Original Research Article

DNA damage protection by fatty acid rich fractions from *Andrographis Paniculata Nees (AP)*: An *in vitro* study

Badhe Pravin [1,2,3,*], Nadaf Kajal [1], Deshmukh Srushti [1], Otari Kishor [3], Badhe Ashwini [1,2]

[1]Swalife Biotech Ltd Unit 3D North Point House, North Point Business Park, Ireland; [2]Swalife foundation India; [3]Navshyadri Biotechnology research group, Navshayadri Institute of Pharmacy, India.

***Corresponding author:** Badhe Pravin, Navshyadri Biotechnology research group, Navshayadri Institute of Pharmacy, India.E-mail: drbadhepravin@gmail.com*

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ABSTRACT

As antioxidants prevent free radical caused DNA damage, they lower the risk of developing chronic diseases. The present study evaluated the impact of fatty acid-rich fractions of *Andrographis Paniculata Nees* (AP) on free radical-induced DNA damage. We prepared fatty acid-rich fractions with dried AP plants and analyzed them qualitatively using gas chromatography. In order to determine their cytotoxicity and free radical scavenging activities, MTT and Viacount assays were performed. *In vitro* proliferation assay was performed to assess the cell proliferation after treating with hydrogen peroxide (H_2O_2) and Ultra Violet-C (UVC). Further testing of the fractions was carried out using a high alkaline comet assay (pH>13) to determine their DNA-protective capacity. Our results indicated that AP fractions containing fatty acids possess DNA protective ability against H_2O_2 and UVC rays in human dermal fibroblasts.

***Keywords*:** Fatty acid, *Andrographis Paniculata Nees*(*AP)*, comet assay, DNA base damage

INTRODUCTION

Plants are an important source of antioxidant compounds [1]. Kalmegh (*Andrographis Paniculata Nees*) is a medicinal herb with an extremely bitter taste that belongs to the family Acanthaceae [2]. *AP* has several compounds that are significant chemical constituents: Andrographolide, 14 deoxy-

11, 12-didehydroandrographolide, 14 deoxyandrographolide, 3,14-dideoxyandrographolide, andrographic acid, 14-deoxy-11 oxoandrographolide, Kalmeghin, 5,7,2,3-tetramethoxyflavanone and 5-hydro-7,2,3-trimethoxyflavone [3].

The herb is said to possess antibacterial, antifungal, antiviral, hepatoprotective, blood pressure and pulmonary tuberculosis effects [4,5,6,7,8]. Extracts from fruit, leaf and stem have shown free radical scavenging activity [9]. It also scavenges free radicals from blood circulation, decreasing the number of transporter proteins necessary to uptake glucose from the blood stream or efficient plasma membrane receptors and control abnormal lipid metabolism [10]. It supports the idea that *AP* is useful for free radical scavenging.

Free radical can be produced by internal factors (metabolic) and external factors (chemicals, radiation, pollution, drugs, and ultra violet rays). Free radical produced by external factors can damage proteins and DNA. Ultra Violet (UV) rays are an important external factor [11]. UV rays are a real ecological factor with great effects on living organisms. The sun emits electromagnetic radiation in the ultraviolet, visible, and infrared spectrum. Ultraviolet radiation (UVR) has wavelengths between 200 and 400nm, a relatively shorter range than visible light (400-700nm). UVR are further divided into UVC (200–280 nm), UVB (280–320 nm), and UVA (320–400 nm). Among these, UVC is successfully trapped by ozone in the atmosphere. Unintentional exposure to UVC can occur from man-made sources, like germicidal lights [12].

The UV produces Reactive Oxygen Species (ROS), which cause DNA Double-Strand Breaks (DSBs) [13]. A Double-Strand Break (DSB) occurs when two corresponding strands of the double helix of DNA are damaged simultaneously in close proximity to each other. It is the most harmful type of DNA damage because even one unrepaired DSB is sufficient to initiate the cell death process [14,15].

When reactive oxygen breaks DNA, it recruits H2ax and PCNA proteins. Breaks can also play a role in the DNA repair pathway. A more specific marker of oxidative damage is the development of 8-oxo-7,8-dihydroguanine (8-oxogua). Among the DNA bases, Guanine has the

lowest oxidation potential since it is effectively oxidizable by singlet oxygen and OH [16]. Upon Guanine association with OH at C8, 8-oxo-G is produced, which further oxidized to 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG) [17]. By far the most common DNA lesions caused by free radicals are 8-oxoG and its altered items.

There have been reports of higher amounts of oxidized bases associated with diabetes, Cancer, arthritic, cardiovascular and neurodegenerative infections [18]. A universal antioxidant that can address all free radicals is as yet undiscovered. So, the task of identifying the particular antioxidant to combat particular free radicals is enormous.

Free radicals play a significant role in distinct diseases, such as skin diseases, Alzheimer's illness, arthritis, asthma, Parkinson's disease, heart attack, kidney and liver damage, retinopathy and cancer is currently broadly accepted to be involved [19,20,21]. Free radicals play a significant role in various diseases, making it necessary to seek compounds that protect us from these free radicals.

Antioxidants are substances that postpone, prevent or evacuate oxidative damage [22]. An antioxidant is a molecule that can prevent oxidative damage, compensating for the destructive effects and neutralizing them, while generating a steady product from its oxidation [23].

A long-chain polyunsaturated fatty acid might act as an indirect antioxidant rather than a pro-oxidant in vascular endothelial cells, so as to reduce inflammation and, therefore, the risk of atherosclerosis and cardiovascular disease [24]. The Conjugated Linoleic Acids (CLAs) are combinations of positional and geometric isomers of Linoleic Acid (LA). All tested CLAs have exhibited radical scavenging activities in a dose-dependent manner and were observed to immediately react with and quench DPPH radicals at all levels [25].Hydrogen peroxide (H_2O_2) radicals rank as one of the most reactive species in the ROS since they are relatively stable [26] and are a byproduct of aerobic metabolism. In biological systems, H_2O_2 causes immediate damage to DNA molecules, leading to DNA degradation and cancer.

Therefore, this study investigated the effect of fatty acid enrich fractions from *AP* on free radical caused DNA damage induced by H_2O_2 and UVC radiation.

MATERIALS AND METHODS

Dryer, grinder, sieve and shaker were available in the lab. Petroleum ether, acetone, hexane, dichloromethane, ethanol and phosphoric acid, were bought from Sigma Aldrich. GC column was bought from SGE USA. Methanol, formic acid, reserpine and phosphoric acid of analytical grade were bought from Sigma Aldrich (UK). *A.Paniculata* leaves/stems were brought from Bioprex Labs Pune (India). Ethyl acetate, sodium bicarbonate, hydrochloric acid, aqueous ammonia and sodium hydroxide of analytical grade were purchased from Sigma Aldrich (UK).

Modified Eagle's Minimal Essential Medium (DMEM), Phosphate buffer (pH 7.4), trypsin-EDTA, penicillin, streptomycin, Glutamine, Fetal Bovine Serum (FBS), Dimethyl sulfoxide, Trypan blue, Thiazolyl Blue Tetrazolium Bromide were obtained from sigma aldrich (UK). Guava instrument cleaning fluid and Guava ViaCount reagent were brought from Millipore corporation (UK). Human Dermal Fibroblasts (HDF) and Human liver carcinoma, $HepG_2$ cells were purchased from Health protection agency culture collection. Disodium phosphate (Na_2HPO_4), Monopotassium phosphate (KH_2PO_4), Ethylenediaminetetraacetic acid (EDTA) were purchased from sigma Aldrich (UK).

Hydrogen peroxide was bought from sigma Aldrich (UK) and Comet assay kit was purchased from Trevigen, Inc., (Gaithersburg, MD).

GC was performed on Petroleum ether (PE), Acetone (Ac), Dichloromethane (DCM) and Hexane (Hex) fractions. Fractions were transferred to its Fatty Acid Methyl Esters (FAMEs) [27].

FAMEs were prepared as follow:

The fractions were dissolved in 1mL hexane and put in a water bath at 60 ^{0}C for 10 minutes. Then 2 ml of 0.01 M sodium hydroxide in methanol was added to the tube containing the extracts. 1 ml of Boron trifluoride in methanol was added and then placed in water bath at 60 ^{0}C for further 10 min. Samples were cooled under running water and then 2ml of 20 % (w/v) sodium chloride and 1ml of hexane was added. After complete mixing, separation of the hexane layer containing FAMEs was performed by centrifugation at 1000 rpm for 3 min at room temperature. The samples were then proceed for GC analysis.

The FAMEs were analyzed by GC. The GC instrument was equipped with a flame ionization detector and a split/split less injector. A 50 mm 0.22 mm, 0.25 µm film thickness fused-silica capillary column BPX70 (SGE, Austin, TX, USA) was used for analysis. Oven conditions were 120 ^{0}C increased to 180 ^{0}C at a rate of 2 ^{0}C/min and maintained for 5 min. Helium was used as carrier gas and nitrogen as the make-up gas at a flow rate of 30 ml/min. The injector temperature was 200 ^{0}C and the detector temperature was 280 ^{0}C. The qualitative analysis of FAMEs composition was realized by peak retention time and comparing their retention times with standards methyl esters. All the GC analyses were run in triplicate. The retention time of standard fatty acids was compared with the extracts sample retention time.

Cell culture

Human Dermal Fibroblast (HDF) and human liver carcinoma cells (Hepg2) were grown with the help of culture media.

All ingredients listed in table-1 were pre-warmed at 37 ^{0}C before starting to prepare the medium. All the materials placed in the cabinet were sprayed with bio guard. Hand gloves were used while working in the cabinet to maintain the aseptic conditions.

Ingredients 2-5 from table-1 were measured and added to Dulbecco's Modified Eagle's bottle. Medium was filtered using a 0.2 µ filter. The cell vials were removed from nitrogen freezer and placed in a 37 ^{0}C water bath to rapidly defrost the suspension. Cells were plated in 90mm petri dishes and placed into a humidified incubator at 37 ^{0}C with 5 % carbon dioxide. Medium was changed on Tuesday and Friday. The cells were passaged twice every week. The medium was removed and the plate with culture was washed using versene (KCl 0.02 % (w/v), NaCl 0.8 % (w/v), KH2PO4 0.02 % (w/v), Na2HPO4 0.0115 % (w/v), and 0.2 % EDTA (w/v)). The cultures was then treated with a solution of 0.25 % trypsin: versene (1:10, v:v) to detach the cells from the tissue culture flasks (approximately 3-5 minutes). The effect of trypsin was neutralized by addition of an equal volume of DMEM medium. This cell suspension was centrifuged at 1000 rpm for 5 min. The supernatant was removed and the cells were re-suspended in a known volume of fresh medium.

Prior to performing any cell base analysis, the haemocytometer method was used to count the cells.

Cytotoxicity Assay

MTT Assay

MTT Assay was performed on the Hepg2 cells to find the cytotoxicity of the extracts on the cells. The plant extracts are applied in serial dilution. Cytotoxicity is determined by plotting the graph of Cell Viability Vs Concentration.

Cell viability formula = (Absorbance of sample/ Absorbance of positive) x 100

Cells were seeded on 96 well plates at a final concentration of approximately

1.5 x 10^4 cells per 200µl medium per well 24 hours before the assay. 96 well plates with cell suspensions were then incubated at 37 ^{0}C for 24 h.

After 24 hours the cell media was removed and the cells were treated with different concentration of extracts and incubated at 37 ^{0}C for 24 h.

After 24 h 20 µL MTT (5 mg/mL) dye solution in PBS was added to 96 well plates and incubated with cells for 3h at 37 ^{0}C. After 3 h the media containing MTT was removed and the plates were washed with 100 µl of PBS. After washing with PBS the solution of DMSO (200 µl) was added to the wells and kept on shaker for 5 to 10 min. The absorbance was measured at 580 nm using microplate reader [29].

Viacount Assay

Cells were seeded on 96 well plates at a final concentration of approximately 1.5 x 10^4 cells per 200 µl medium per well 24 h before the assay. 96 well plates with cell suspensions were then incubated at 37 ^{0}C for 24 h.

After 24 h the cell media was removed and the cells were treated with different concentration of extracts. The 96 well plates which are treated with different concentration were incubated at 37 ^{0}C for 24 h.

After 24h the supernatant was removed from the plate and the plate was wash twice with PBS solution and then Trypsin-EDTA (90 µl) was added for 1min to detach the cells. New media (90 µl) was added to the wells and 20 µl of viacount dye was added and incubated for 5 to 10min. Viacount assay was performed with "Guava" flowcytometer [30].

Antioxidant Assay (DPPH assay 2,2-diphenyl-1-picryl-hydrazyl-hydrate)

DPPH was performed using a Microplate Reader (BMG BMG LABTECH Instrument). The reaction mixture in each one of the 96-wells consists of extract solution, aqueous methanol solution, and 70 % ethanol as a blank containing DPPH radical's. The mixture was left to stand for 60 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 517 nm [31].

Plant fractions were subjected to primary screening using the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay to identify the cell viability, Viacount assay to estimate the viable cells and 2,2-Diphenyl-1- Picrylhydrazyl (DPPH) assay to identify the free radical scavenging activity.

Proliferation Assay

Proliferation Assay was performed on the Human Dermal Fibroblast (HDF) cells to find the effect of the fractions on the cells before and after exposing to hydrogen peroxide and UVC. The plant extracts are applied in serial dilution. Activity is determined by plotting the graph of Cell Viability Vs Concentration.

Cell viability formula = (Absorbance of sample/ Absorbance of positive) x 100

Same procedure was applied as used in MTT assay. The absorbance was measured at 580nm using microplate reader [29].

Single Cell gel electrophoresis (COMET) Assay

Cells were grown in 35mm petri dishes with normal cell culture protocol for a week. Low serum media (0.5 %) was added to the dishes and left for 7 days to make them Quiescent Fibroblast Cells (QFC). Cells respond differently to DNA damage dependent upon cell cycle position. Therefore, by using only quiescent cells, which incidentally better reflects the cells natural state in vivo, the cellular response is no longer affected by cell cycle events. On the experiment day the medium was removed and cells were washed twice with PBS. The QFC were first treated with samples (fractions) for one hour. After that media and sample was removed and exposed to damage causing substances such as Hydrogen peroxide (30 min) or ultra violet light such as UVC(25 J/m2). After that samples were added to the medium once more and left for 30 min.

After half an hour cells were washed twice with pre-warmed PBS. Trypsin- EDTA was added to coat the entire monolayer of cells. Cells were incubated for 2 min at 37

°C or until the cells easily detached upon tapping. 2 mL of complete media (containing foetal bovine serum) was added to inactivate the trypsin. Cells were transferred to a centrifuge tube and re-suspended at 1.5 x 10^5 cells/mL in ice cold 1X PBS.

This centrifuge tube with cells was further preceded to Comet assay. The tubes were placed in 37 ℃ water bath. The lysis solution was placed in fridge. Low Melting Agarose (LMA) is melted and place in 37 ℃ water bath. Amount of cells and LMA ratio used 1:10 (v/v) and 40 µL of this mixture is added onto the FLARE slide and spread with pipette tip. The FLARE slides were then placed in refrigerator (in dark) for 20 min. After 20 min the slides were removed and immersed in pre-chilled lysis solution and placed in refrigerator for 60 minutes. The slides were removed from the fridge and excess buffer was tap off. The slides were then immersed in 1X FLARE Buffer 1 changing solution three times over a 30 min period at room temperature to equilibrate the slide.

The assay was also adapted to detect Oxidative base damage by adding enzyme 75 µL Fpg enzyme (1:75 Fpg in Fpg Flare reaction buffer) is added to one sample area, which combines a specific glycosylase activity, removing the damaged base and creating an Apurinic/Apyrimidinic (AP) site, and an AP lyase which converts the AP site to a break. Without Fpg only strand breaks are identified. Addition of Fpg enzyme help to detect addition extra strand breaks caused by conversion of oxidised bases to cuts.

Two sets were prepared one with Fpg enzyme and one without Fpg. The slides were placed in 37 °C incubator for 60minutes. In meantime two litre of alkali solution (pH>13) was prepared (500mM EDTA 2mL, NaOH 8 gm in 1 L deionized water) and placed in cold room at 2-8 °C. The slides were removed from the incubator and transfer to a coplin jar containing alkali solution and incubate for 30 minutes at room temperature in the dark, changing the solution once. After alkali treatment the slides were transfer to horizontal electrophoresis chamber. The apparatus was then filled with chilled alkali solution until the level just to cover the slides. The voltage was set to about 22 volts for 30 min with the electrophoresis apparatus.

Slides were dried overnight at 37 °C. Drying brings all the cells to a single plane which facilitates observation. Samples slide may be stored at room temperature with desiccant before scoring. 100 µL of diluted SYBR Green I was added onto each circle of dried agarose and placed in a refrigerator for 15-30 min before taking images.

RESULTS

Gas Chromatography (GC) analysis of fractions

GC helped to identify different fatty acids including methyl palmitate, methyl stearate, methyl oleate and methyl Linoleate present in fractions of *A.Paniculata*.

GC analysis of phytochemicals from fractions was performed after converting them to Fatty Acid Methyl Esters (FAMEs).

The chromatogram in figure 2 derived from a standard mixture of four fatty acids (methyl palmitate (4.753), methyl stearate (8.369), methyl oleate (8.645) and methyl Linoleate (9.632), prepare by dissolving 1mg of the mixture in 1ml of solvent. The four fatty acids were first run separately as per the method described above and then the mixture of four fatty acids was run.

The retention time shown on the x-axis of the chromatogram is unique to the specific compound run with the specified method in that specific instrument.

Using the same method and conditions developed for standard mixture FAMEs analysis of *A.Paniculata* fractions of PE (Figure 3), and Ace, Hex DCM (data in supplement), were also run under the same conditions. The results obtained were compared with standard mixture peak (Figure 2).

Methyl palmitate was found in high concentration in PE, Ace and Hex fractions of *A.Paniculata*, while Methyl linoleate was found high in concentration in DCM extract.

The chromatogram in figure 3 is a fame analysis of the PE fraction of *A.Paniculata*, identify the four major fatty acids that are Methyl Palmitate (4.783), Methyl stearate (8.405), Methyl Oleate (8.670) and Methy Linoleate (9.667).

Methyl Palmitate, Methyl Oleate and Methy Linoleate are the major fatty acid present in the petroleum ether fraction.

Cytotoxicity Assay

To determine the cytotoxicity of the fractions and to identify the non-cytotoxic extracts concentration it was screen with MTT and Viacount assay. Fractions were screen in the range of 1.25 µg/ml to 0.156 µg/ml.

Each experiment was done in triplicate. Data were represented as means ± Standard Derivation. Statistical analysis was performed by Anova followed by post-hoc tukey test. Tukey test compare std with different concentrations and the stars indicate a significant difference ($^{***}P<0.0005$, $^{**}P< 0.005$ and $^{*}P<0.05$) to cells treated with different concentration of fractions. Where P= cells without samples, PE *Andrographis Paniculata Nees* (*AP)* fraction, *Ac Andrographis Paniculata Nees (AP), Hex Andrographis Paniculata Nees* (*AP)* fraction and DCM *Andrographis Paniculata Nees* (*AP)* fraction.

Andrographis Paniculata Nees (*AP)* fractions were screened with MTT assay in Hepg2 cells. Percentage of cell viability in petroleum ether and acetone was observed to be in the range of 75-95 % at applied concentration. While in hexane and dichloromethane the observed percentage of cell viability was in the range of 60-95%. Significant cell viability was observed in all *Andrographis Paniculata Nees (AP)* fractions at applied concentrations.

Andrographis Paniculata Nees (AP) fractions were also screened with the viacount assay (data in supplement) to confirm the MTT results. It helps to confirm that as concentration was increased the cell viability decreased. P value was also observed less than 0.05.

Antioxidants Assay

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) assay was first developed by Dr Blois in 1958 to determine the antioxidant activity using stable free radical α, α-diphenyl-β-picrylhydrazyl ($C_{18}H_{12}N_5O_6$, M=394.33) [32]. Mechanism is very simple which measure the scavenging capacity of antioxidants. Hydrogen atom from antioxidants reduces the odd electron of nitrogen atom in DPPH to the corresponding hydrazine [33].

Each experiment was done in triplicate. Data were represented as means ± Standard Derivation. Statistical analysis was performed by Anova followed by post-hoc tukey test. Tukey test compare std with different concentrations and the

stars indicate a significant difference ($^{***}P<0.0005$, $^{**}P< 0.005$ and $^{*}P<0.05$) to free radicals treated with different concentration of extracts. Where Neg Std= free radical solution without samples, PE *Andrographis Paniculata Nees (AP)* fraction, Ac *Andrographis Paniculata Nees (AP)* fraction, Hex *Andrographis Paniculata Nees (AP)* fraction and DCM *Andrographis Paniculata Nees (AP)* fraction.

Andrographis Paniculata Nees (AP) fractions were screened at same concentrated used in MTT assay. Significant scavenging activity was observed at low concentration that is 0.1562 µg/ml. High activity was observed in Ac *Andrographis Paniculata Nees (AP)*> DCM *Andrographis Paniculata Nees (AP)*> PE *Andrographis Paniculata Nees (AP)*> Hex *Andrographis Paniculata Nees (AP).*

Proliferation Assay

The *in vitro* proliferation assay was performed to determine whether or not cells are triggered to divide after treating with fractions and Hydrogen peroxide/UVC and assess differences between cell populations.

Each experiment was done in triplicate. Data were represented as means ± Standard Derivation. Statistical analysis was performed by Anova followed by post-hoc tukey test. Tukey test compare std with different concentrations and the stars indicate a significant difference ($^{**}P< 0.005$ and $^{*}P<0.05$) to cells treated with different concentration of fractions. Where Std= cells without samples, PE *Andrographis Paniculata Nees (AP)*, Ac *Andrographis Paniculata Nees (AP)*fraction, Hex *Andrographis Paniculata Nees (AP)*fraction and DCM *Andrographis Paniculata Nees (AP)* fraction.

Andrographis Paniculata Nees (AP) fractions were further tested to identify its effect on normal Human fibroblasts. Fractions were applied in same concentration used in previous experiments. It was observed that at all applied concentrations viability was maintained. High significant activity is observed at low concentration and it was decreased as the concentration was increase. *Andrographis Paniculata Nees (AP)* fractions help to maintain the cell

viability of HDF at applied concentration shown in figure 5. Now to see the effect of fractions in hydrogen peroxide treated or UVC expose experiments cells were treated with the fractions.

Andrographis Paniculata Nees (AP) fractions were then further tested to see what effect they produced when applied to HDF after treating to hydrogen peroxide and exposing to UVC. Significant activity was observed in all fractions at all applied concentration. As in previous experiment high significant activity was observed at low concentration (Data in supplement). This help to conclude that *Andrographis Paniculata Nees (AP)* fractions show high significant protective activity against hydrogen peroxide and UVC rays.

Single cell gel electrophoresis (SCGE)/ COMET assay

The most promising fractions were further tested for any effect on levels of DNA Damage suffered by HDF following exposure to hydrogen peroxide/UVC. Control, untreated cells or cells treated with fractions were irradiated with UVC or hydrogen perioxide and then levels of DNA damage estimated within individual cells using the COMET assay. To begin with, we tested fractions alone for any genotoxic effects on cells. Neither methanol nor acetone alone caused any detectable increase in DNA damage on cells.

Determination of Genotoxicity of selected A.Paniculata fractions

To test whether the extracts on its own produce any DNA damage, cells were treated with extracts for 60mins, before being prepared for the comet assay. Three independent experiments were performed and in total 200 cells were scored for each experiment. We estimated percentage DNA in comet Heads for WE (positive standard without extracts), 70 % ethanol(solvent to dissolve the extracts) S1, S2, S3, and S4.

Effect of plant fractions on DNA of QFC was studied with single cell gel electrophoresis. The effects measured were oxidative base damage and Strand breaks of DNA. Three independent experiments were performed and in total 200 cells were scored for each experiment. We estimated the percentage DNA in the comet Head for WE (positive standard without fractions), 70% Eth, S1, S2, S3 and S4.

These results suggest that all selected fractions from *Andrographis .Paniculata*

Nees (AP) do not induce any oxidative damage and strand breaks in QFC.

Effect of Plant fractions against Hydrogen peroxide induce DNA Damage

To test whether or not the fractions are able to protect cell from oxidative DNA damage caused by H_2O_2, cells were treated with fractions for 60mins, then washed and treated with H_2O_2 (0.2M) for 30 minutes, then washed and incubated with fractions again for further 30 minutes before being prepared for the comet assay. Three independent experiments were performed and in total 200 cells were scored for each experiment. We estimated percentage DNA in comet Heads for WE (positive standard without fractions and without H_2O_2), Eth-70 % Ethanol (solvent used to dissolve samples), S1, S2, S3 and S4

Cells treated with fraction alone didn't show any DNA damage. The next step then was to test the ability of extracts to protect cells from DNA damage deliberately caused by treatment of cultures with H_2O_2. QFC were treated with 0.25 µg/mL of fractions from AP. S4 fractions showed significant activity against oxidative damage (93 %) and Strand breaks (92 %) protection in H_2O_2 damage cells, While S1, S2 and S3 fractions showed significant activity against oxidative damage (75,80 and 82 %) and strand breaks (75,85 and 78 %) protection in H_2O_2 damage cells.

Table 1. Composition of cell culture after modification adopted from [28]

Sr No	Name of Ingredient	Quantity
1	Dulbecco's Modified Eagle's Medium with a high glucose content (4.5 g l−1)	500 mL
2	10 % v/v Foetal Bovine serum	56.5 mL
3	100 U ml−1 Penicillin and 10µg ml−1 streptomycin	6mL
4	1 % Glutamine	6mL
5	1 % non-essential amino acids	6mL

Figure 1. *Andrographis Paniculata Nees* (Kalmegh) plant (Akbar, 2011).

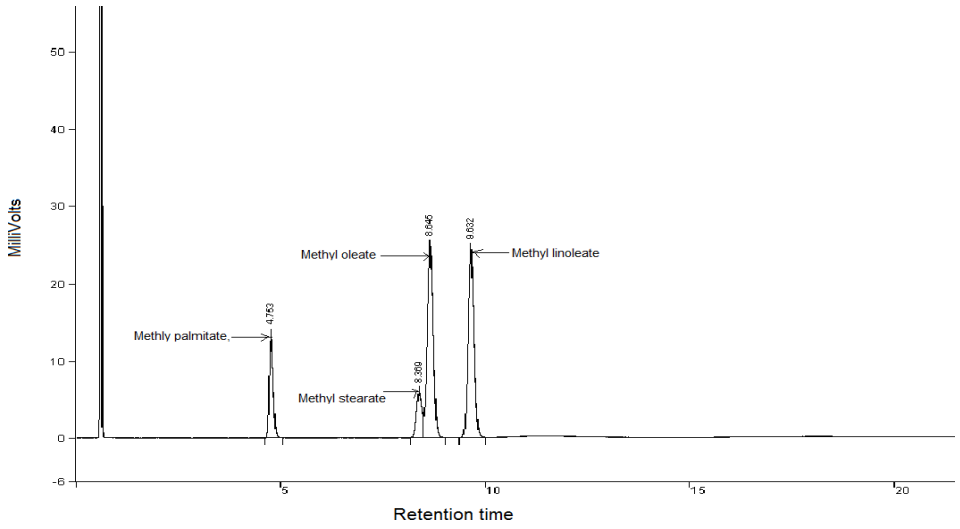

Figure 2. GC chromatogram of standard mixture of methyl palmitate (4.753), methyl stearate (8.369), methyl oleate (8.645) and methyl linoleate (9.632).

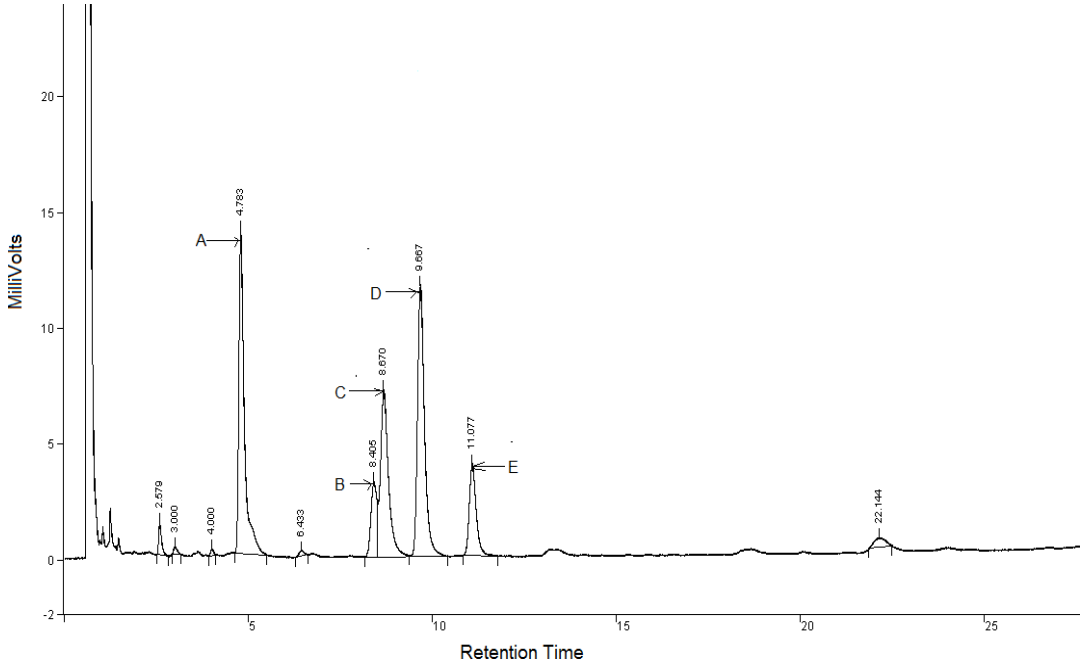

Figure 3. FAME analysis of *A.Paniculata* PE fraction A= Methyl palmitate (4.783); B= Methyl stearate (8.405); C= Methyl oleate(8.670) and D= Methyl linoleate(9.667).

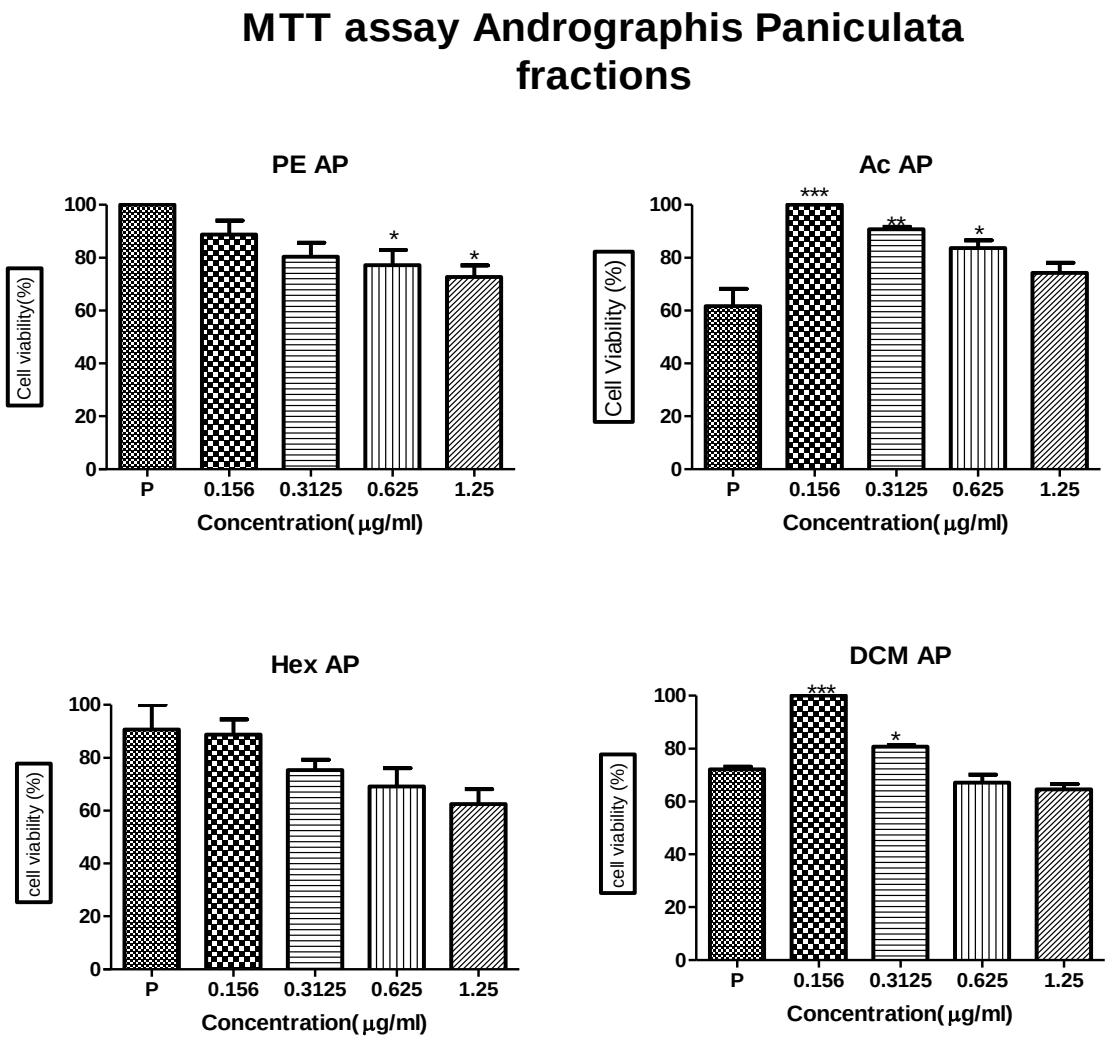

Figure 4. *Andrographis.Paniculata Nees (AP)* fractions were screened with MTT to identify its toxicity.

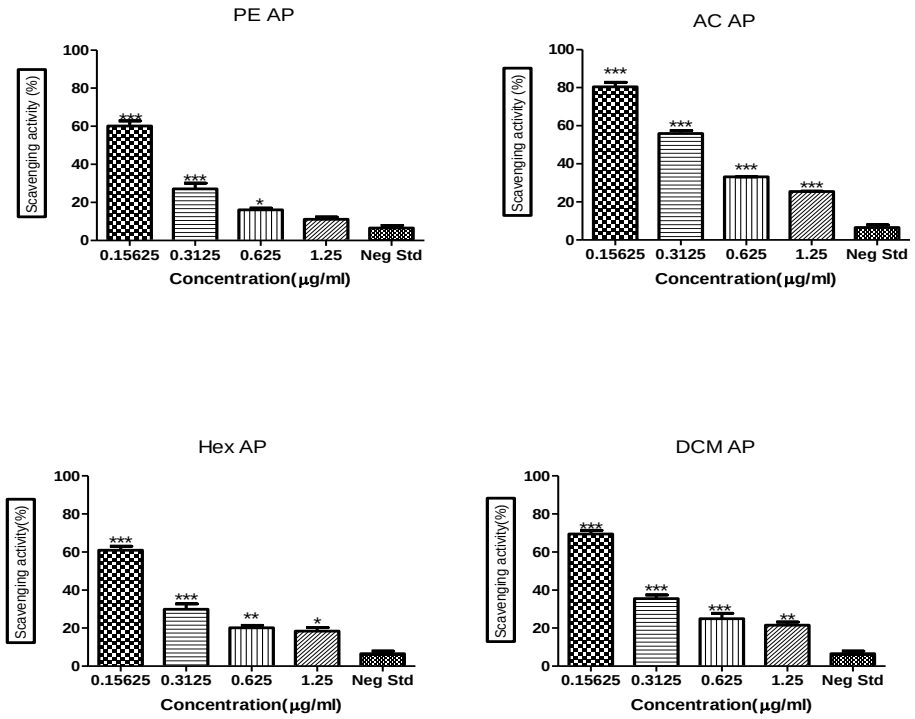

Figure 5. DPPH assay of *Andrographis.Paniculata Nees (AP)* fractions from both methods was screened to identity the free radical scavenging activity.

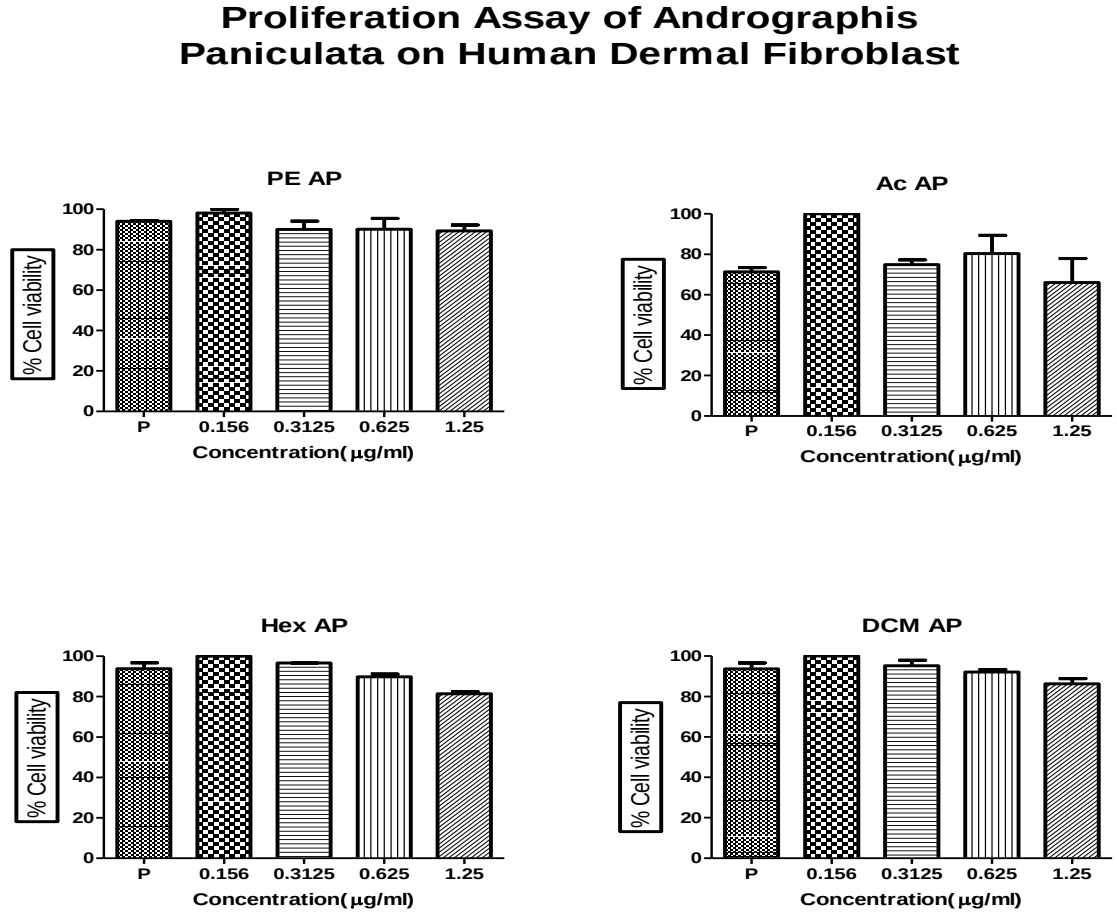

Figure 6. MTT Proliferation assay of *Andrographis.Paniculata Nees (AP)* fractions from both methods was screen to identify its effect on HDF.

Effect of Plant extracts against Ultra violet C induce DNA Damage

To test the fractions abilities to reduce levels of DNA damage caused by UVC rays on QFC, cell monolayers were treated for 60 min with fractions, washed with PBS to remove the medium then irradiated with 25JM-2 UV-C. Cells were refed with medium containing fractions then returned to the incubator for 30 min before being prepared for the comet assay. Three independent experiments were performed and in total 200 cells were scored for each experiment. The percentage DNA in the comet Head was estimated for WE (positive standard without fractions and without exposure), Eth-70 % Ethanol (solvent used to dissolve samples), S1, S2, S3 and S4.

In each case, cells were treated with same concentration of fractions as in previous experiments. All fractions showed significant protective activity against oxidative damage (60-75 %) While fractions also showed DNA protection against strand break (65-80 %) in UVC rays damaged cells.

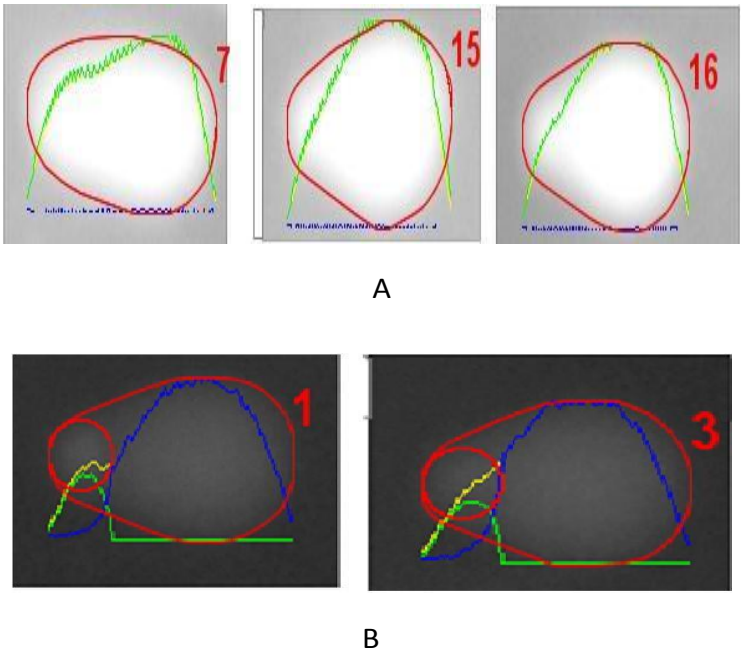

Figure 7. A- Representative Images of quiescent fibroblast cells from untreated slides analysed by OpenComet plugin in ImageJ. Cell without COMET. B- Representative Images of quiescent fibroblast cells from treated slides analysed by OpenComet plugin in ImageJ. Cell with COMET.

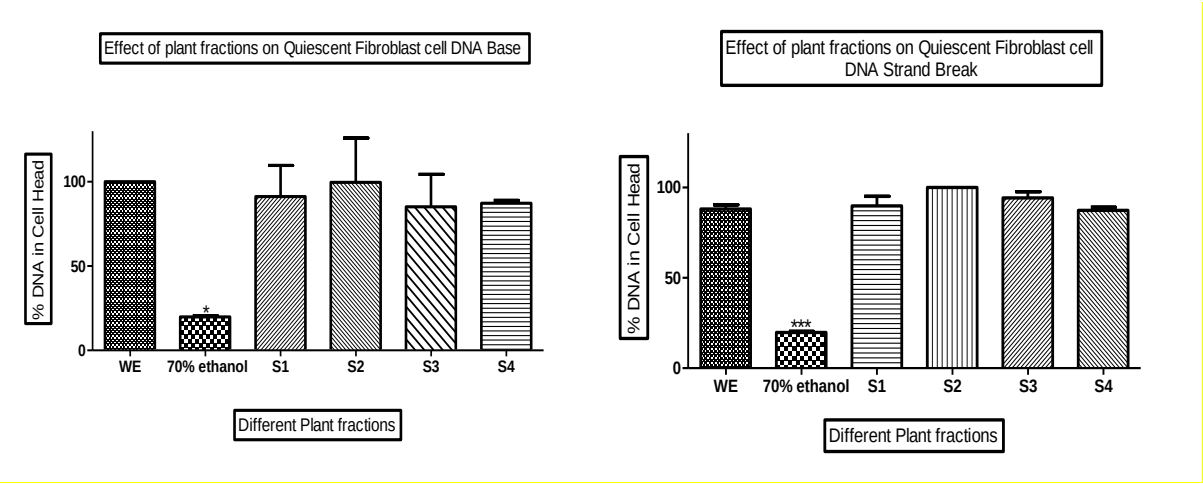

Figure 8. COMET assay of plant fractions with QFC. DNA in comet head was analysed in 200 cells. Cells were treated with 0.25 µg/mL of fractions. Results expressed as percentage of DNA in cell head in WE=without fractions, Eth- 70 % Ethanol, S1= *Andrographis Paniculata Nees (AP)*PE, S2= *Andrographis Paniculata Nees (AP)* Acetone fraction, S3= *Andrographis Paniculata Nees (AP) Hex* fraction and S4= *Andrographis Paniculata Nees (AP)* DCM fraction. Values shown are the averages of three replicates per samples +/-SD. Statistical analysis was performed by Anova followed by post-hoc tukey test. Tukey test compare std (WE) with different fractions. It was observed that there was no significant difference between WE cells and cells treated with fractions

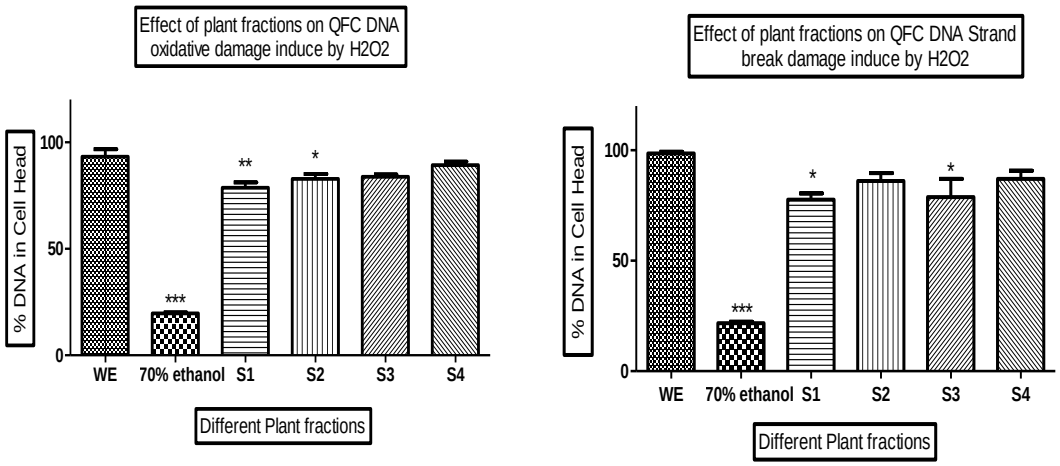

Figure 9. COMET assay of QFC damage with H_2O_2 and treated with plant fractions. DNA in comet head was analysis in 200 cells. Cells were treated with 0.25µg/mL of fractions. Results expressed as percentage of DNA in comet head in WE=without fractions, Eth-70 % Ethanol, S1= *Andrographis Paniculata Nees* PE, S2= *Andrographis Paniculata Nees* Acetone fraction, S3= *Andrographis Paniculata Nees Hex* fraction and S4= *Andrographis Paniculata Nees* DCM fraction. Value is three replicates +/-SD. Statistical analysis was performed by Anova followed by post-hoc tukey test. Tukey test compare std (WE) with different fractions and the stars indicate a significant difference ($^{***}P<0.0005$, $^{**}P< 0.005$ and $^{*}P<0.05$) to cells treated with different fractions.

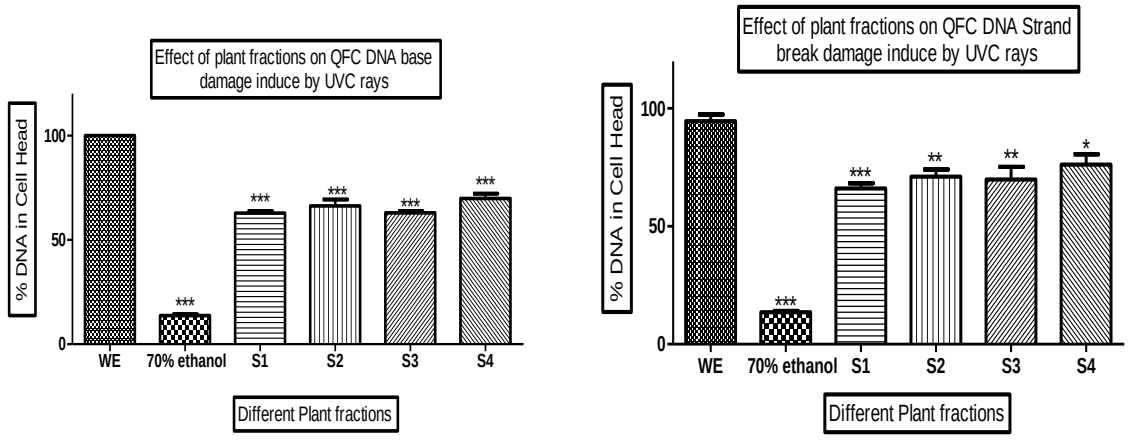

Figure 10. COMET assay of QFC damage with UVC rays and treated with plant fractions. DNA in comet head was analysis in 200 cells. Cells were treated with 0.25 µg/mL of fractions. Results expressed as percentage of DNA in comet head in WE=without fractions, Eth-70 % Ethanol, S1= *Andrographis Paniculata Nees* PE, S2= *Andrographis Paniculata Nees* Acetone fraction, S3= *Andrographis Paniculata Nees Hex* fraction and S4= *Andrographis Paniculata Nees* DCM fractions. Value is three replicates +/-SD. Statistical analysis was performed by Anova followed by post-hoc tukey test. Tukey test compare std (WE) with different fractions and the stars indicate a significant difference ($^{***}P<0.0005$, $^{**}P< 0.005$ and $^{*}P<0.05$) to cells treated with different fractions.

DISCUSSION

We selected *Andrographis.Paniculata Nees* (AP) plant based on ethnobotanical and ethnopharmacological approaches [34,35]. The ethnobotanical approach to select plants has proven very successful in discovering new compounds.

Andrographis.Paniculata Nees (AP) plants have shown antioxidant activity, but have not yet been studied for bioactive protection against oxidative DNA damage caused by UVC and hydrogen peroxide [36].

The *Andrographis.Paniculata Nees* (AP) fractions were further processed for qualitative analysis to identify the fatty acids. GC analysis of the fractions from *Andrographis.Paniculata Nees* (AP) was performed to identify different fatty acids present in the fractions. Methyl palmitate, methyl stearate, methyl oleate and methyl linoleate were identified in PE, Ace, Hex, DCM fractions of *Andrographis.Paniculata Nees* (AP). Our result confirms the presence of palmitic acid, Stearic acid oleic acid and linolenic acid in *Andrographis.Paniculata Nees* (AP) fractions.

Toxicity is one of the leading causes of attrition at all stages of the drug development process [37]. So performing *in vitro* cytotoxicity testing becomes an essential aspect of drug discovery. *In vitro* cytotoxicity testing is a cost effective, predictive and convenient means of characterizing the toxic potential of compounds [38].

In the MTT assay, MTT dye is reduced by mitochondrial dehydrogenase in living cells to produce insoluble purple formazan crystals which can be quantitatively measured using a spectrophotometer [39,40]. MTT assay is widely used in the high throughput screening for drug discovery to assess cell proliferation and cytotoxicity [38,41,42]. However, it exhibits some limitations in the presence of some compounds and can produce false results under certain conditions [43,44,45]. So to overcome and check this limitation with our plant extracts, the Viacount assay was also performed. Both assays confirm that at low concentrations extracts showed high viability and as the concentration was increased viability of cells started decreasing.

There is growing interest in the antioxidant studies due to the implication of free radicals in the development and progression of cardiovascular, neurodegenerative, ageing and cancer disease [46]. To test the free radical scavenging activity extracts were screened with a DPPH assay. The DPPH assay is single electron transfer method in which one electron is transfered to reduce any compound, including metal ions, carbonyls and radicals to measure the

ability of a potential antioxidant [47,48,49]. DPPH assay showed high significant activity at low concentration and it was decrease as the concentration was increase similar to the viability.

In vitro proliferation assay was performed on the human dermal fibroblast cells after treating with hydrogen peroxide and exposing to UVC to understand its effect on cell population. Plant extracts help to restore the damage produce by hydrogen peroxide and UVC by prevention and treatment.

Genotoxicity has been one of the problems in the drug discovery process. Generally mutagenic and thus potentially carcinogenic activity has been observed in genotoxic compounds [50].

In our experiments, no genotoxicity has been observed in extracts from *Andrographis.Paniculata Nees* (AP) plant. One of the previous study perform on the standardized extracts of *Andrographis.Paniculata Nees* (AP) did not show any genotoxicity while different studies have been performed to look toxicity of *Andrographis.Paniculata Nees* (AP) [51-55]. Our results of genotoxicity correlate to the result of chandrasekaran.

At low concentrations, H2O2 damages DNA through the Fenton reaction [56]. H2O2-treatment is a model for oxidative damage because it can lead to mutation in cells. In general, protection from this damage is very important. Here we report that fractions from *Andrographis.Paniculata Nees* (AP) can directly modulate hydrogen peroxide induced DNA damage in QFC. *Andrographis.Paniculata Nees* (AP) fractions suppress base damage, strand break damage produced by hydrogen perioxide and UVC. DCM fraction is most effective in both cases.

UVC was used to induce damage to DNA in QFC, since UVC induces the same lesions as UVB (and to some extent UVA), although the kinetics of their formation and their relative proportions may differ [57,58]. We report here that all fractions from *Andrographis Paniculata Nees* (AP) show strong protection activity in both type of damage.

Genotoxic data of new drugs is a requirement of regulatory authorities, as part of the safety evaluation. Generally mutagenic and thus potentially carcinogenic activity has been observed in genotoxic compounds [50]. Pre-clinical

studies give basic toxicological data that can be used to evaluate the safety and efficacy of NCE [59]. Genotoxicity testing can be studied by looking at different mechanisms and endpoints- DNA breaks, altered DNA bases, mutations and chromosomal alterations [60].

CONCLUSION

In conclusion, the GC analysis agrees that the fractions are rich in fatty acids. Fractions of *Andrographis.Paniculata Nees* (AP) don't exhibit any toxicity at low concentration (0.15 g/mL) and also demonstrate antioxidant activity at that concentration.

In vitro proliferation assays confirm the protection of the cell population at all concentrations of extract treated with hydrogen peroxide and UVC. In a high alkaline comet assay (pH >13), the extracts demonstrate DNA protection against H_2O_2 and UVC. Human Dermal Fibroblast cells ($1x10^5$cells/ml) were pre-incubated with fatty acid rich extracts (250 µg/ml) for one hour prior to an exposure to 50 µM H_2O_2 and a 254 nm UVC germicidal lamp for DNA damage induction.

Andrographis.Paniculata Nees (AP) fractions counteract UVC and H_2O_2 damage by 30-60 %. In human dermal fibroblasts, fatty acid fractions from *Andrographis.Paniculata Nees* (AP) protect against DNA damage produced by hydrogen peroxide and ultra violet C rays.

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