INDO AMERICAN JOURNAL OF PHARMACEUTICAL RESEARCH

ISOLATION AND STRUCTURAL ELUCIDATION OF BIOLOGICAL AND PHARMACOLOGICAL ACTIVITIES COMPOUNDS FROM *CANTHIAM COROMENDELICUM*

Arulprakash. P, Sasidharan. P, Jayachitra. A

Department of Plant Biotechnology, School of Biotechnology, Madurai Kamaraj University, Madurai, India.

ARTICLE INFO

Article history
Received 09/03/2017
Available online
31/03/2017

Keywords
Canthiam Coromendelicum,
Cytotoxicity Assay,
Squalene,
Gas Chromatography And
Mass Spectrometry.

ABSTRACT

The present study deals with the identifying a Squalene from medicinal plants for pharmaceutical application. Squalene was isolated from *Canthiam coromendelicum* using soxhlet apparatus. Methanol is a good solvent for extraction of the Squalene. The crude extractions of *C. coromendelicum* shows many Secondary metabolites present in the extracts such as Squalene, phytol, n-Butyl are major compounds to find out from the plant profile using GC-MS, UV-Vis, FT-IR, HPTLC, NMR and Mass. Then it was used for further pharmaceutical applications such as antioxidant, antibacterial and cytotoxicity assay. The Squalene possess enhanced characteristic of antioxidant activity against for their free radicals (DPPH, NO, SO, H_2O_2), antibacterial activity against *Salmonella typi*, *E. Coli, Pseudomonas putida*, *Staphylococcus aureus.* It shows good cytotoxicity activity against Hep-2 cells. The characterization analysis proved that the isolated Squalene would be a good and potential drug for cancer. In future it will be useful in many biomedical applications.

Corresponding author
Dr. A. Jayachitra, P.Arulprakash
Assistant Professor,
Dept of Plant biotechnology,
School of Biotechnology,
Madurai Kamaraj University, Madurai-625 021.
jchitralab@gmail.com

*Please cite this article in press as **P. Arul prakash** et al. Isolation and Structural Elucidation of Biological and Pharmacological Activities Compounds from Canthiam Coromendelicum. Indo American Journal of Pharmaceutical Research.2017:7(03).*

Copy right © 2017 This is an Open Access article distributed under the terms of the Indo American journal of Pharmaceutical Research, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

INTRODUCTION

Natural products principally medicinal plants have long been prescribed in traditional medicine for centuries for treating different diseases. The significance of herbs in the management of human ailments cannot be overemphasizing (1) .Plant derived compounds have got an increasing interest throughout the world as they possess potent, less or no toxic pharmacological compound, economic viable, safer and more dependable (2). Triterpenes are a group of biologically active compounds which are present in plant tissues in almost every geographical region in the world. The group consist of approximately 30 000 identified compounds (3,4) Triterpenoids are abundant in nature, particularly in resins and may occur as either esters or glycosides (5). In most of these compounds, two or more isoprene units are joined together in a head-to-tail configuration of the carbon atoms, either in open chain or in cyclic systems containing one or more rings. The main exception to the head-to-tail arrangement involves the formation of artemisia ketone (and related mono In most of these compounds, two or more isoprene units are joined together in a head-to-tail configuration of the carbon atoms, either in open chain or in cyclic systems containing one or more rings. The main exception to the head-to-tail arrangement involves the formation of artemisia ketone (and related monoterpenoids), squalene, gossypol, and the carotenoids (6,7,8,9) .Chemically triterpenoids are compounds with a carbon skeleton based on six isoprene units which are derived biosynthetically from the acyclic C30 hydrocarbon, squalene. They have relatively complex cyclic structures, most being either alcohols, aldehydes or carboxylic acids. Triterpenoids can be divided into at least four groups of compounds, true triterpenes, steroids, saponins and cardiac glycosides (10). Squalene is biosynthetically derived Chemical triterpenoids compounds with a carbon skeleton based on six isoprene units. They have relatively complex cyclic structures, most being either alcohols, aldehydes or carboxylic acids.Intermediate for the biosynthesis of phytosterol or cholesterol in plants/animals and humans. In human, squalene is synthesized in the liver and the skin, and then transported in the blood (11).It is an important intermediate in the sterol biosynthetic pathway an essential natural antioxidant to protect the cells from free radicals and reactive oxygen species. It plays a major role in releasing oxidative stresses, such as sunlight exposure (12). Squalene is also effective in decreasing serum cholesterol level and has cardio protective effect on myocardial infarction in experimental animals (13, 14).Squalene is used in clinical and daily usage such as detoxification factor, skin and eye antioxidant, provide cells with oxygen, bactericidal and fungicidal agent, antistatic and emollient in pharmaceutical and cosmetics, fine chemical, magnetic tape and also as low temperature lubricants (15).

This terpenoid hydrocarbon (C30H50, 6,10,15,19,23-hexamethyl- 2,6,10,14,18,22-tetracosahexaene) has been associated with a chemo preventive effect on colon cancer upon consumption of virgin olive oil, a decrease in chemotherapy-induced side effects, scavenging properties for toxic metabolites, and effective inhibition of photoperoxidation. Squalene has also been reported to have important beneficial effects on health, such as decreasing the risk for various cancers (11, 16). Recent epidemiological studies have indicated that squalene can effectively inhibit chemically-induced lung, colon and skin tumorigenesis in animals under experimental conditions (17). The traditional sources of squalene are liver oils of deep-sea sharks and whales. Large-scale extraction of squalene frommarine animals, however, is nearly impossible as a result of international concern over the protection of endangered marine animals (18) With regard to squalene occurrence and biosources, marine sources are currently exhausted, so other sources of animal, plant, or microbe origin have to be evaluated. Well-investigated inedible sources are the distillates of the olive oil industry and Amaranthus seed oil (19).The GC-MS analysis revealed the presence of 26 compounds from the methanolic leaf extract of *canthium coromandelicum.* the major components were Squalence and other compounds Z-8-Methyl-9-tetradecenoic acid, Heptadecanoic acid,3,7,11,15-Tetramethyl -2-hexadecen-1-ol, heptadecyl ester. Chromatographic methods (column chromatography, HPTLC) were applied to monitor the squalene content and the structure of purified squalene was verified by its UV spectra. The UV spectrum of the squalene fraction displayed a strong absorbance only at 214 nm in the UV range.

The structure of the purified squalene was further confirmed by FTIR its 1H and 13C NMR spectrascopic data. Squalene has been reported to possess antioxidant properties. In vitro experimental evidence indicates that squalene is a highly effective oxygen-scavenging agent. The antimicrobial activity of isolated squalene carried out against four different bacterial Strains such as *Staphylococcus aureus* , *Salmonella typhimurium* , *Escherichia coli* , *Streptococcus pyogenes.* The percent viability was analyzed by MTT assay after treatment with different concentration of Squalene and Morphological changes of apoptosis in Hep 2 cells. The main aim of this presents study to investigate antimicrobial, anti-oxidant and anticancer activities of active compound Squalene isolated from extracts of *Canthium coromandelicum.*

MATERIALS AND METHODS

Collection of Plant Material

The fresh plant leaves of *Canthiam coromendalicum* were collected randomly from ponnamaravathy, pudukkottai District, Tamil Nadu. Plant samples were authenticated by Dr. M. Palanisamy (ScientistD), The specimen voucher number SC/SRC/5/23/2015/Tech/1415. Botanical survey of India, Coimbatore, India.

Preparation of extracts

Plant materials were washed under running tap water, air dried and then homogenized into fine powder and stored in airtight bottles in refrigerator. About 50gm of powdered plant material was uniformly packed into a thimble and extracted with 300 ml of methanol using Soxhlet extraction method. The Soxhlet extraction method has to be continued for 24 hours or till the solvent in siphon tube of extractor become colourless. After that the extract was taken in a beaker and kept on a hot plate and heated at 30-40ºC till all the solvent got evaporated. Dried extract was kept in refrigerator at 4ºC for further use.

Qualitative phytochemical screening

The major classes of secondary metabolites such as alkaloids, anthocyanins, anthraquinones, flavonoids, polyphenols, saponins,tannins, steroids and triterpenes be screened according to the common phytochemical methods described by Harborne with some modifications. The methanolic extract showed higher positive test when compared to other extracts. Based on the higher active principle crude methanolic extract of C. coromandelicum selected for further studies.

Purification by chromatography techniques

Column Chromatography

The extracts were further purified for squalene by column chromatography on a silica gel (24 g, 70- 230 mesh, Sigma Co.) column. A solution of 1.0 g of plant extract in 5 ml of petroleum ether was loaded and eluted by washing the column with 1% diethyl ether in petroleum ether, at a flow rate of approximately 1.0 ml/min. Test tubes (10 ml with screw cap) were used for fraction collecting. Thin-layer chromatography (TLC) was used for detection. Squalene appeared completely in the 1% diethyl ether in petroleum ether elute. The elute was evaporated by vacuum evaporation to give colourless squalene liquid. The residues in the column were washed out by chloroform.

HPTLC

From the successful development of TLC plate with the prepared solvent system, the Preparative High Performance Thin Layer Chromatography of the sample was carried out on the CAMAG HPTLC System. Prior to sample application, HPTLC plate (HPTLC Silica gel 60 F254, Merck) was activated at 110°C for 30 min. 2 μL of the sample was then applied as a single band of 8 mm length on the activated HPTLC plate using a CAMAG automatic TLC sampler III (CAMAG, Switzerland). The plate was then developed with the 10 ml of standardized solvent system, Hexane:Ethyl acetate (6:1) in the twin trough chromatographic chamber. After the successful development, the plate was examined under the UV Chamber at 254 and 366 nm.
Characterization techniques

Gas Chromatography–Mass Spectrometry (GC/MS) analysis

GC/MS analysis of this extract was performed using a Perkin Elmer GC Claurus 500 system and Gas Chromatograph interfaced to a Mass Spectrometer (GC/MS) equipped with a Elite-1 fused silica capillary column (30 m × 0.25 mm ID. ×1 μMdf, composed of 100% Dimethyl poly siloxane). For GC/MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium gas (99.999%) was used as the carrier gas at a constant flow rate of 1 ml/min. and an injection volume of 2 μl was employed (split ratio of 10:1).Injector temperature 250°C, Ion-source temperature 280°C. The oven temperature was programmed from 110°C (isothermal for 2 min.), with an increase of 10°C/min, to 200°C, then 5°C/min to 280°C, ending with a 9 min. isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 seconds and fragments from 45 to 450 Da. Total GC running time was 36 min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Software adopted to handle mass spectra and chromatograms was a TurboMass Ver 5.2.0.

Mass spectrometer

The identification of the compounds was accomplished by coupling the chromatograph with a 5989A HP Mass Spectrometer (electron energy: 70 eV; MS source: 250 ◦C; MS quadrupole: 110 ◦C) through a particle beam Hewlett-Packard HP 59980B LC/MS interface (desolvationChamber temperature: 55 ◦C; helium inlet pressure: 40 psi).

UV –Vis spectroscopic technique

UV-visible spectroscopy analysis was carried out on a SHIMADZU UV-visible absorption spectrophotometer 117 with a resolution of +1nm between 200-1000nm processing a scanning speed of 200nm/min. equal amounts of the suspension (0.5ml) were taken and analysed at room temperature. The progress of the reactions was monitored by UV-visible spectra of Squalene in aqueous solution with wavelength 200-850nm.

FT-IR analysis

Infrared light from a suitable source passes through a scanning Michelson inferometer and Fourier transformation gives a plot of intensity versus frequency. When a sample is placed in the beam, it absorbs particular frequencies, so that their intensities are reduced in the inferogram and the ensuing Fourier transform is the infrared absorption spectrum of the sample. For FTIR measurements, SHIMADZU -spectrum model was used. The TLC isolated and purified squalene was diluted in chloroform. Fouried transformed infrared spectra is generated by the absorption of electromagnetic radiation within the frequency range 400 to 4000cm-1.

NMR analysis

The powdered squalene was also subjected to ^{1}H-NMR (Bruker, 200 MHz in $CdCl_3$; internal standard TMS) and ^{13}C-NMR (Bruker, 75 MHz in $CdCl_3$; internal standard TMS). The chemical shift values were recorded as δ (delta) value/ppm, relative to the TMS.

Antioxidant Assay

DPPH radical-scavenging activity

Different concentrations of squalene (10 µl -50 µl) were added, at an equal volume, to the methanolic solution of DPPH (100 µM). The experiment was repeated for three times and allowed to stand at room temperature for 30 minutes. Methanol served as the blank. After 30 minutes, the absorbance was measured at 518nm and converted into percentage radical scavenging activity as follows:

$$\textbf{Scavenging activity (\%)} = \frac{A_{518}[\text{Control}] - A_{518}[\text{Sample}]}{A_{518}[\text{Control}]} \times 100$$

Scavenging of Hydrogen peroxide

The reaction mixture (0.1ml) contained 0.1ml of deoxyribose, 0.1ml of $FeCl_3$, 0.1ml of EDTA, 0.1ml of H_2O_2, 0.1ml of ascorbate, 0.1ml of KH_2PO_4-KOH buffer and (10µl -50 µl)of squalene in a final volume of 1.0ml. The reaction mixture was incubated for 1 hour at 37˚C. Then 1.0ml of TBA was added and heated in a boiling water bath for 20 minutes. The pink colour produced was measured at 535nm in a spectrophotometer. Deoxyribose degradation was measured as TBARS and the percent inhibition was calculated.

Nitric oxide-scavenging activity

The reaction mixture containing 0.3ml of sodium nitroprusside, 2.68ml PBS and (10µl-50 µl) of squalene was added and incubated at 25˚C for 15 minutes. Control tubes (100% generation) were prepared without the sqaulene. After incubation, 0.5ml of the Griess reagent was added. The absorbance of the chromophore formed, indicative of the quantum of NO generated, was read at 546 nm.

Scavenging activity of Superoxide

The assay tubes contained 0.02ml of the sample (corresponding to 10-50 µl of squalene) with 0.2ml EDTA, 0.1 ml nitroblue tetrazolium, 0.05ml riboflavin and 2.64ml phosphate buffer. The control tubes were set up without the squalene, where DMSO was added. The initial optical densities of the solutions were recorded at 560nm and the tubes were illuminated uniformly with the fluorescent lamp for 30 minutes. A_{560} was measured again and the difference in O.D was taken as the quantum of superoxide production. The percentage inhibition by the leaf samples **was calculated by comparing with the O.D of the control tubes.**

Determination of Antimicrobial Activity

The squalene was tested on the following strains *Staphylococcus aureus* (ATCC 25923), *Salmonella typhimurium* (ATCC 14028), *Escherichia coli* (ATCC 25922), *Streptococcus pyogenes* (ATCC 19615). The antibacterial activity of the squalene was determined by using the Kirby-Bauer method (Bauer and Kirby, 1966). Briefly, the test was performed in sterile Petri dishes (100 mm diameter) containing an appropriate solid sterile media. The Gram positive and negative bacteria were cultivated on Luria bertania agar. The surface of the plates was inoculated with 200 µL of bacterial suspension. Sterile filter paper (Whatman No. 1) discs (6 mm in diameter) containing 10, 20, 30 µL of the tested squalene were placed in the centre of the agar surface. A disc containing 10 µL of sterile broth media was used as the negative control. Reference antibiotics tetracycline at 100 µg/mL^{-1} concentrations, were used as the positive control for comparison. Each individual Petri dish was covered to prevent eventual evaporation. After allowing the squalene to diffuse across the surface for 1 h at room temperatures, the plates were sealed with sterile parafilm and immediately incubated at 37°C for 24-48 h. The antibacterial activities of the squalene and antibiotics were demonstrated by a clear zone of inhibition around the disc. The zone of inhibition was measured using electronic digital Vernier calipers. Each test was performed in triplicate on at least three separate experiments.

Anticancer activity

Culturing of cells

Hep-2, (human epithelial larynx cancer cell line) was obtained from the National Centre for Cell Sciences (NCBS), Pune, India, and were grown in MEM media supplemented with 10% FBS 100 IU/ml, penicillin 100 mg/ml, streptomycin 20 mg/ml. The cells were maintained as monolayers in 25cm^2 plastic tissue culture flasks at 37°C in a humidified atmosphere containing 5% CO_2 in air. Exponentially growing cells were used in all the experiments.

In-vitro cell viability studies

The MTT assay is a simple, nonradioactive colorimetric assay to measure cell viability. Metabolically active cells are able to convert this dye into a water-insoluble dark purple formazan by reductive cleavage of the tetrazolium ring. Formazan crystals, then, can be dissolved in an organic solvent such as dimethylsulphoxide (DMSO) and quantified by measuring the absorbance of the solution at 545 nm, and the resultant value is related to the number of living cells.

To determine cell cytotoxicity /viability, the cells were plated at a density of 1x10 [6] cells/well in a 96-well plate at 37˚C in 5% CO_2 incubator. After 24 h of culture, the medium in the wells was replaced with the fresh medium containing squalene in varying concentrations. After 24 h, 20 µl of MTT dye solution (5 mg/ml in phosphate buffer pH 7.4) was added to each well. After 4 h of incubation at 37˚C and 5% CO_2, the medium was removed and formazan crystals were solubilized with 200 µl of DMSO and the solution was vigorously mixed to dissolve the reacted dye. The absorbance of each well was read on a microplate reader at 545 nm. The spectrophotometer was calibrated to zero absorbance, using culture medium without cells. The relative cell viability (%) related to control wells containing cell culture medium without squalene was calculated by the following formula:

% of cell viability = 100 x (Sample absorbance / Control absorbance)

Determination of Morphological changes in the Hep-2 cells:

The morphological and nuclear changes associated with apoptosis were studied using various staining techniques. The features of apoptotic cells include shrinkage, condensation of chromatin and cytoplasm, detachment of the cells from the neighbouring cells, fragmentation of the nucleus and membrane blebbing.

Propidium Iodide

Hep-2 cells were grown in a 6 well plate, Squalene and control were added and incubated for 24 hrs and then cells were trypsinised and collected in Microcentrifuge tubes. Cells were resuspended in 50 µl of PBS. 5 µl of RNase (1 mg/ml) and 5 µl of Propidium Iodide (25µg/ml in PBS) was added and incubated at 37°C for one hour by using Fluroescence microscopy.

Hep2 cells were grown in a 6 well plate, with Squalene and without squalene considered as a control were added and incubated for 24 hrs and then cells were trypsinised and collected in Microcentrifuge tubes. Cells were resuspended in 50 µl of PBS. 5 µl of RNase (1 mg/ml) and 5 µl of Ethidium bromide (25µg/ml in PBS) was added and incubated at 37°C for one hour.

Giemsa Staining

The cells were cultured. The isolated compound (squalene) were exposed for 24 h to Hep-2 cells. The IC-50 Concentration of isolated compound used for the entire assay was 15µg. The nuclear changes that are characteristic of apoptotic events resulted after the treatment due to the effect of isolated compound were validated by the Giemsa Staining technique.

RESULTS AND DISCUSSIONS

Qualitative Phytochemical Screening

The phytochemical investigations of C*anthium coromendelicum* plant leaf methanolic extract Conform the presence of various secondary metabolites such as alkaloids, flavonoids, terpenoids, saponin, tannin, carbohydrates were present. The results were qualitatively analysed.(Figure 1) shows the observed colour change in the various phytochemical tests. The methanolic extract showed higher positive test when compared to other extracts. Based on the higher active principle crude methanolic extract of C. coromandelicum selected for further studies.

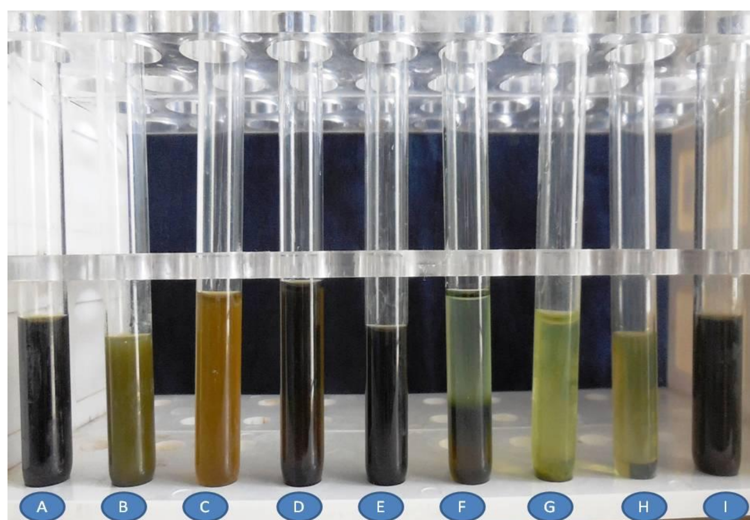

Figure 1: Phytochemical Screening of *Canthium coramandelium.*

Ligand: (A) Control, (B) The yellow, cream precipitate indicates the presence of alkaloids. (C) Orange colour indicates the presence of Flavonoids, (D) Violet to blue or green colour indicate the presence of steroids, (E) The appearance of pink-red trun blue-violet colour indicates the presence of Anthocyanins, (F) reddish brown colour indicates the presence of Terpenoids, (G) A yellow colour precipitate indicates the presence of Phenols, (H) Formation of frothing (appearance of creamy, stable, persistent of small bubbles) shows the presence of Saponins, (I) A dark green colour formation indicates the presence of tannins.

Purification by chromatography techniques

Chromatography a general technique used to separate components of a mixture based on intermolecular forces. Sometimes it is performed on a small scale for the purpose of analysis or detecting the presence or absence of a particular compound. Other times it is done pre operatively to obtain pure samples. All chromatography involves interaction with (and equilibrium between) the stationary phase and the mobile phase.

Column Chromatography

The extract were eluted by column chromatography shown (Figure 2) .The eluent was collected in a fraction of 5ml and tentative identification has been carried out Thin-layer chromatography (TLC) and HPTLC was used for detection. Squalene appeared completely in the 1% diethyl ether in petroleum ether elute. The Elute was evaporated by vacuum evaporation to give colourless Squalene liquid. The residues in the column were washed out by chloroform.

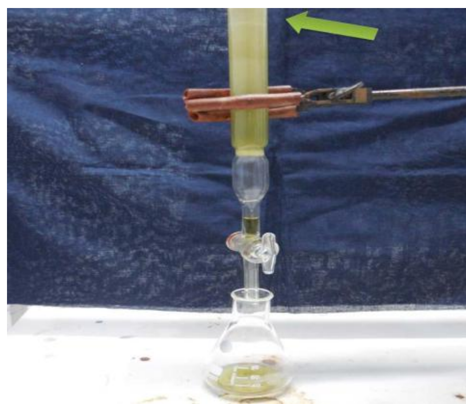

Figure 2 Column Chromatography analyses for Squalene.

The fraction is separated in four different Colours.

HPTLC

The co-chromatographic screening of HPTLC with the Chloroform dissolved compound revealed the sharp, single, blue fluorescent band which was considered of squalene only at Rf = 0.68 cm under 254nm and 366 nm shown (Figure3). This resolves the approximate true confirmation of isolation of compound.

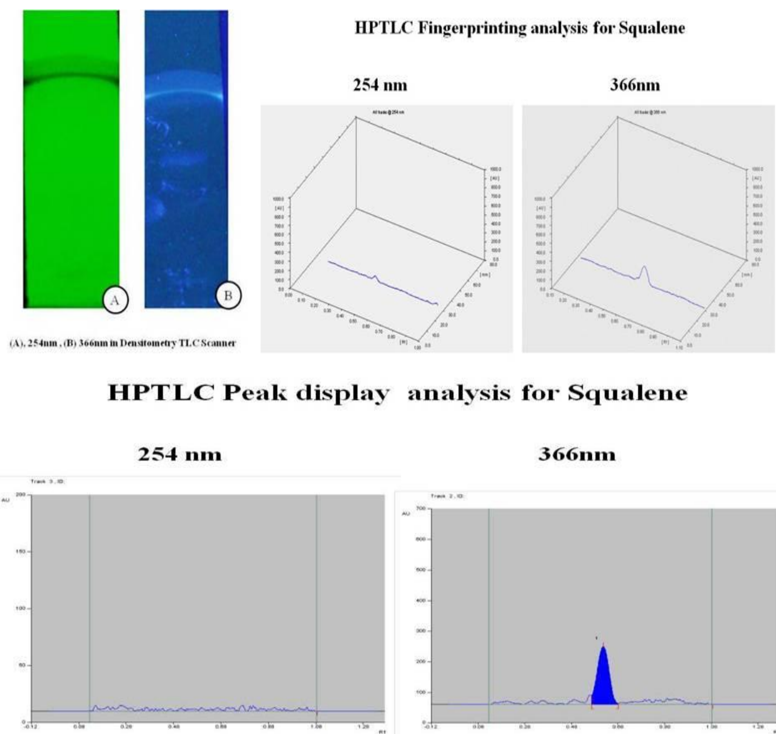

Figure: 3 High Performance thin layer chromatography analysis for Squalene.

Liebermann- Burchard test for Triterpinoid (squalene)

The Isolated squalene was mixed with chloroform and concentrated. H_2SO_4 it was formed reddish brown colour layer. (Figure 4) shows confirmation the presence of squalene.

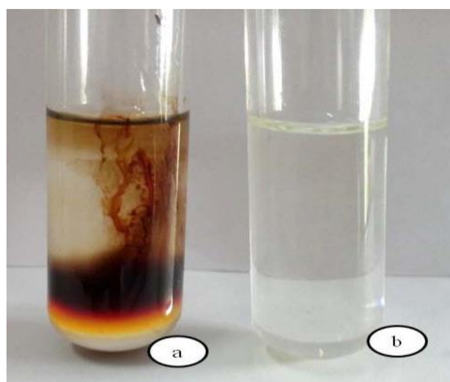

Figure: 4 Confirmatory Tests for Squalene.

Lingad : (a) reddish brown colour layer indicates the presence of Squalene, (B) control

Gas Chromatography–Mass Spectrometry (GC/MS) analysis

The GC-MS analysis revealed the presence of 26 compounds from the methanolic leaf extract of *Canthium coromandelicum.* The Name, Molecular weight and Structure of the components of the test materials were ascertained. Among the 31 components Squalene (C30H50) is the major component available at RT 29.50 and 27.69%, Phytol 21.17%,n-Butyl 11.51%peak area Along with another miner constituents were also present. The GC-MS chromatogram (Figure 5 & 6) shows the peak area separation. Along with other miner constituents were also present.

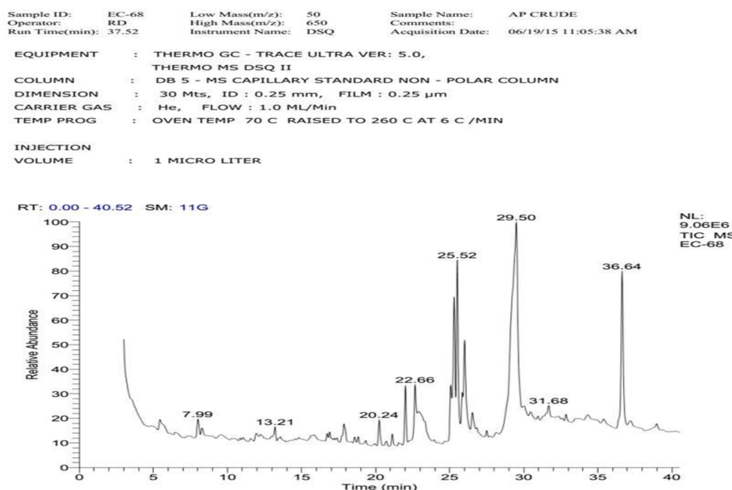

Figure: 5 Gas Chromatography analysis for Squalene.

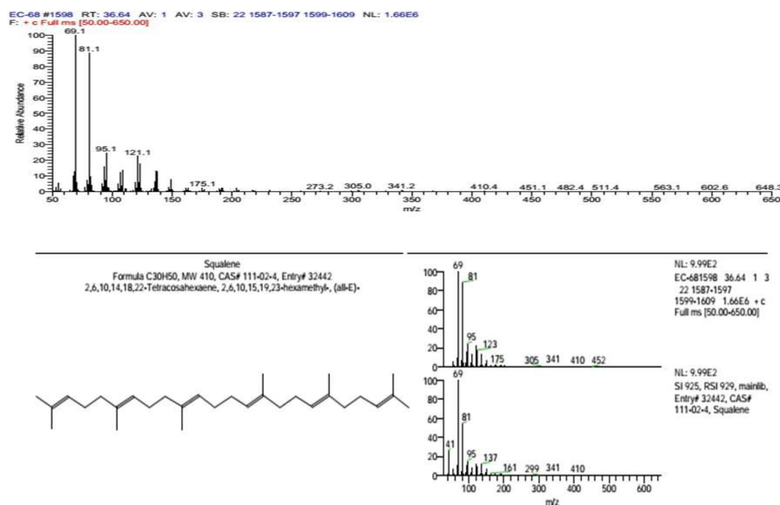

Figure 6 Mass Spectrometry (GC/MS) analyses for Squalene.

UV –Vis spectroscopic technique

The structure of the purified squalene bioactive compound was verified by its UV spectra. The UV spectra enriched fractions were compared to determine possible impure compounds with similar physical/chemical properties. The samples with relatively high concentration was used for UV measurement to increase the concentration of potential impurities in the enriched sample. The UV spectrum of the bioactive compound fraction displayed a strong absorbance at 214nm in the UV range, well indicated that no impurities were observed in the UV range (Han-Ping He et al., 2002) shown (Figure 7).

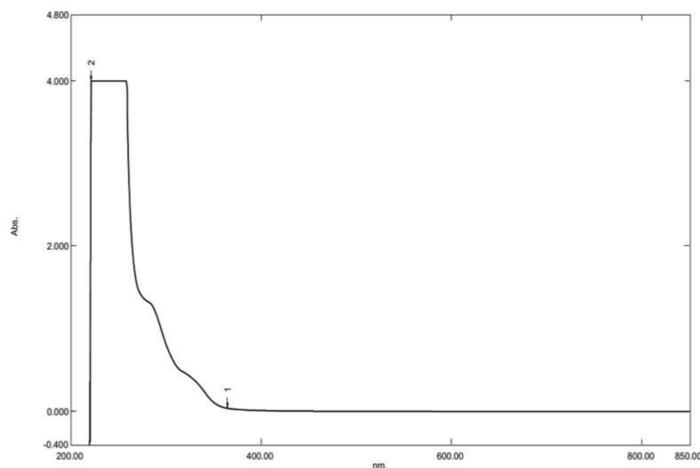

Figure 7: UV –Vis spectroscopy analysis for Squalene.

FT-IR analysis

FTIR measurement was carried out to identify the potential of isolated bioactive compound shows the FT-IR specterum shown (Figure 8). A strong, sharp peak was obtained at 2906.62 cm^{1}, 2920.32 cm^{1} and 2852.81 cm^{1} corresponds to alkyl C-H stretch and H consisting of alkenes . A strong band at 1741.76 cm^{1} indicates Aldehyde C=O stretch and its shows to carbonyl group. A strong peak at 1664.62 cm^{1} denotes Amide C=O stretch, stretch and containing carbonyl groups. 1516.10 cm^{1}this peak indicate Aromatic C=C Bending. Peak in the fingerprint regions were observed at 1446.66 cm^{1}, 1379.13 cm^{1}, 1217.12 cm^{1} and 1153.47 cm^{1} corresponds to groups like alkenes, carboxylic acid, alkynes. These evidences show the compound could be squalene.

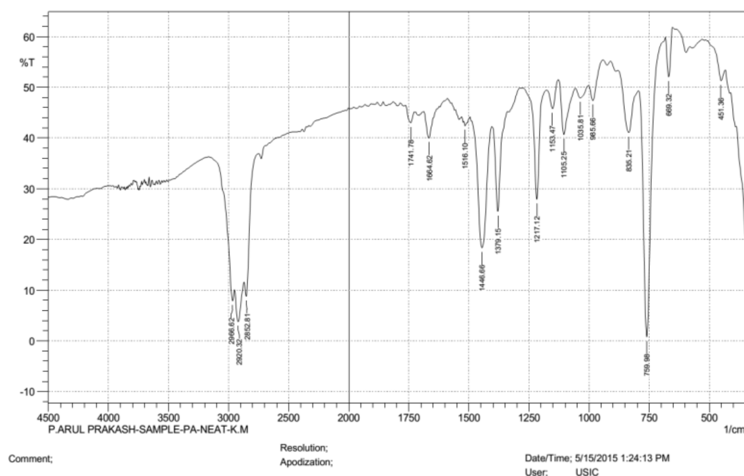

Figure 8: FT-IR spectrum analysis for Squalene.

NMR analysis

The isolated compound from the extract was identified as squalene which was characterized by 1H NMR and 13C NMR

^{1}H NMR

All the peaks appeared in proton NMR was in aliphatic region, which evidently confirms there are no aromatic units in this compound. The peak appeared in 5.35 ppm indicates the presence of alkaline (double bond unit). The sharp peak appeared 1.3 indicates the presence of methyl units. The peaks appeared before 3.0ppm which indicates which contains the aliphatic chains. These evidences show the compound could be squalene.

^{13}C NMR

The squalene was additionally confirmed by ^{13}C NMR. The peak appeared around 120 ppm indicates the presence of the double bonded unit. There is no aromatic carbon, which clearly visible in ^{13}C NMR spectrum. All the peaks

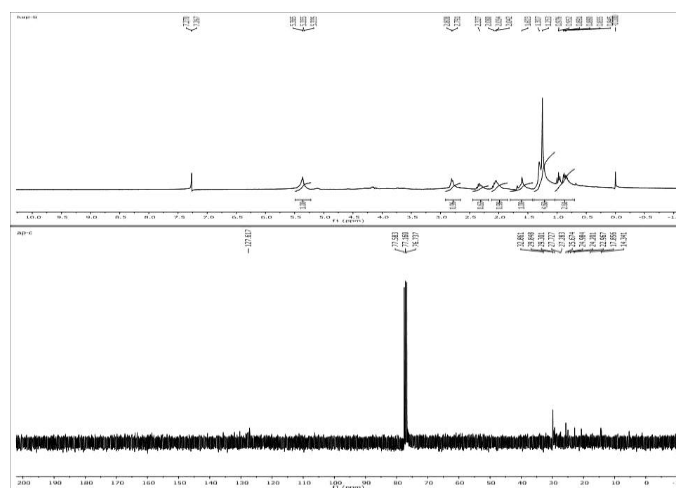

Figure: 9 NMR analysis for Squalene.

Appeared in this NMR shows the compound should be aliphatic chained. The above details also shown the compound could be squalene shows (Figure 9). The NMR spectra were consistent with the published literature.

Antioxidant activity

In vitro experimental evidence indicates that squalene is a highly effective oxygen-scavenging agent. Subsequent to oxidative stress such as sunlight exposure, squalene functions as an efficient quencher of singlet oxygen and prevents the corresponding lipid peroxidation at the human skin surface.

DPPH radical-scavenging activity

The radical scavenging activity of isolated squalene from *canthium coromendelicum* leaf extract estimated by comparing the percentage inhibition of DPPH radicals with ascorbic acid. (Figure 10) shows The DPPH activity of squalene increased with increasing concentration of squalene.

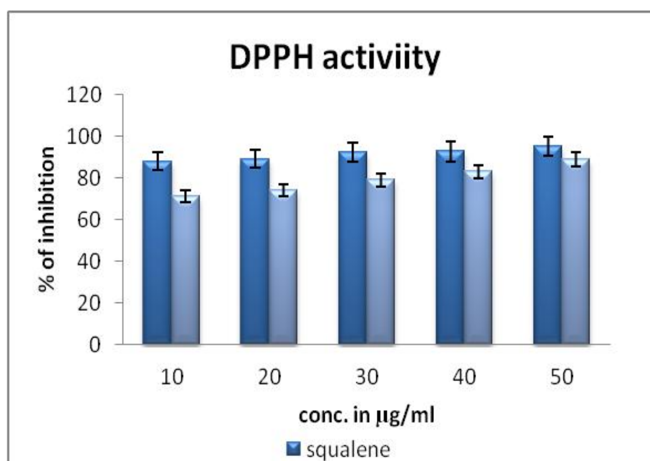

Figure: 10 DPPH scavenging activity for Squalene.

Scavenging of Hydrogen peroxide

Hydrogen peroxide, a weak oxidizing agent and cross cell membrane rapidly, reacts with Fe2+ and possibly Cu2+ ions to form the damaging toxic hydroxyl radical.(Figure 11) shows Hydrogen peroxide scavenging activity of the isolated Squalene from *canthium coromendelicum* methanlic extract and ascorbic acid standard. squalene caused a strong dose-dependent inhibition of hydrogen peroxide. The squalene showed a good scavenging ability compared to the standard.

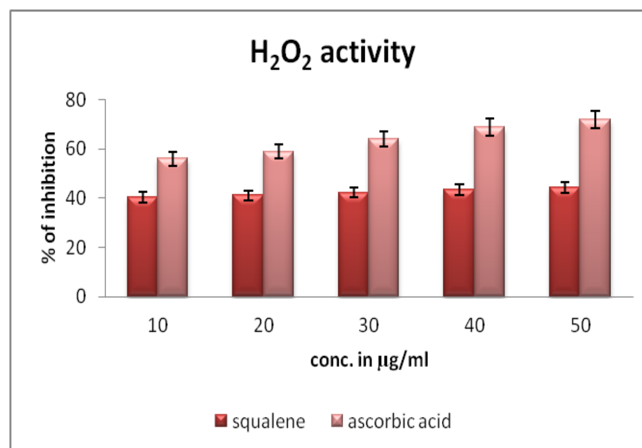

Figure 11 Scavenging of Hydrogen Peroxide assay for Squalene.

Nitric oxide-scavenging activity

Nitric Oxide scavenging by the squalene the change of optical density of NO was monitored**. (Figure 12**) shows the comparative NO scavenging activity of the squalene and ascorbic acid standard. Nitric oxide or reactive nitrogen species, formed during their reaction with oxygen or with superoxides, such as N02, N204, N304, N03. And N02 are very reactive. These compounds are responsible for altering the structural and functional behaviour of many cellular components. The squalene showed a good scavenging ability compared to the standard.

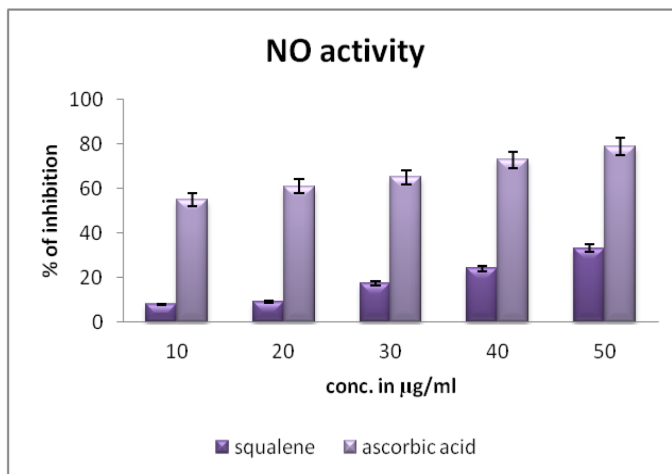

Figure 12 Nitric oxide-scavenging activities for Squalene.

Scavenging of super oxide

The super oxide scavenging activity of isolated squalene from *canthium coromendelicum* leaf extract estimated by comparing the percentage inhibition of super oxide radicals with ascorbic acid. (Figure 13) shows the scavenging activity of squalene increased with increasing concentration of squalene. The squalene moderately inhibit the superoxide radical generation.

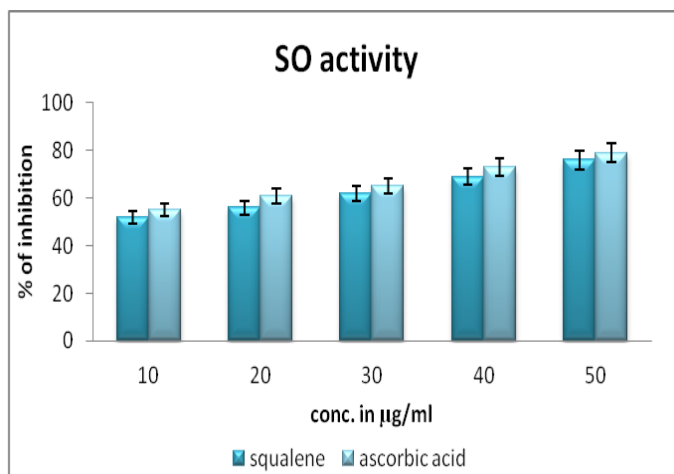

Figure 13 scavenging of Super oxide assay for Squalene.

Antibacterial activity

The antimicrobial activity of isolated squalene against four different bacterial Strains such as *Staphylococcus aureus*, *Salmonella typhimurium*, *Escherichia coli*, *Streptococcus pyogenes* A clear zone of inhibition was seen around the disc containg is isolated squalene different concentrations and standard antibiotic tetracycline these results are shown in (Figure 14(a) & 14 (b)).

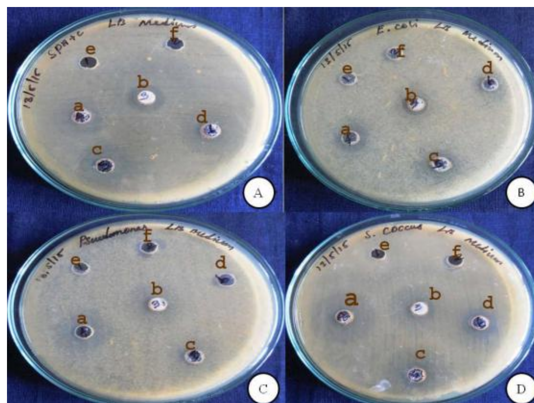

Figure 14 (a) Antibacterial Activity for Squalene.

Lingand : (A) *salmonella typi*, (B) *E. Coli,* (C) *Pseudomonas putida*. (D*) Staphylococcus aureus,*

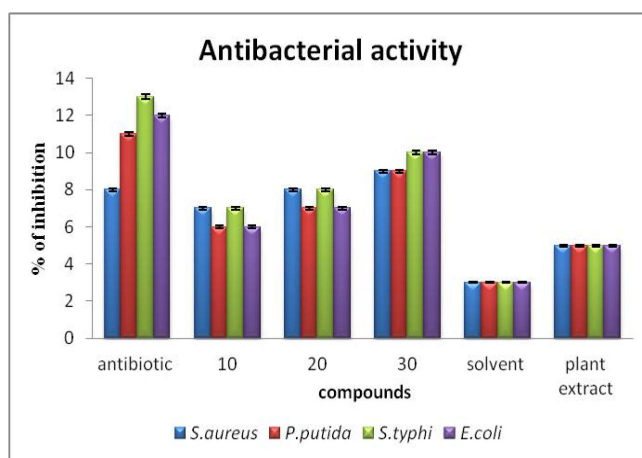

Figure 14 (b) Antibacterial Activity for Squalene.

Detection of apoptosis

The percent viability was analyzed by MTT assay after treatment with different concentration of Squalene.The results obtained for the cell survival as determined by MTT assay, The cytotoxic assays have been carried out in order to assess the optimal concentration at which, the compound gives maximum protection to the cells. (Figure 15) shows Cytotoxic assays MTT were used to analyze the cytotoxic effects of squalene in Hep-2 cells. The compound was added in 5 different concentrations namely 10, 20, 30, 40, 50µg/ml. The treated Hep-2 cells contained more apoptotic cells when compared to untreated monolayer (Fig 4). There was characteristic nuclear fragmentation of nuclei in treated HEp2 and the untreated control cells did not show any nuclear fragmentation. The apoptotic cells displayed the characteristic features of condensed nuclear chromatin and formation of membrane blebs.

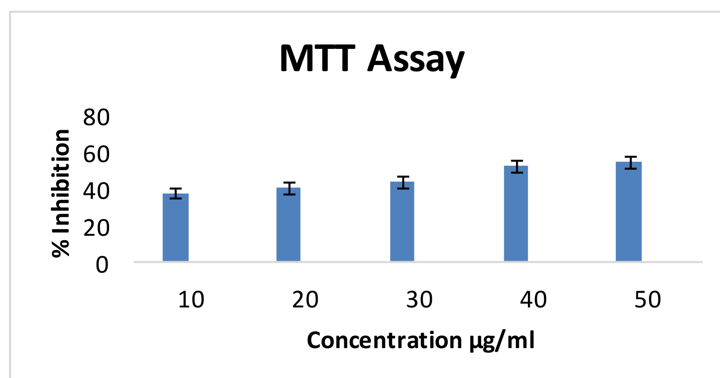

Figure 15 Cytotoxicity assay.

Cell morphology Assay

Morphological changes of apoptosis in Hep 2 cells

The characteristic features of apoptotic cells are cell shrinkage, membrane blebbing and cell swelling were analysed.

Propidium Iodide staining

The nuclear morphology that characterize apoptosis are chromatin condensation, nuclear fragmentation and cornering of the nuclear contents were analyzed using Propidium Iodide staining. The nuclear changes were observed in the Hep-2 cells exposed to presence and absence of squalene compound. (Figure 16) shows the apoptotic changes in the cells.

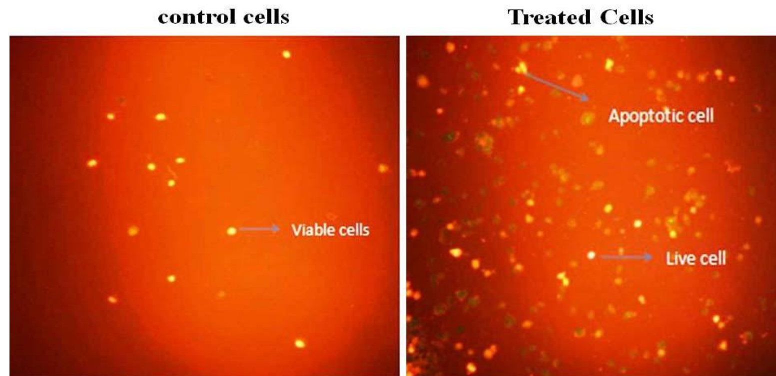

Figure 16 Nuclear morphology of control and treated cells by propidium iodide staining in Hep-2 cells.

Ethidium bromide staining

The characteristic features of apoptotic cells are cell shrinkage, membrane blebbing and cell swelling were analysed. These morphological and nuclear changes that resulted from treating the cells with the presence and absence of the squalene were analysed by Ethidium bromide staining. **(Figure 17)** shows the apoptotic changes in the cells.

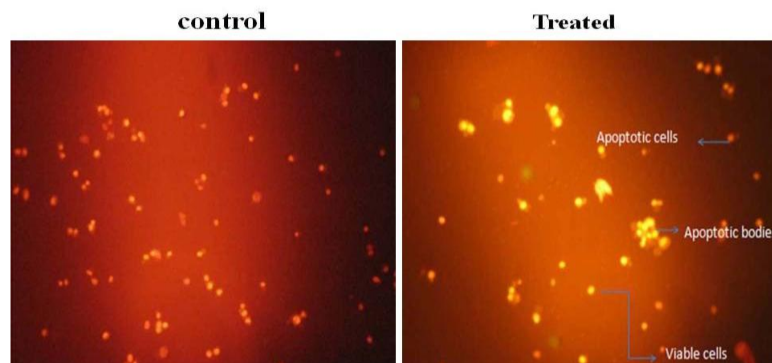

Figure 17 Ethidium bromide staining showing Control and Treated (apoptotic cells) in Hep-2 cells.

Giemsa Staining

The nuclear morphology that characterize apoptosis are chromatin condensation, nuclear fragmentation and cornering of the nuclear contents were analyzed using Giemsa staining. (Figure 18) shows the nuclear changes were observed in the Hep-2 cells exposed to presence and absence of squalene compound.

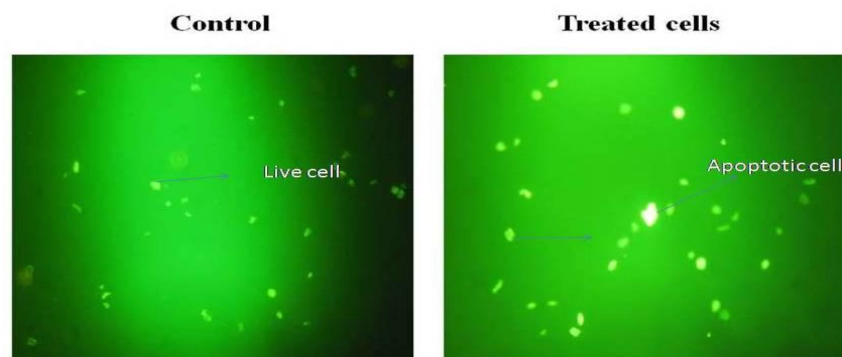

Figure 18 Giemsa staining showing Control and Treated (apoptotic cells) in Hep-2 cells.

Conflict of interest

The authors declare no conflict of interest.

ACKNOWLEDGEMENT

Authors are thankful to Madurai Kamaraj University to carry out this work

REFERENCE

1. Aliyu AB, Musa AM, Abdullahi MS, Oyewale AO (2008) Phytochemical and antibacterial properties of Ludwigia suffruticosa (Willd.) Oliv (Onagraceae). Int J Pure Appl Sci 2:1-5.
2. Prashant KR, Dolly J, Singh KR, Gupta KR, Watal G (2008) Glycemic properties of Trichosanthes dioica leaves. Pharm. Bio 46(12):894-899. http://dx.doi.org/10.1080/13880200802370167
3. Dzubak P, Hajduch M, Vydra D, Hustova A, Kvasnica M, Biedermann D (2006) Pharmacological activities of natural triterpenoids and their therapeutic implications. Nat Prod Rep 23: 394–411. doi:10.1039/b515312n
4. Mufflera K, Leipolda D, Schellera MC, Haasb C, Steingroewerb J, Bleyb T, Neuhausc HE, Miratad MA, Schraderd J, Ulbera R (2011)Biotransformation of triterpenes. Proc Biochem46: 1–15. doi:10.1016/j.procbio.2010.07.015
5. Evans WC, (2005) Saponins, cardioactive drugs and other steroids In Trease and Evans Pharmacognosy. Fifteenth Edition, published by Elsevier ; pg. no, 324.
6. Waller, (1970) Metabolism of plant terpenoids. Prog Chem Fats Other Lipids10:153-212
7. Chatterjee P, Kouzi SA, Pezzuto JM, Hamann MT(2000) Biotransformation of the antimelanoma agent betulinic acid by Bacillus megaterium ATCC 13368. Appl Environ Microb 66: 3850–3855. 0099-2240/00/$04.0010
8. Parra A, Rivas F, Garcia-Granados A, Martinez A (2009) Microbial transformation of triterpenoids. Mini-Rev Org Chem 6: 307–320. doi: http://dx.doi.org/10.2174/157019309789371569
9. Marienhagen J, Bott M, (2013) Metabolic engineering of microorganisms for the synthesis of plant natural products. J Biotech 163:166– 178. doi:10.1016/j.jbiotec.2012.06.001
10. Harborne, JB (1998) Methods of extraction and isolation. In Phytochemical Methods. London, UK: Chapman & Hall :60-64.
11. Reddy HL, Couvreur P (2009) Squalene: A triterpene for use in disease management and therapy. Advanced Drug Delivery Review 61:1412-1426. doi:10.1016/j.addr.2009.09.005
12. Kohno Y, Egawa Y, Itoh S, Nagaoka S, Takahashi M, Mukai K, (1995) Kinetic study of quenching reaction of singlet oxygen and scavenging reaction of free radical by squalene in n-butanol. Biochim Biophys Acta 1256:52–6. PMID:7742356
13. Trichopoulou A, Lagiou P, Kuper H, Trichopoulou D (2000) Cancer and Mediterranean dietary traditions. Cancer Epidemiol Biomarkers Prev. 9:869–73. PMID:11008902
14. Aguilera Y, Dorado ME, Prada FA, Martı´nez JJ, Quesada A, Gutie´ rrez VR (2005) The protective role of squalene in alcohol damage in the chick embryo retina. Exp Eye Res 80:535–43. doi:10.1016/j.exer.2004.11.003
15. Bhattacharjee P, Singhal, RS (2003) Extraction of squalene from yeast by supercritical carbon dioxide. Journal of Microbiology and Biotechnology 19: 605-608. doi:10.1023/A:1025146132281
16. Rao CV, Newmark HL, Reddy BS (1998) Chemopreventive effect of squalene on colon cancer. Carcinogenesis19:287–290. PMID:9498278
17. Nergiz C, Celikkale D (2011) The effect of consecutive steps of refining on squalene content of vegetable oils. J. Food Sci. Technol 48:382-385. doi:10.1007/s13197-010-0190-2
18. Ko TF, Weng YM, Chiou RYY (2002) Squalene content and antioxidant activity of *Terminalia catappa* leaves and seeds. J Agric Food Chem 50:5343–8. PMID:12207472
19. He HP, Corke H, (2003) Oil and Squalene in Amaranthus grain and leaf. J. Agric. Food Chem 51:7913–7920. doi:10.1021/jf030489q
20. Han-Ping He, Vizhong Cai,Mei sun,(2002) Harold Corke. Extraction and Purification of Squalene from Amaranthus *Grain*. J. Agric. Food Chem 50:368-372. doi: 10.1021/jf010918p
21. Pouchert CJ, Behnke J (1996). The Aldrich Library of 13C and 1H FT NMR Spectra. Aldrich Chemical Co Milwaukee WI: 46.

22. Saint-Leger D, Bague A, Cohen E, Chivot M (1996) A possible role for squalene in the pathogenesis of acne. I. In vitro study of squalene oxidation. Br. J. Dermatol 114:535- 542. doi: 10.1111/j.1365-2133.1986.tb04060.
23. Santhosh Kumar Chinnaiyan, Mohan Raj Subramanian S, Vinoth KumarAtul N, Chandu , Karthikeyan Deivasigamani (2013) Antimicrobial and anti-HIV activity of extracts of Canthium coromandelicum (Burm.f.) Alston leaves. Journal of pharmacy research 7:588-594. doi:10.1016/j.jopr.2013.06.026.
24. Suriyavathana Muthukrishnan, Sumathi Selvaraj, Kavitha RaniMari (2014) Phytochemical Characterization Using Various Solvent Extracts and GC-MS Analysis of Methanolic Extract of Canthium Coromandelicum (Burm. F) Leaves. International Journal for Pharmaceutical Research Scholars 3(3):178-185. ISSN No: 2277 – 7873.

Submit your next manuscript to IAJPR and take advantage of:
Convenient online manuscript submission
Access Online first
Double blind peer review policy
International recognition
No space constraints or color figure charges
Immediate publication on acceptance
Inclusion in ScopeMed and other full-text repositories
Redistributing your research freely
Submit your manuscript at: editorinchief@iajpr.com