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Comprehensive Assignments of Extraction, Isolation and Characterization of Taraxerol from Bark *Annona reticulata L.* and Chemopreventive Effect on Human Prostate Cancer Cell Lines (lncap and pc-3)

Saritha Surapaneni and Prakash T*

Department of Pharmacology, Acharya and BM Reddy College of Pharmacy, Bangalore, India

***Corresponding author:** Dr. T. Prakash, Department of Pharmacology, Acharya and BM Reddy College of Pharmacy, Bangalore-560 107, Karnataka, India, Tel: +918892943090; E-mail: prakasht@acharya.ac.in

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Abstract

Prostate Cancer (PC) ruins a foremost cause of death of males in the US as well its growth rate is increased in the rest of the world. The current learning aims to perform a preliminary photochemical analysis by the successive extraction of the bark of *Annona reticulata L.* using petroleum ether, chloroform, and ethanol. The isolation, structure elucidation and identification of Taraxerol and check up *in vitro* study in prostate carcinoma. The structure was elucidated by spectroscopic techniques included Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC), UV and Gas Chromatography-Mass Spectrometric (GC-MS). The prostate cell lines, LNCaP and PC-3 cell lines was cultured and antiproliferative effect by MTT Method, Neutral red cytotoxicity, measurement of LDH release, determinations of apoptosis by Acridine Orange (AO) and Ethidium Bromide (EB) double staining. Inhibition of protein denaturation, caspase levels by indirect ELISA and DNA fragmentation was performed. Investigation of the phytochemical summary on the bark of *A. reticulata L.* reports the occurrence of flavonoids, saponins, triterpenoid and tannins. *In-vitro* experiments show the selected compound exhibited of cytotoxicity against the cancer cell lines. An increase in caspase activity or caspase levels is generally considered as indicators of cellular apoptosis. The compounds to prevent heat associated denaturation of albumin are measured as a screening method for assessing anti-inflammatory potential of compounds.

Keywords: *A. reticulata L*; Triterpinoid; Taraxerol; Gas chromatography-mass spectrometric (GC-MS); MTT assay; DNA fragmentation; Caspase-3/7

Abbreviation:

ANOVA: Analysis of Variance; DHT: Dihydrotestosterone; TLC: Thin Layer Chromatography; HPLC: High Performance Liquid Chromatography; GC-MS: Gas Chromatography-Mass Spectrometric; AO: Acridine Orange; EB: Ethidium Bromide

Introduction

Phytochemical and pharmacological activities of components imply a broad range of clinical application in lieu of cancer therapy. Some of these plants exhibited a wide range of biological activities such as cytotoxicity [1]. The current investigation deals with the qualitative and quantitative pharmacognostical assessment of the bark of *A. reticulata L.* investigation reports the isolation of a triterpenoid glycoside from the bark of *A. reticulata L.* The compound gave positive colour reactions with specific spray reagents indicating that it may be a triterpenoid. It gave a pink spot on spraying with Liebermann-Burchard reagent with Vanillin-sulphuric acid reagent the compound gave a violet colour. *A. reticulata L.* (Family: Annonaceae) small deciduous green tree commonly called as bullock's heart widely distributed in tropical and subtropical regions [2]. *In vivo* cycling of cancer cells has been established to be a useful method to select for extremely aggressive cell lines. The human prostate cancer cell lines, PC-3 and LNCaP, were beforehand cycled *in vivo* to choose for tremendously metastatic variant from sentinel lymph node metastasis. These human cancer models contain proven highly advantageous to the prostate cancer research community [3]. Human prostate cancer cell lines are one of the cell lines used in prostate cancer research. These cells are useful in investigating the biochemical changes in advanced prostatic cancer cells and in assessing their response to chemotherapeutic agents. Moreover, they can be used to create subcutaneous tumors in mice in order to investigate a model of the tumor environment in the context of the organism [4]. Taraxerol, a triterpenoid compound, has potent anti-inflammatory effects according to review of literature statistics suggest that Taraxerol down regulates the turn of phrase of proinflammatory mediators in macrophages by interfering with the activation of TAK1 and Akt, thus prevent NF-κB activation [5].

Materials and Methods

Collection and authentication of the plant material

The fresh bark of *Annona reticulata L.* were collected washed and dried from the plant in the month of July 2014 and authenticated by Dr. Ajmeera Ragan and the voucher specimen was preserved within the herbarium of the university.

Preparation of successive extracts

The mature bark of collected locally were cleaned with water, shade dried and grounded into fine powder 200 g of *A. reticulata L.* the dried bark powder using Soxhlet apparatus. Each extract obtained following successive extraction was filtered using Whatman No. 1 filter paper, dried to a semisolid mass using water bath and the yield of each extract

thus obtained was recorded and stored in a refrigerator at 40°C till further use [6].

Photochemical analysis

A stock focus on 1% (W/V) of every successive extract obtain using petroleum ether, chloroform; ethanol was geared up using the respective solvent. These extract beside with positive controls were tested for the existence of active phytochemicals of tannins, alkaloids, phytosterols, triterpenoid, flavonoids, cardiac glycosides, anthraquinone glycosides, saponins, carbohydrates, proteins, amino acids and fixed oils and fats following standard methods [7,8]. The crude extract was suspended in water and n-hexane successively to afford n-hexane water soluble, respectively. Purification of n-hexane soluble fraction by column chromatography.

Isolation of taraxerol

Preparation of Column-Thin-layer chromatography was adopted by the Suvarchala. Liquid Chromatography Profiling of Taraxerol in crude extract materials used and Taraxerol HPLC grade methanol, Sonicator, Thermo fisher's instruments with PDA detector (200 nm-600 nm), Syncronis C18 (250 × 4.6) mm with particle size of 5 µ. Mobile phase-(1) Buffer: Methanol in the rate of 0.5: 9:5 at the flow rate of 1.2 ml/min (2) Method: Isocratic (3) Injection volume 10 µl. Sample Preparation-10 mg of sample is dried and crushed and dissolve in 10 ml of HPLC grade methanol.

Standard Preparation: Taraxerol used as standard and was preparation run under similar condition. The wave length for maximum absorption of Taraxerol is 211 nm and the flow rate was maintained at 1-2 ml/min. Sample and standards solution as well as the mobile phase were degassed and filtered through 0.45 µm membrane filter (Millipore). A chromatographic operation was carried out at ambient temperature.

Method: Separation was done in the isocratic mode using potassium di hydrogen Orthophosphate buffer KH2B4: Methanol (0.5: 9.5%) at a flow rate of 1.2 ml/min with an injection volume of 10 µl: UV detection was at 211 nm. Identification of the compound was done by comparison of that retention's time and UV absorption spectrum with those of standards.

Solubility: Methanol [9].

General experimental procedure: The HPLC UV spectrum was recorded on a thermo fisher. The FT-IR spectra obtained, on a Bruker Tensor 27 FT-IR type spectrometer, 1H and 13C NMR were recorded on Bruker 400 MHz (1H:400 MHz, 13C:400 MHz)Spectroscopy analytical test facility, Society for innovation and development (SID), NMR data centre, Indian institute of Science Bangalore.

In vitro Cell culture PC3 (human prostatic carcinoma) LNCaP (human prostate adenocarcinoma cells) cell line was initially procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained Dulbecco's modified Eagles medium (Gibco, Invitrogen). The cell line was cultured in 25 cm^2 tissue culture flask with (Dulbecco's modified Eagles medium) DMEM supplemented with 10% (Fetal bovine serum) FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin (100 U/ml), Streptomycin (100 µg/ml), and Amphotericin B (2.5 µg/ml). Cultured cell lines were kept at 37°C in a humidified 5% CO_2 incubator (Thermo Fisher Scientific, Bangalore). The viability of cells were evaluated by direct observation of cells by Inverted phase contrast microscope and followed by MTT assay method. Antiproliferative effect by MTT Method For the MTT assay Fifteen mg of MTT (Sigma, M-5655) of reagent was used as previously described in Deep et al. The absorbance values were measured by using micro plate reader at a wavelength of 570 nm and discussed [10,11]. The percentage of growth inhibition was intended using the formula: % of viability=Mean OD Samples × 100 Mean OD of control group. Neutral Red Assay was performed according to the method adopted from Zhang. The absorbance was measured using micro plate's reader at 540 nm and the percentage viability was calculated [12]. LDH (Lactate Dehydrogenase Assay) release assay was performed with cell free supernatant collected from culture plates exposed to different concentration of silver nanoparticles (5, 14, 28, 56, 200 µg/ml) and the method adopted from Laurenzana et al. [13].

Determinations of apoptosis by acridine orange (AO) and ethidium bromide (EB) double staining

DNA-binding dyes AO and EB (Sigma, USA) are used for the morphological detection of apoptotic and necrotic cells. AO is taken up by both viable and non-viable cells and emits green fluorescence if intercalated into double stranded nucleic acid (DNA). EB is taken up only by non-viable cells and emits red fluorescence by intercalation into DNA. The cells were cultured in Dulbecco's modified Eagles media and grown to 60-70% confluency and treated at final concentrations. The cells were washed by cold PBS and then stained with a mixture of AO (100 µg/ml) and EB (100 µg/ml) at room temperature for 10 min. The stained cells were washed twice with 1X PBS and observed by a fluorescence microscope in blue filter of fluorescent microscope (Olympus CKX41 with Optika Pro-5 cameras). The cells were divided into four categories as follows: living cells (normal green nucleus), early apoptotic (bright green nucleus with condensed or fragmented chromatin), late apoptotic (orange-stained nuclei with chromatin condensation or fragmentation) and necrotic cells (uniformly orange-stained cell nuclei) [14].

Caspase levels by indirect ELISA

Cell lysate (100 µl each) was added to the 96 well plate/and kept in incubation for 37°C overnight. The following day, the wells were drained and washed with PBS for 3 to 5 times Added 200 µl of blocking buffer (1 h at room temperature) Composition-0.2% gelatin in 0.05% tween 20 in PBS (freshly prepared) and washed with PBS TWEEN (2 times). Add 100 µl of primary antibody (Anti human, caspase 9 Santhacruz, USA) and kept for 2 hours at room temperature. The antibody was washed in PBS (2 times). Added secondary antibody (antihuman HRP conjugated secondary antibody-100 µl) and left for 1 h at room temp. Washed with PBS TWEEN for two times and added with 200 µl of chromogen for 30 min at room temperature in dark conditions. The colour was developed using O dianizdine hydrochloride and the reaction was stopped by adding 5N HCL (50 µl) OD was read at 415 nm in an ELISA reader and relative absorption.

O-dianizdine (composition–1 mg/100 ml methanol+21 ml citrate buffer pH-5+60 ml H_2O_2 Percentage increase of caspase activity=(OD of test-OD of control)/OD of test X 100.

Caspase-9 (also known as Mch6, Apaf-3 or IC E-LAP-6) is a member of the Interleukin-1β Converting Enzyme (ICE) family of cysteine proteases. Caspase-9 exists in cells as an inactive 46 kDa proenzyme, called pro-Caspase-9. Pro-Caspase-9 is cleaved to active 35 and 10 kDa subunits by complexing with a cofactor, Apaf-1,

through a caspase recruitment domain (CARD) and with cytochrome c during apoptosis. The downstream substrates of Caspase-9 include Caspases-3 and -7. Caspase-9 plays a critical role in the survival of neuronal progenitors within the embryonic forebrain and cortex. Phosphorylation of Caspase-9 by Akt, a serine/threonine kinase, inhibits its protease activity. The Caspase-9 colorimetric protease assay provides a simple and convenient means for quantitating the enzyme activity of caspases that recognize the amino acid sequence, LEH. The cells were treated with 97.2 µg/ml of sample and incubated for 24 h. The cells were digested after incubation with cell lysis buffer and the supernatant was coated on to a 96 well plate. 100 µl of primary antibody (Invitrogen, USA) was added to each well and incubated for 2 h at room temperature. After PBS washing nonspecific binding was avoided by using 3% BSA in PBS. The wells were washed with PBS and added with 100 µl (1:1000) HRP conjugated secondary antibody (Santa Cruz, USA). Color was developed using Danisidine-H_2O_2 substrate and read using an ELISA reader at 410 nm [15].

DNA fragmentation

The cell was implanted in 6 well plates. After attaining 60% confluency, in PC-3 and LNCaP cell lines was added, an untreated control cells site also maintained and incubated. The cell was totally centrifuged for 5 min at 3,000 rpm in a micro centrifuge. The pellets thus obtain and washed twice through PBS (137 mM NaCl, 27 mM KCl, 100 mM NaHPO, 2 mM KHPO, pH 7.4). Then the pellet was resuspended in 0.5 mL of lysis buffer (10 mM Tris-HCl, pH 8.5, 5 mM EDTA, 200 mM NaCl, 0.2% SDS) and incubated at 60°C for 5 min. 2.5 µL of Thermo Scientific Proteinase K (Cat #EO0491, EO0492) and 5 µL of Thermo Scientific RNase A/T1 Mix (Cat #EN0551) were added to it. It was incubated at 60°C for 1 h. 250 µL of 5 M NaCl was added drop by drop and mixing was done properly follow by incubation on ice for 5 min to precipitate protein and centrifugation done for 15 min at 10,000 rpm in a micro centrifuge and the supernatant transferred to a fresh tube then equal volume of isopropanol added to it and mixed well still the precipitate of the DNA is formed The cells were again centrifuged for 10 min at 10,000 rpm in a micro centrifuge complete supernatant was discarded and the pellet was washed by means of 1.2 mL 70% cold ethanol the DNA pellet is dried and suspended in TE buffer.

Loading order: M–Marker, Lane 1-Control untreated PC-3 cells, Lane 2-Control untreated LNCaP cells Lane 3-PC-3 cells treated with compound, Lane 4: LnCap cells treated with compound [15].

Results and Discussion

The proportion of yield of petroleum (6.58), chloroform (8.20), and ethanolic (18.26) was recorded. Phytochemical investigation of petroleumether, chloroform, etanolic extract of *A. reticulata L.* were recorded and bark extract show the presence of phytochemical constituents namely, Pet ether extract gives: +ve test for Sterols, Fats and Fixed oils. Chloroform extract gives: +ve test for alkaloids, non-sugar moieties for glycosides, triterpenoid and tannins. Ethanolic extracts gives +ve test for flavanoids saponins, triterpenoid tannins. Sample was crystallized from n-hexane/acetone (15:1) as white needles, Melting Point: 303-305°C. It gave pale of colors' (pink to blue to green) to Liebermann-Burchard test. Hence, the compound was identified. TLC analysis of the fractions using solvent systems n-hexane/acetone (15:1), exposed the presence of shows potential spots as shown in TLC no. of spots-1, Rf values-0.59. While the IR spectrum exhibit peak at 3494.51 (OH) stretching, 2941.11 (C-H) alkyl stretching, 1681.65 (C=C) cyclic bending cm-1 and 1043.9 (C-H) cyclic stretching (Figure 1). Broadband at 3419.2 cm1 indicate its triterpenoid property [16]. Based upon the HPLC it can be finished that retention of sample and Taraxerol was initiate to be 3.13 and 3.11 respectively observed in (Figure 2) by compare the preservation factor and the reaction of the peak in the chromatogram of the standard by means of the illustration chromatogram, the classification of the isolated compound as Taraxerol based upon retention of the paradigm and sample λ max of sample and standard was establish to be 211 nm based on UV spectroscopic studies explained in (Figure 3). The use of a mixture of chromatographic and spectroscopic techniques is necessary. With the application of various stationary phases, solvent systems and spray reagents, TLC proved to be a significant method for the characterization of sample mixtures [17]. HPLC was an effective and quicker instrument for the complete or at least partial quantitative separation of individual samples. For qualitative separation and quantitation GC is exceedingly capable whereas for identification and structure elucidation GC-MS and NMR are important methods. 21 Steroidal based nuclei is confirmed by (UV, IR and 1H NMR, 13C NMR, GCMS are explained [18]. Basic nuclei occurrence of functional groups like OH stretching, C-C aryl bending, C-H alkyl stretching, C-H aryl stretching like OH and Cyclic rings being confirmed by IR stretching and bending vibrations. Nature of proton of CH_3, CH_2, CH, OH, types being identified HNMR1 spectroscopy explained in (Figures 3-5) [16]. The nature of tertiary, secondary, primary and methyl carbon the corresponding delta values in association with expected structures further the structure is confirmed by mass spectrum the m/e value observed (73, 147, 207, 221, 281, 341, 355, 401, 426) was in connection with parallel fragmentation patterned explained in (Figure 6) [17-19]. In order to assess the *in-vitro* analysis of Taraxerol in prostate cancer cells, the androgen-dependent LNCaP and androgen independent PC-3 cells (Figure 7) were treated with Taraxerol and the analysis of proportion of viable cells revealed that at a concentration of 100 µg/ml sample Taraxerol, there were nearly 49.8% viable cells. The IC 50 of the sample Taraxerol was estimated to be 97.26 µg/ml in PC-3 and LNCaP is estimated as 80.61 µg/ml on the cultured cells. MTT results (Figures 8-10) exhibits a substantial decrease in cell viability in a concentration dependent manner [20]. 100 µg of compound reduced the cell viability to 47.87% with IC 50 value of 80.16 µg/ml suggest significant anticancer activity. This can be outstanding to the effect of sample Taraxerol, which induces apoptosis. And it can be conditional that the sample at a lower concentration might show good anti-cancer activity. The neutral red uptake assay delivers a quantitative estimation of the amount of viable cells in the treated cell lines. LDH is used as a quantitative enzyme for the intact cells; quantity of LDH leakage is an important test for cellular membrane permeabilization since it indicates several irreversible cell damage. LDH is an intracellular enzyme and leakage of LDH to media depicts loss of membrane integrity and in the current study treatment with Taraxerol has increased LDH leakage to media confirming apoptosis/necrosis can be interrelated with (Figure 11) [21]. Neutral red cytotoxicity test was based on the aptitude of living cell to uptake and bind neutral red is a positively stimulating dye that easily diffuses through cellular cytoplasm and stores in the acidic atmosphere of lysosomes. Principle of test is that neutral red is absorbed and bound only by live cell, while this ability is declined or damage in lifeless cells explained in (Figure 12) [22]. The amount of stored neutral red was thus right proportional to the sum of live cells in cell culture. In neutral red it can be seen that according to a dose-dependent manner the proportion of viable cells are getting decreased. Double staining method (AO/Et-Br staining) for evaluating the cytotoxic impact of Taraxerol revealed the statistic that

the Taraxerol is capable of inducing apoptosis. The proportion of live cells decreased on increase in concentration. The reduction in live cells and increase in apoptotic cells indexed the toxic effect of Taraxerol exerted on the Taraxerol treated PC-3 cells. Apoptosis popularly take place within developmental circumstances in reaction to physiological stimuli such as hormones and is dependent on de novo gene expression. In malice of the perceived omnipresence of this observable fact, little is known about what tells a cell to die and less still about the physiological and molecular mechanisms that bring about death. Treatment with compound shows in LNCaP cells with red fluorescent nuclei an indicator of apoptosis in (Figure 13). Inhibition of protein denaturation of the sample is assessed the length of with the diclofenac sodium the capability of compounds to prevent heat associated denaturation of albumin is measured as a screening method for evaluation anti-inflammatory potential of compounds. Diclofenac sodium is measured as a potent standard and the ability of compound to resist protein denaturation is expressed in standardising of percentage inhibition shows effective in (Figure 14). DNA fragmentation is used to confirm initiation of apoptosis by compound treated. The results clearly depict increased fragmentation in both PC3 and LNCaP upon compound treatment which can be attributed to the apoptotic movement of compounds in (Figure 15). Caspases mainly considered as major indicators in apoptosis as pro caspases are initiated during apoptosis. An raise in caspase activity or caspase levels is generally considered as indicator of cellular apoptosis. In our study it can be observed that treatment of compounds increased the relative caspase 3 levels to 49% suggesting cellular apoptosis (Figure 16).

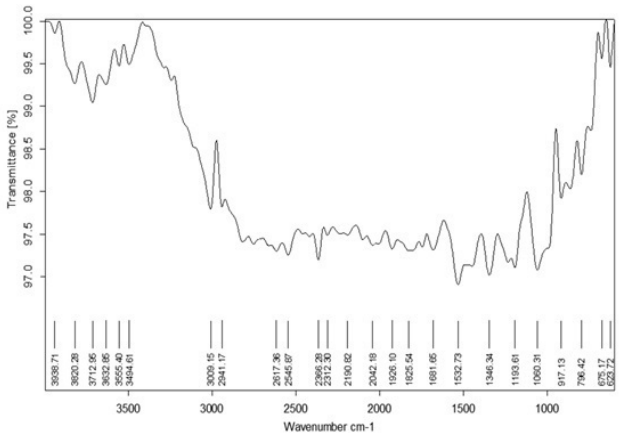


Fig. 1. Fourier transform infrared spectroscopy (FTIR) of Sample

Figure 1: Fourier transform infrared spectroscopy (FTR) of sample.

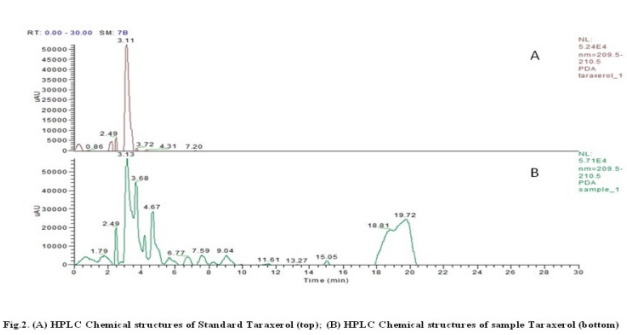


Fig.2. (A) HPLC Chemical structures of Standard Taraxerol (top); (B) HPLC Chemical structures of sample Taraxerol (bottom)

Figure 2: A) HPLC Chemical structures of standard Taraxerol (top). B) HPLC Chemical structures of sample Taraxerol (bottom).

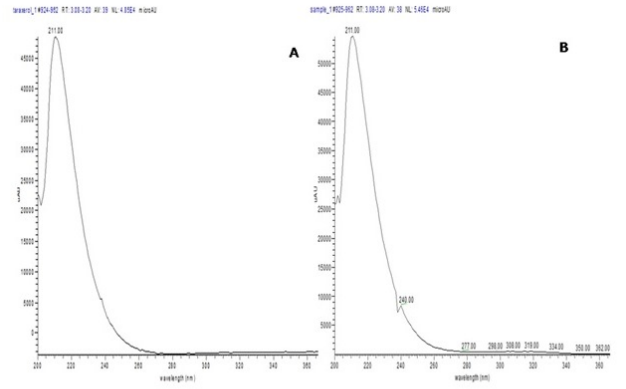
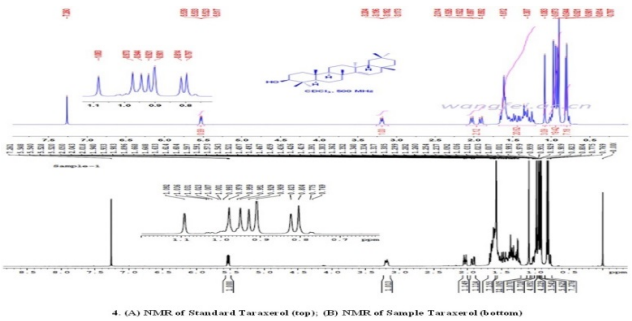


Fig. 3. (A) HPLC UV chromatograms Chemical structures of Standard Taraxerol (top); (B) HPLC UV chromatograms Chemical structures of sample Taraxerol (bottom)

Figure 3: A) HPLC UV chromatograms Chemical structures of Standard Taraxerol (top). B) HPLC UV chromatograms Chemical structures of Sample Taraxerol.



4. (A) NMR of Standard Taraxerol (top); (B) NMR of Sample Taraxerol (bottom)

Figure 4: A) NMR of Standard Taraxerol (top). B) NMR of Sample Taraxerol (bottom).

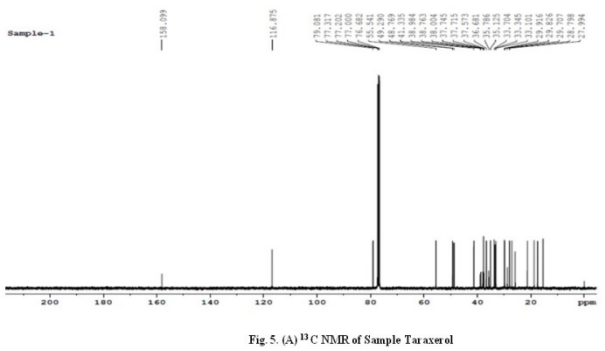


Fig. 5. (A) ^{13}C NMR of Sample Taraxerol

Figure 5: 13C NMR of Sample Taraxerol.

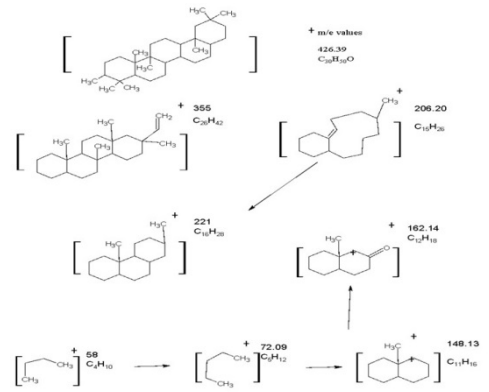


Fig. 6. (A) Schemes 1- Fragmentation

Figure 6: Schemes 1-Fragmentation.

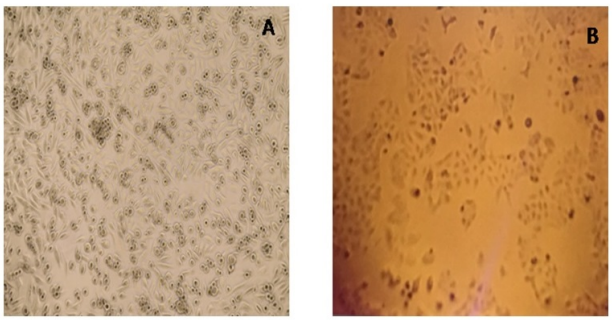


Fig. 7. (A) PC-3 Cells at confluency 80% picture (B) LNCaP Cells at confluency 80% picture

Figure 7: A) PC-3 Cells at confluency 80% picture. B) LNCaP Cells at confluency 80% picture.

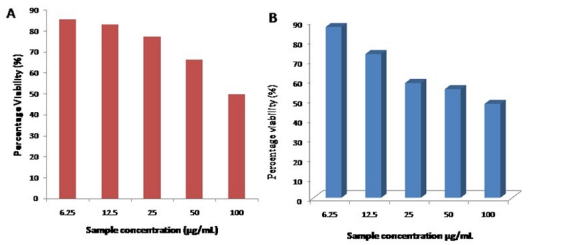


Fig. 8. Effect of Taraxerol on cell viability measured by MTT assay along X axis percentage cells viability (%) and X axis concentration of sample (µg/ml). (A) PC3 cells (B) LNCaP cells, were treated with Taraxerol at various concentrations (6.25, 12.5, 25, 50, 100 µg/mL for 48 h. The percentage of viable cells was determined as the ratio between treated cells and untreated controls. Results were expressed as mean ± standard deviation (S.D.) of three independent experiments.*p<0.05 as determined by a Student t-test compared to the untreated control.

Figure 8: Effect of Taraxerol on cell viability measured by MTT assay along X axis percentage cells viability (%) and X axis concentration of sample (µg/ml). A) PC3 cells .B) LNCaP cells, were treated with taraxerol at various concentrations (6.25, 12.5, 25, 50, 100 µg/ml for 48 h. The percentage of viable cells was determined as the ratio between treated cells and untreated controls. Results were expressed as mean ± standard deviation (SD) of three independent experiments *p<0.05 as determined by a student t-test compared to the untreated control.

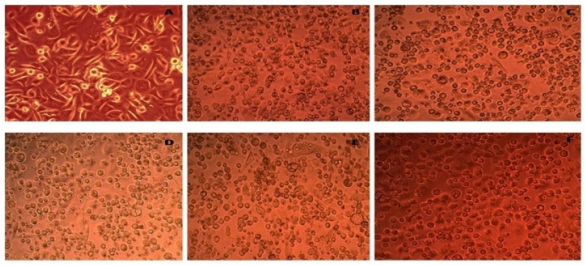


Fig. 9. MTT Phase contrast analysis of PC3 cells treated with taraxerol. Treatment with sample decline cells morphology and nuclear changes observed (A) Control pc-3 cell line (B) 6.25 µg in 100 µl of 5% DMEM (C) 12.5 µg in 100 µl of 5% DMEM (D) 25 µg in 100 µl of 5% DMEM (E) 50 µg in 100 µl of 5% DMEM (F) 100 µg in 100 µl of 5% DMEM

Figure 9: MTT phase contrast analysis of PC3 cells treated with taraxerol.treatment with simple decline cells morphology and nuclear changes observed. A) Control pc-3 cell line. B) 6.25 µg in 100 µl of 5% DMEM. C) 12.5 µg in 100 µl of 5% DMEM. D) 25 µg in 100 µl of 5% DMEM. E) 50 µg IN 100 Ml of 5% DMEM. F) 100 µg in 100 µl of 5% DMEM.

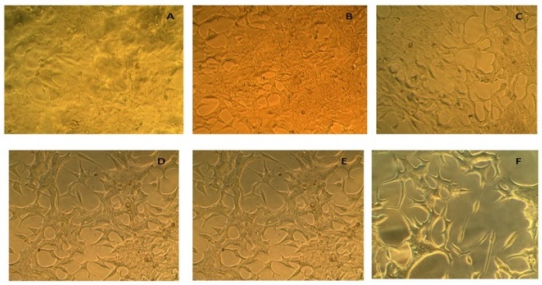


Fig. 10. MTT Phase contrast analysis of LNCaP cells treated with taraxerol. Treatment with sample decline cells morphology and nuclear changes observed (A) Control LNCaP cell line (B) 6.25 µg in 100 µl of 5% DMEM (C) 12.5 µg in 100 µl of 5% DMEM (D) 25 µg in 100 µl of 5% DMEM (E) 50 µg in 100 µl of 5% DMEM (F) 100 µg in 100 µl of 5% DMEM

Figure 10: MTT phase contrast analysis of LNCaP cells treated with taraxerol. Treatment with sample decline cells morphology and nuclear changes observed. A) Control LNCaP cell line. B) 6.25 µg in 100 µl of 5% DMEM. C) 12.5 µg in 100 µl of DMEM. D) 25 µg in 100 µl of 5% DMEM. E) 50 µg IN 100 µl of 5% DMEM. F) 100 µg IN 100 Ml of 5% DMEM.

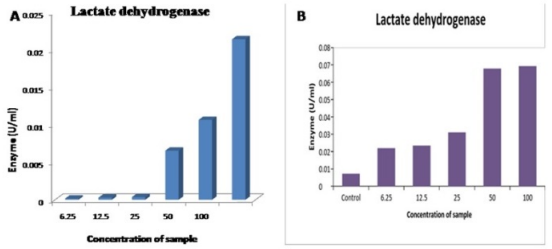


Fig. 11. LDH leakage showing increase membrane permeability: Along *Y axis* enzyme Units /ml and *X axis* concentration in µg/ml

Figure 11: LDH leakage showing increase membrane permeability. Along Y axis enzyme Units/ ml and X axis concentration in µg/ml.

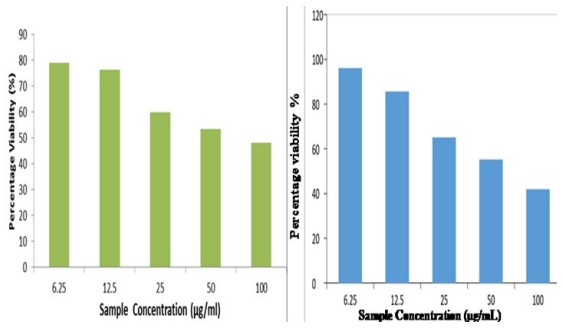


Fig. 12. Neutral red uptake assay showing decreased: Along *Y axis* enzyme units /ml and *X axis* concentration in *µg/ml*

Figure 12: Neutral red uptake assay showing decreased. Along Y axis enzyme units/ml and X axis concentration in µg/ml.

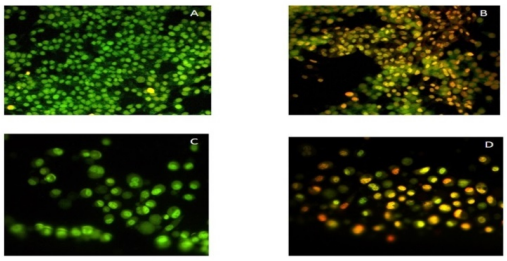


Fig. 13. Determination of analysis of Taraxerol. (A) Untreated PC-3 Cell lines before treatment control cells showing intact cells with Acridine orange stained green nucleus (B) cells treated with 6.25 *µg/ml* taraxerol increased apoptosis. (C) LNCaP cells before treatment (D) LNCaP cells after treatment under Fluorescence analysis revealed caspase activity in Green staining indicates the localization of AO, red staining represents EtBr and yellow staining represents the co-localization of both AO and EtBr.

Figure 13: Determination of analysis of Taraxerol. A) Untreated PC-3 Cell lines before treatment control cells showing intact cells with acridine orange stained green nucleus. B) cells treated with 6.25 µg/ml taraxerol increased apoptosis. C) LNCaP cells before treatment. D) LNCaP cells after treatment under fluorescent analysys revealed caspase activity in Green staining indicates the localization of AO red staining represents EtBr and yellow staining represents the colocalization of both AO and EtBr.

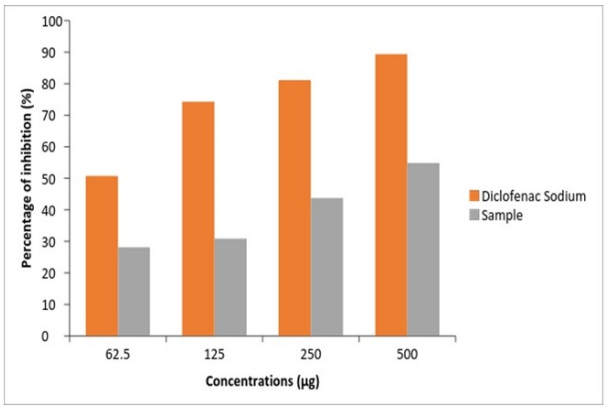


Fig. 14. Inhibition of Protein denaturation

Figure 14: Inhibition of protein denaturation.

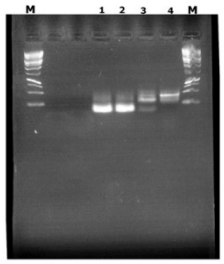


Fig. 15. Loading order, M – Marker, Lane 1- Control untreated PC3 cells, Lane 2- Control untreated LNCaP cells, Lane 3- PC3 cells treated with compound, Lane 4- LNCaP cells treated with compound.

Figure 15: Loading order, M-Marker, Lane 1-Control untreated PC3 cells,Lane 2-Control untreated LNCaP cells, Lane 3- PC3 cells treated with compound, Lane 4-LNCaP cells treated with compound.

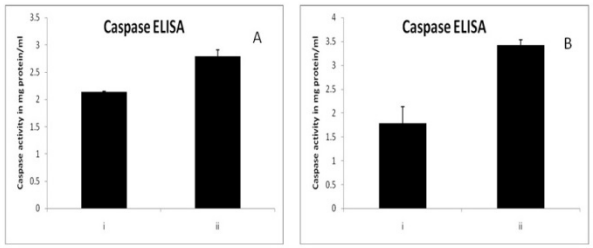


Fig. 16. Caspase activity in activity units per mg protein , Along X axis Samples (I – untreated control , ii- Compound treated (A) PC-3 and (B) LNCaP at IC50 values)

Figure 16: Caspase activity in activity units per mg protein. Along X-axis samples (I-untreated control, ii- compound treated. A) PC-3. B) LNCaP at IC50 values).

Conclusion

The chemical components of *A. reticulata L.* is investigated which resulted in the separation of Taraxerol. The occurrence of Taraxerol is reported from *A. reticulata L.* bark species. So that it can be relayed that the sample Taraxerol is effective against the PC-3 and LNCaP cell lines loss of lysozymal function with treatment of compound was observed in a dose dependent manner suggesting anticancer activity. Results postulated that the Taraxerol is more cytotoxic and effective in inducing apoptosis.

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