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Antioxidant and anti-proliferative activity of different solvent extracts of *Casuarina equisetifolia* needles

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Abstract

The context and purpose of the study: Plants that have been used for traditional medicines are very good sources of phyotochemicals. There are many plants like *Casuarina equisetifolia*, which are still unexplored for their medicinal properties. In the present study, we have elucidated the in vitro antioxidant and antiproliferative activity of different solvent extracts (both polar and non polar) of *C. equisetifolia* needles.

Main findings: In vitro antioxidant activity of different solvent extracts of *C. equisetifolia* needles was studied by analyzing the total polyphenols, flavonoids, total antioxidant capacity and free radical scavenging activity. The polar solvent extracts showed significantly high amount total polyphenols, flavonoids, antioxidants and free radical scavenging activity compared to the non polar solvent extracts. The cytotoxic and apoptosis inducing activity of the different solvent extracts were analyzed on MCF-7 cells by MTT assay, acridine orange/ethidium bromide staining, DAPI staining and caspase-3 release. The polar solvent extracts did not show any growth inhibition in MCF-7 cancer cells. But the non polar solvent extracts are very good in inducing cell death by inducing apoptosis which involves DNA fragmentation and release of caspase 3. Using silica gel fractionation and RP-HPLC analysis the active component present in non-polar solvent extracts was identified as ascorbic acid.

Brief summary and potential implications: Our results indicated that the needles of *C. equisetifolia* are rich sources of antioxidants and also contain potential anticancer agents. Detailed study on the mechanism of action of purified compound on inhibition of cancer cell growth may provide some potential anticancer molecule from natural source.

Key words : *Casuarina equisetifolia*, Antioxidant, Anticancer, Apoptosis, Caspase-3.

Introduction

Oxidative stress, results from an imbalance between formation and neutralization of pro-oxidants has an effect on a number of lifestyle diseases [1]. Consumption of dietary bioactive compounds and microelements from functional foods, herbs and neutraceuticals can alleviate these diseases to a greater extent and results in good health [2]. Cancer is a complex disease affecting public health worldwide. A number of naturally occurring compounds such as alkalods, terpenoids and polyphenols from vegetables and herbs exert antitumor and chemopreventive effects [3, 4]. Up to 70% of the approved anticancer agents (vincristine, vinblastine, taxol, camptothecin) were derived directly or indirectly from natural sources. The discovery of new lead molecules and elucidation of their underlying mechanisms will lead to the development of alternative and complementary means for cancer prevention and treatment.

Casuarina equisetifolia belongs to the family Casuarinaceae is found in the dry hill sides and open forests of India, Sri Lanka and Australia. The phenolic compounds from the needles (branchlets) and bark showed significant antioxidant activity [5]. Its bark is astringent and the seeds are antihelminthic, antispasmodic and antidiabetic [6]. However, no information is available on the in vitro antioxidant or antiproliferative activity of *C. equisetifolia*. So the present study focused on the detailed analysis of in vitro antioxidant and antiproliferative activities of different solvent extracts of *C. equisetifolia* needles.

Materials and Methods

Reagents and Chemicals

All the chemicals used for the study were of high quality analytical grade reagents. Methanol, Ethanol, Ethyl acetate and Hexane were purchased from Sisco Research Laboratory, India. Gallic acid, ascorbic acid, DPPH, Ethidium bromide, MTT, Acridine orange,

DAPI, paraformaldehyde, caspase-3 assay kit were purchased from Sigma Chemical Co., USA. DMEM, Penstrep solution and FBS were from Himedia, India. MCF-7 cells were purchased from NCCS, Pune.

Plant material and Preparation of extracts

Cassuarina equisetifolia needles were collected from Kerala during August-September season and identified by Dr. Toji Thomas. A voucher specimen (No.MGU-968) is deposited in the herbarium collection of plant biology department in MG University, Kerala, India. 5g of the needles were extracted (suceessive) with 20 ml of different solvents (water, methanol, ethanol, ethyl acetate, and hexane). The extracts were filtered through cheese cloth, centrifuged 5000 rpm for 15 minutes. From the supernatants, the solvents were evaporated under reduced pressure and dried extracts were stored at 4°C until use.

Estimation of total polyphenols

Total phenolic content of each extract was determined by the Folin–Ciocalteu micro-method [7] using gallic acid as standard.

Estimation of total flavonoids

Total flavonoid content present in various solvent extracts were determined by aluminium chloride colorimetric assay [8] using quercetin as standard.

Total antioxidant capacity

This assay is based on the reduction of Mo (VI) to Mo (V) by the sample and the subsequent formation of a green phosphate/Mo (V) complex at acidic pH [9] using ascorbic acid as standard.

Free radical scavenging activity by DPPH method

The free radical scavenging activity of different solvent extracts of Casuarina and trolox was measured by DPPH method [10]. The results are expressed as EC_{50} value (the amount of extract in µg needed to decrease the initial OD of DPPH to 50%).

DNA protection assay

Potential DNA damage inhibition by various solvent extracts of *C. equisetifolia* was analysed by photolysing H_2O_2 by UV radiation in presence of pBR322 plasmid DNA and performing agarose gel electrophoresis with the irradiated DNA [11]. The gel was stained with ethidium bromide (1ug/ml) and photographed on BioRad gel documentation system.

Cell culture

The cells were maintained in DMEM supplemented with 10% FBS and 100mg/l streptomycin and 100U/l penicillin. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Cell viability assay

MTT assay was used to measure the effect of different solvent extracts of *C. equisetifolia* on human breast cancer MCF-7 cell's viability [12]. Viability of the cells was calculated as,
% viability = [(absorbance of treated cells)/(Absorbance of untreated cells)]x 100.
Cisplatin was used as a positive control for cell vibility studies.

Apoptosis assay by Acridine orange/Ethidium bromide staining

MCF-7 cells were treated with different solvent extracts of Cassuarina for 48 hours were washed with PBS and trypsinised. 25 µl of cell suspension ($1x10^4$ cells/ML) were incubated with 1µl of acridine orange/ethidium bromide (one part each of 100 µg /ML of acridine orange and 100 µg/ML of ethidium bromide in PBS) just prior to microscopy (13). A 10 µl of gently mixed suspension was placed on a microscope slide covered with glass slips and examined under fluorescent microscope (Olympus 1X 71) connected to a digital imaging system.

Nuclear fragmentation assay-DAPI staining

Exponentially growing MCF-7 cells ($1x10^4$/ML) were incubated for 48 hours with different solvent extracts of Cassuarina. Apoptotic nuclear morphology was visualized by DAPI staining (14). Cells were fixed with 4% paraformaldehyde and the fixed cells were stained with DAPI (10 µg/ML) under dark for 10 minutes. After staining, the cells were washed twice with PBS and visualized using fluorescent microscope (Olympus 1X 71).

Caspase 3 activity assay

Caspase 3 activity of cell lysates of both treated and untreated conditions were measured by colorimetric assay kit according to the manufacturer's protocol (Sigma). Samples were read at 405 nm in a microplate reader and expressed as µM of paranitroaniline released per minute per mg cell protein (15).

Identification of active components in non-polar solvent extracts

The non-polar solvent extracts were fractionated using silica gel low pressure column chromatography. 200 mg of sample dissolved in hexane has been loaded on a 15cm x 1.2cm i.d. silica gel column (6-120 mesh size) which was preconditioned with hexane. Elution was started with pure hexane and thereafter increased the polarity of the eluent by adding 1%, 2%, 3%, 4%, 5% of ethyl acetate in hexane and finally 100% of ethyl acetate.
The identification of the active component in the fraction showing positive cytotoxicity was done using Waters 1525 HPLC system equipped with a C-18 column (150x4.6nm, I.D. 5 µM), a binary pump system, UV visible detector (Waters model 2487) and an auto sample injector. The mobile phase was consisted of 0.1 %v/v acetic acid (solvent A) and HPLC grade methanol (solvent B). Total running

time was 20 min and the gradient programme was as follows: 90 % A and 10% B for 0- 5 min, 70 % A to 30 % B for 3 min, 55 % A to 45 % B for 3 min, 30% A to 70%B for 2 min, 20% A to 80% B for 2 min, and 100% B for 5 min. The sample injection volume was 20 µl and the wavelength of the UV-VIS detector was set at 255 nm. The retention time the standard was also determined using the same protocol.

Statistical Analysis

Results were collected from three independent experiments performed in triplicates. Data were analyzed using SPSS statistical analysis programme and are expressed as means ± standard deviation (SD).

Results and discussion

Phytochemicals are rich sources of antioxidants which will fall into different categories such as phenolics, steroids, alkaoids and terpenoids (16). In the present study we have used different solvent extracts (water, methanol, ethanol, ethylacetate and hexane) of *C. equisetifolia* needles to elucidate its in vitro antioxidant and anti proliferative property. The total yield of each solvent-extractable matter was more in methanol (77.2%), followed by ethanol (39.2%), water (31.2%), ethyl acetate (1.2%) and hexane (0.8%).

Total phenolic content, flavonoid content and antioxidant activity of each solvent extracts of *C. equisetifolia* needles were analyzed and the results are given in Table 1. The ethanol extract showed maximum phenolic content (67.14±0.535 mg/g eq. gallic acid) and the least was for hexane (26.30±0.301 mg/g eq. gallic acid). Total flavonoid content of water, methanol, ethanol, ethyl acetate and hexane extracts were 24.25±0.858, 37.75±0.351, 46.02±0.593, 17.18±0.522 and 14.05±0.525 mg/g quercetin respectively. Total antioxidant activity was maximum for ethanolic extract (30.48±0.522 mg/g eq. ascorbic acid) and least for hexane extract (10.16±0.350 mg/g eq. ascorbic acid). There was a linear correlation between the phenolic content and antioxidant activity. Higher antioxidant activity of ethanolic extract may be associated with its high polyphenol content (17).

Table 1: Total polyphenols, flavonoids and antioxidants present in various solvent extracts of *C. equisetifolia* needles. Values are mean of triplicate determinations ± standard deviation.

Sample	Total polyphenols (mg/g gallic acid eq.)	Total flavonoids (mg/g quercetin eq.)	Total antioxidants (mg/g ascorbic acid eq.)
Water	36.40±1.045	24.25±0.858	20.29±0.497
Methanol	44.26±0.742	37.75±0.351	27.09±0.156
Ethanol	67.14±0.535	46.02±0.593	30.48±0.522
Ethyl Acet.	34.70±0.815	17.18±0.522	14.99±0.601
Hexane	26.30±0.301	14.05±0.525	10.16±0.350

The free radical scavenging activity was analysed by DPPH radical scavenging method. The results expressed as EC_{50} (µg) values were shown in Fig. 1(A). The EC_{50} value was less for water extract (341 µg) and high for ethyl acetate extract (3088 µg). This radical scavenging activity might prevent ROS from damaging biomolecules in the system (18).

The DNA protective effect of various solvent extracts of *C. equisetifolia* on plasmid DNA pBR322 was analyzed (Fig. 1(B). The plasmid DNA when treated with H_2O_2 or UV alone (lane 2 and 3) showed an electrophoresis pattern compared to the control (Lane 1). The OH radical generated by the photolysis of H_2O_2 upon UV irradiation resulted in the cleavage of supercoiled DNA to open circular and linear form, indicating DNA strand scission (lane 4). 10 µg of different solvent extracts of *C. equisetifolia* protected pBR322 plasmid DNA from this free radical induced DNA cleavage (lane 5 to 9). Extracts from medicinal plants were reported in the protection of DNA against free radical induced cleavage (19).

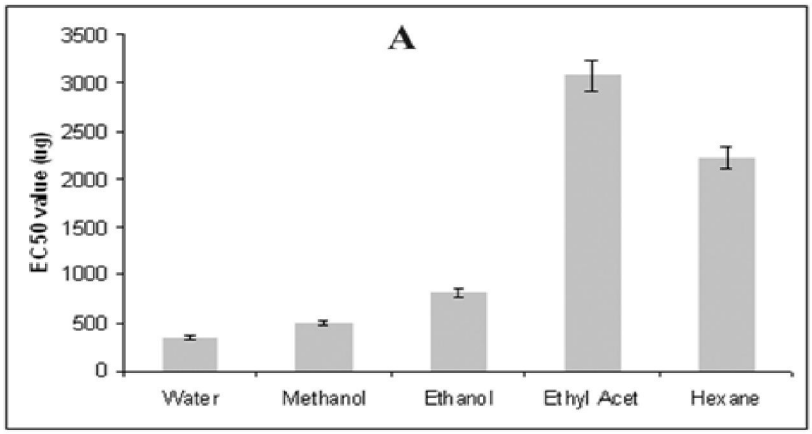


Figure 1(A): DPPH free radical scavenging activity of different solvent extracts of *C. equisetifolia*. µg of sample required to reduce initial OD of DPPH by 50% (EC_{50}). Values are mean of three independent experiments ± standard deviation.

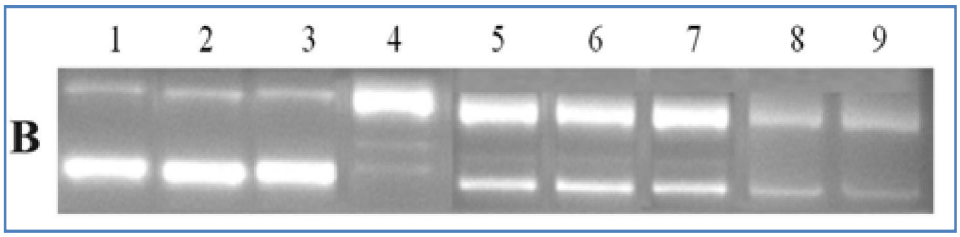


Figure 1(B): Effect of different solvent extracts of *C. equisetifolia* on the protection of supercoiled DNA against hydroxyl free radical generated by photolysis of H_2O_2. Lane 1: Plasmid DNA alone, Lane 2: Plasmid DNA+UV, lane 3: Plasmid DNA+30 mMH_2O_2, lane 4: . Plasmid DNA+30 mMH_2O_2+ UV, lane 5: Plasmid DNA+30 mMH_2O_2+ UV+ 5µg water extract, lane 6: Plasmid DNA+30 mMH_2O_2+ UV+ 5 µg Methanol extract, lane 7: Plasmid DNA+30 mMH_2O_2+ UV+ 5 µg Ethanol extract, lane 8: Plasmid DNA+30 mMH_2O_2+ UV+ 5 µg Ethyl Acet. extract, lane 9: Plasmid DNA+30 mMH_2O_2+ UV+ 5 µg Hexane extract.

The effects of different solvent extracts of *C. equisetifolia* on cell proliferation was studied by treating MCF-7 cells with different concentrations of (0.5, 1, 2, 5, 10 µg) extracts for 24 hours (Fig. 2A). The polar solvent (water, methanol, ethanol) extracts did not show any cytotoxic effects. But the non polar solvent (ethyl acetate and hexane) extracts showed significant reduction in cell viability. The GI_{50} (the amount of extract needed to reduce the viability of cells to 50%) value of ethyl acetate extract was found to be 1.5 µg whereas for hexane it was 0.5 µg. Cisplatin was used as the positive control which gave a GI_{50} value of 0.05 µg. This results correlates with many previous reports regarding the antiproliferative activity of non polar solvent extractable active principles from other plants (20, 21). Many plant extracts exert antiproliferative activity by inducing apoptosis (22). Acridine orange/ethidium bromide staining of MCF-7 cells treated with GI_{50} concentrations of non polar solvent extracts for 48 hours showed significant apoptosis as cells stained as yellow to orange color because of the incorporation of ethidium bromide along with acridine orange due to membrane breakage (Fig. 2B). 10 µg of polar solvent extract treated cells were similar to control cells with identical green in color. Nuclear fragmentation, a hallmark of apoptosis has been visualized by DAPI staining on cells treated with non polar solvent extracts, indicating that these extracts can induce apoptosis in MCF-7 cells (Fig. 2C). Apotosis occurs after a series of events which starts with the release of cytochrome c from mitochondria and finally the fragmentation of chromosomal DNA (23). Regardless of which pathway is initiated (extrinsic or intrinsic), the apoptosis eventually converge in the activation of the execution caspases, caspase 3 and 7. The assay performed for caspase-3 activity showed significant increase in caspase-3 activity in cell lysates of ethyl acetate and hexane extract treated MCF-7 cells (Fig. 2D). This clearly indicate that the active constituents present in the non polar solvent extracts of *C. equisetifolia* are involved in the activation of caspase 3, which subsequently results in the cleavage of poly ADP ribose polymerase (PARP) leading to DNA fragmentation during the later stages of apoptosis. This is the first report on the antiproliferative and apoptosis inducing activity of *C. equisetifolia* but the hepatoprotective activity of this plant was previously reported (24).

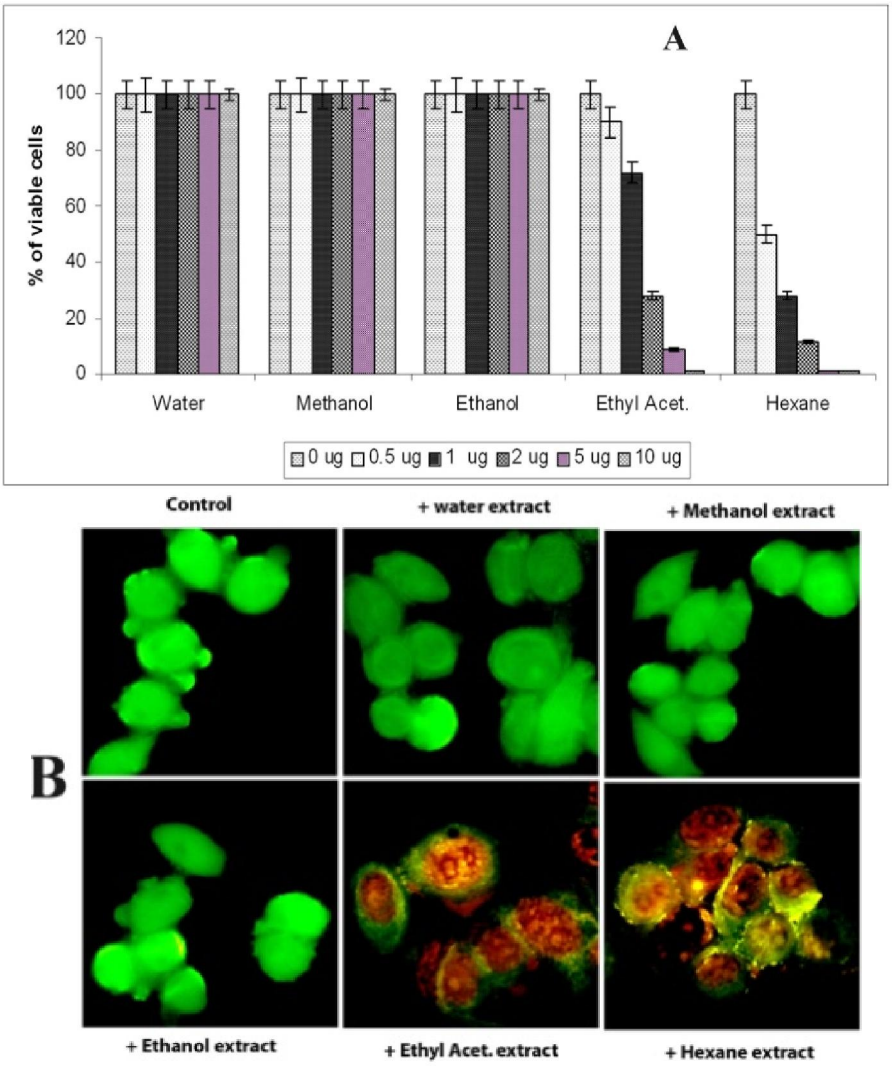
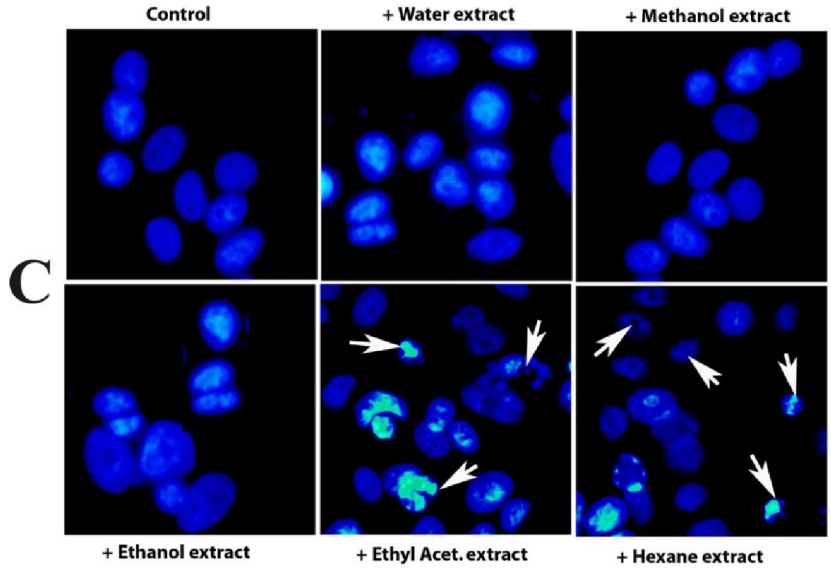


Figure 2(A): Effect of various solvent extracts of *C. equisetifolia* on proliferation of MCF-7 cells in vitro: cells were treated with different concentrations of extract for 24 hours and the cell viability was determined by MTT assay. Values are mean± standard deviations from three independent experiments.**(B):** Acridine orange/Ethidium bromide staining of MCF-7 cells to detect apoptosis induced by different solvent extracts of *C. equisetifolia*. Live cells are uniformly green, whereas apoptotic cells are characterized by yellow-orange staining by the co-staining with ethidium bromide due to the loss of membrane integrity. Magnification-10X. Results are representative of three experiments.



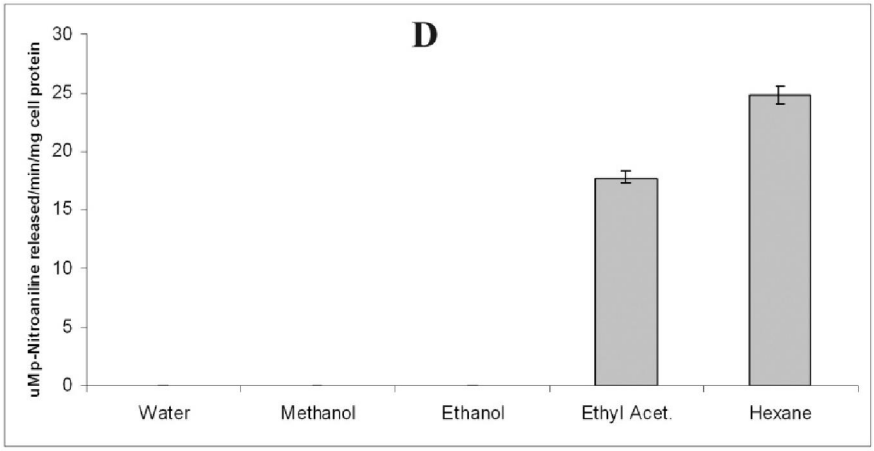


Figure 2(C): Nuclear morphology observation of MCF-7 cells treated with different solvent extracts of *C. equisetifolia* by DAPI staining. Arrows indicate apoptotic features (condensed chromatin and nuclear fragmentation). Magnification-10X. Results are representative of three experiments.**(D):**After treatment with different solvent extracts of *C. equisetifolia* for 48 hrs, the cell lysates were analysed for changes in caspase 3 activity using colorimetric assay. Results are expressed as the µM of pNitroaniline released per minute per mg cell protein. Values are mean of three experiments ± standard deviation.

The active components present in the non-polar solvent extracts which exert apoptosis in MCF-7 cells were identified using silica gel fractionation and HPLC as described in the methods. Residues collected from the eluted fractions of 1% ethyl acetate in hexane of both hexane and ethyl acetate extracts from silica gel column showed significant cytotoxic effects. To identify the active component in these two residues it was subjected to RP-HPLC analysis as described in the methods. The results (Fig. 3) indicate that the major peak obtained from both hexane and ethyl acetate extract was corresponding to ascorbic acid as indicated by the retention time of the standard ascorbic acid. From the literature it was found that ascorbic acid was capable of inducing apoptosis in variety of cancer cells such as melanoma and T cell leukemia (25, 26).

Conclusions

In conclusion, our observations indicated that the polar solvent extracts of C.Equistifolia needles are very good sources of antioxidants and the non polar solvent extracts are very good in

inducing apoptosis in cancer cells. The active component responsible for the apoptosis inducing effect was identified as ascorbic acid. Further studies are necessary for detailed mechanism of action and elucidate the biological pathway responsible for apoptosis induction.

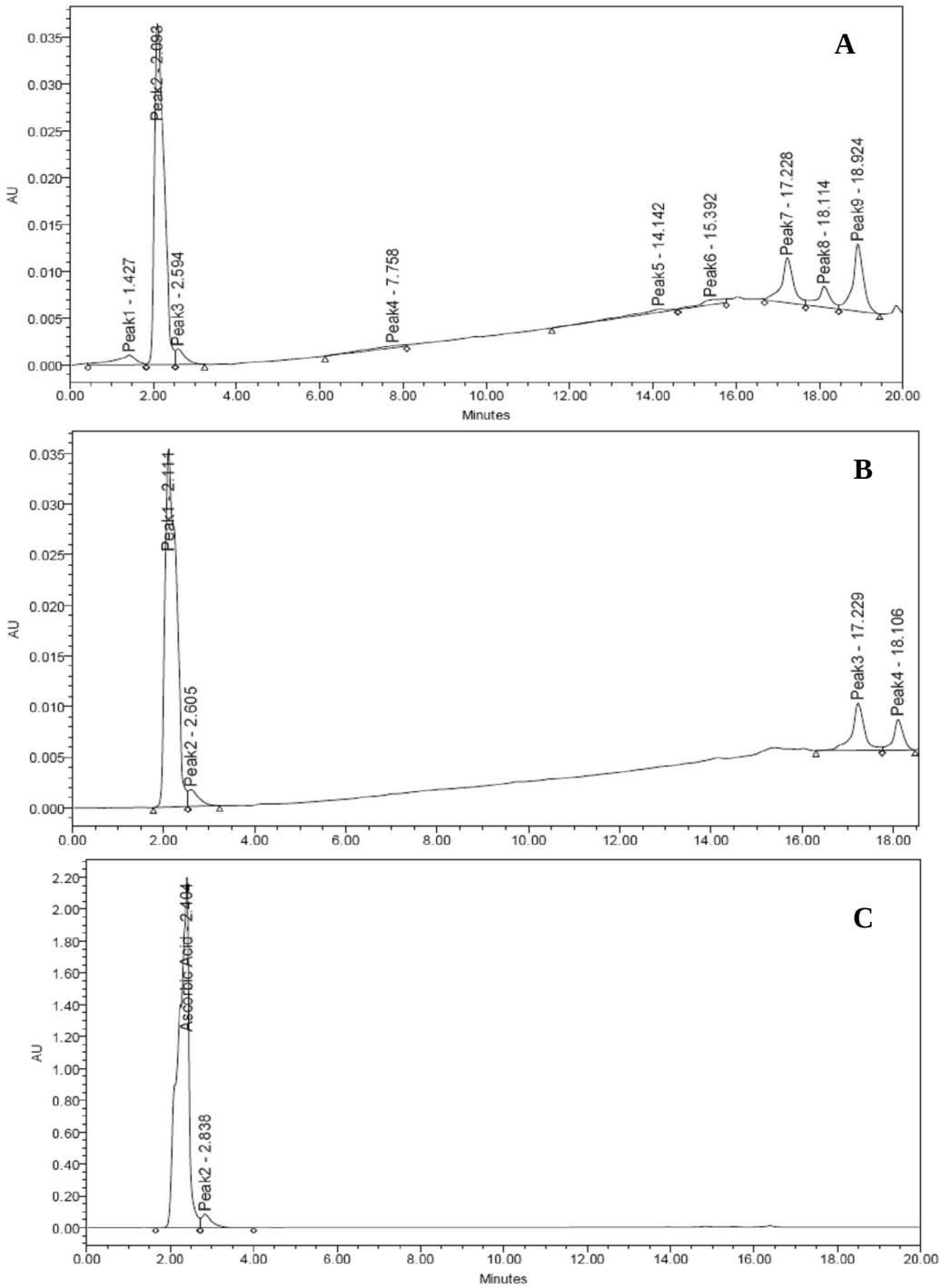


Figure 3:RP-HPLC profile of active fractions of ethyl acetate (A) and hexane (B), which induced cytotxicity in MCF-7 cells. Retention time of standard Ascorbic acid (D) has been given as reference.

List of Abbreviations

DPPH- α, α -diphenyl-β-picrylhydrazyl
MTT- (4, 5-dimethylthiazoly-2)-2, 5-diphenyltetrazolium bromide
DAPI- 4', 6-diamidino-2-phenylindole

Author's contributions

SPK designed the experiments. SP and AN did all the experimental works. SP wrote the manuscript and other two authors corrected and proof read it. The final version of the manuscript was equally approved by all the three authors.

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