0.69[a]	1.82 ±0.92[a]
Betulinic Acid	1.98±0.32[a]	1.96±0.65[a]

* **Values are Mean ± SD. [a]p<0.001: compared to the control**

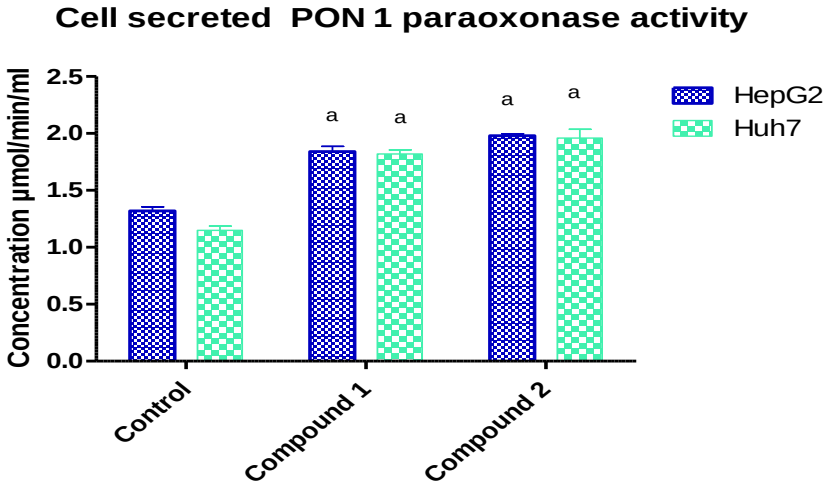

Figure 12: In HepG2 and Huh7 cell lines, isolated chemicals had an effect on cell secreted paraoxonase activity

When comparison to the HFD fed group, there was a substantial reduction (p 0.001) in total triglycerides contains 59 %, VLDL-C has 38 %, cholesterol has 36 % and LDL-C of 18 % and a significant increases (p 0.001) in HDL-C (18 %). Table 15 and Figures 23 - 27 illustrate the results.

4. DISCUSSION

In plant-based research, isolation of lead molecules responsibilities of the various biological functions is a critical step. Two extracts, ethanol and methanol extract of *Cajanus cajan*, were submitted to phytoconstituent extraction depending on the outcomes of anti-oxidant and anti-inflammatory activities. *Cajanus cajan's* Cetyl palmitate and Betulinicacid have been isolated, and their spectrum properties have been examined.

The docking postures of the separated compounds, particularly betulinic acid and cetyl palmitate, were categorized based on binding energy with the target PON1 and scores of docking. The research will also look at the use of atorvastatin. Docking poses for betulinic acid revealed hydrogen bond interactions with Hip184, Lys192, and Asp269, while docking poses for cetyl palmitate revealed hydrogen bond interactions with Asn168 and Asn224. In the instance of atorvastatin, hydrogen bond interactions were detected with Phe292, Asp269, Ile291 and Lys192, as well as a hydrophobic connection with Hip184. PON1 is an enzymes with a sophisticated substrates recognition system and catalytic sites that allow it to function through numerous methods and on multiple residues [18]. PON1's varied actions (lactonase/esterase and phosphotriesterase) are catalysed by residues situated at different locations in the active center, according to studies. The enzyme's His115-His134 dyad increases enzymatic action for a variety of substrates by deprotonating a molecule of water to produce a hydroxide ion. The hydrolytic action of PON1 is mediated by this hydroxide ion [19]. Asp269, His115, His134, Asp169, Phe222,) Leu69, His285, Phe292, Val346, Thr332, and Try281 are the residues involved for catalytic activitives. Any one of these amino acids that have been replaced have resulted in a lack of function. PON1 activity is also dependent on, Asp53, Try280, His133, Glu52, His154, His114,Val167, His184, His284, Lys 192, His242an d Asn168 [20].

The outcomes of betulinic acid are clearly superior to those of cetyl palmitate based on the *in silico* data. Thus, the *in vitro* PON1 paraoxonase activities of betulinic acid and cetyl palmitate are consistent with their *in silico* docking results. Toxicological studies in cell cultures are a quick, effective, and low-cost method. Cells grown in culture are commonly used to determine the possible harmful effects of compounds/plant extracts, and the results could be used to anticipate the performance of the similar in entire animals. This assay is required to categorize the ranges of concentrations for *in vitro* testing of extract and isolated chemicals in greater detail [21]. The MTT test was used to assess the cytotoxicity of various plant extracts. The CTC50 value was used to estimate the cytotoxicity of the most efficient extract.

5. CONCLUSION

Cajanus cajan leaf extract has yielded two compounds: cetyl palmitate and betulinic acid, which are separated and spectral analysis carried out using IR, MS, and NMR studies. The MTT assay method was used to test the cytotoxicity of alcoholic extracts and isolated chemicals in HepG2 and Huh7 cell lines, and they were shown to be toxic over 500 μg/ml. The compounds extracted from HepG2 and Huh7 increased the secretion of the biologically active PON1 enzyme, demonstrating that a methanol extract from the *Cajanus cajan* plant induced the production of physiologically PON1 enzymes which is active. Betulinic acid, one of the separated chemicals, had enhanced secretion. It was revealed that betulinic acid exhibited a greater affinity for the target enzyme paraoxonase (PDB ID: IV04) in a *in silico* docking study of the molecules cetyl palmitate and betulinic acid which is used towards the enzyme paraoxonase (PDB ID: IV04). *In vitro* cells produced PON1 paraoxonase activities,and *in silico* docking research of betulinic acid

and cetyl palmitate demonstrated similarities in their effects with betulinic acid showing superior activity in both approaches.

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