International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 8, Issue 1, 2016

Original Article

GC-MS ANALYSIS AND *IN-VITRO* CYTOTOXIC STUDIES OF *BIXA ORELLANA* SEED EXTRACT AGAINST CANCER CELL LINE

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Received: 24 Oct 2015 Revised and Accepted: 02 Dec 2015

ABSTRACTS

Objective: The present study was carried out to analyze the bioactive component of petroleum ether (PE) extract of *Bixa orellana* seed and its anticancer potential against B16F-10 melanoma cell line.

Methods: The presence of a bioactive component of PE extract was analyzed by GC-MS method. Cell viability was measured using MTT assay. Apoptotic inductive effect of the extract was evaluated by AO/EB staining; DAPI is staining and Annexin V/PI staining. DNA fragmentation analysis of the control and treated cell was carried out by agarose gel electrophoresis and comet assay. *In vitro* prevention of cell proliferation and migration was further estimated by colony forming assay and wound healing assay respectively.

Results: The cytotoxicity of the PE extract against B16F10 melanoma cell was dose and time dependent. The IC_{50} values for the B16F-10 melanoma cell were 145.98±7.49 µg/ml and 121.60±6.20µg/ml for 24h and 48h respectively. DAPI staining showed DNA fragmentation which was further confirmed by ladder-like appearance of DNA by gel electrophoresis and comet assay. PE extract has prevented the cell proliferation and migration of the treated cancer cell. GC-MS analysis shows the presence of three anticancer compound geranylgeraniol, squalene and beta-sitosterol.

Conclusion: The present study established that PE extract of *Bixa orellana* seed possesses potent anticancer and apoptotic inductive potential which can further be explored *in-vivo* model for possible cytotoxic activity.

Keywords: *Bixa orellana*, GC-MS, Geranylgeraniol, Comet assay, Annexin V/PI staining, AO/EB staining.

INTRODUCTION

Natural products as an anti-cancer agent have a long history. Many compounds isolated from plants are being employed in chemotherapy [1]. More than the 50% of the drugs sourced in clinical trials are from natural sources or related to it[2]. Many plant metabolites induce apoptosis in cancer cells but not in normal cells, thus it is a potential drug leads [3-5]. Cancer is a dreadful disease caused by abnormal and uncontrolled cell division and having the potential to invade other parts of the body. About 6 million new incidences of cancer are reported yearly worldwide [6] and more than 70 percent of all cancer deaths occurred in low-and-middle-income countries. Deaths from cancer are projected to continue rising worldwide with an estimated 12 million deaths by 2030 [7]. The number of melanoma cases is increasing world wide faster than any other cancer and estimates suggested a doubling of melanoma incidence every 10-20 y [8, 9]. *Bixa orellana* is a plant native to Brazil and cultivated in other tropical countries. The seeds of this plant produce the dye most frequently used worldwide in the food, textile, paint, and cosmetic industries. The use of this dye has been stimulated due to the ban on the use of synthetic dyes in food and cosmetics, and it is one of the few accepted by the World Health Organization (WHO), since, in addition to being nontoxic, it does not change the food value. Another interesting fact is that 70% of all natural coloring agents consumed world wide are derived from annatto [10]. All parts of the *Bixa orellana* are used in traditional medicine. In Brazil, leaves, roots, and seeds are a remedy for fever, inflammatory conditions and parasitic diseases [10]. Gas chromatography is important techniques which separate the mostly volatile components of the mixture and analysis of the each component was carried out by mass spectroscopy. Long chain unsaturated fatty acids are an important component of a volatile substance that involves in the structural element of many valuable compounds as well as an important source of energy [11]. The present study was aimed to evaluate the anti-cancer activity against B16f-10 melanoma cells and analysis of bioactive component present in the PE extract.

MATERIALS AND METHODS

Chemicals and reagents

DMEM medium, Fetal bovine serum (FBS), penicillin/streptomycin, trypsin-EDTA, PBS (phosphate buffer saline), Acridine orange, Ethidium bromide, MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) used in the work were purchased from HI-MEDIA Pvt. Ltd, Bombay, India. Annexin V/PI kit was obtained from Sigma. The solvents used were of analytical grade.

Cell culture

B16F-10 melanoma cell was obtained from National Centre for cell sciences (NCCS), Pune. Cells were maintained as an adherent cell line in Dulbecco's modified Eagle's medium (DMEM medium, PH 7.4), supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) at 37 °C in a humidified 5% CO_2 atmosphere. Cells were passaged as needed using 0.5% trypsin-EDTA.

Collection and identification of plant material

The seeds of *Bixa orellana* used for the investigation were purchased from a local herbal shop in Pondicherry and later it was authenticated by Professor N. Parthasarathy of Department of Ecology and Environmental Sciences, School of Life Sciences, Pondicherry University, Puducherry-605014, India.

Preparation of extracts

A 100 g sample of dried seed powder of *Bixa orellana* was extracted in 400 ml of petroleum ether in soxhlet apparatus for 72 h. It was evaporated in rotavapor and kept in hot air oven at 40 °C overnight to remove traces of petroleum ether and obtained extract was kept in an airtight container at 4 °C for further use.

Gas chromatography-mass spectroscopic analysis

GC-MS analysis of the PE extract of *Bixa orellana* seed was performed using the equipment GC-MS-5975C (AGILENT). The equipment has a

DB 5–MS Capillary Standard non-polar column with dimensions of 30 mm × 0.25 mm ID × 0.25 µm films. The carrier gas used is Helium with at a flow of 1.51 ml/min. The injector was operated at 250 °C and the oven temperature was programmed as follows: 70 °C for 3 min, then gradually increased to 300 °C at 9 min. The identification of components was based on NIST libraries search.

Measurement of cytotoxicity by MTT assay

This assay was performed according to a slight modification of the procedure reported by Mosmann [12]. All cells were grown as a monolayer in 96 well plate seeded at a density of 5,000 cells/well/200 µl of complete medium. The cells were grown for 24 h before drug treatment. The different concentration of the extract was treated for another 24 and 48 h after that cytotoxicity were determined by MTT assay. 20 µl of MTT (5 mg/ml) were added to each well and kept in the dark for another 4 h at 37 °C. The formazan crystal formed was dissolved in 100 µl of DMSO and reading was taken at 570 nm in Elisa plate reader (Molecular Devices Inc., Sunnyvale, CA, USA). Doxorubicin was used as a positive control.

Acridine orange/ethidium bromide (AO/EB) staining

Cell morphological changes were assessed by differential staining using Acridine orange and ethidium bromide [13]. Approximately 1,00,000 cells were seeded into 24-well tissue culture plates, incubated for 24h, treated with IC_{50} drug concentration and incubated for 24 h. Untreated cells were used as negative control. After 24 h of treatments, cells were stained with AO/EB prior to microscopic examination. The stained cells were examined under a fluorescence microscope using a UV filter (450-490 nm).

DAPI staining

Detection of apoptosis by DAPI staining were performed by the method of Machana *et al.*[14]. 1,00,000 cells were seeded into 24 well culture plate for 24 h and treated with IC_{50} drug concentration for another 24h. Untreated cells were used as negative control. The cells were fixed in 4.0% paraformaldehyde for 20 min and then stained with 5µg/ml DAPI dye at 37 °C for 10 min. The image was taken with a Nikon Eclipse Ti fluorescence microscope (Nikon Instruments Inc., NY, USA) with ultraviolet illumination.

DNA fragmentation analysis by agarose electrophoresis

DNA fragmentation assay was performed as described by Sheng *et al.*[15]. The B16F-10 melanoma cells were plated in 60 mm culture dish at a density of 2 x 10^6 cells for 24 h and treated with PE extracts (121 µg/ml and 145 µg/ml) for 48 h. After that cells were collected and centrifuged to 1,500 rpm for 5 min at 4 °C to make pellet. DNA was prepared from the pelleted cells. Briefly, cells were lysed with 200 µl of lysis buffer containing 200 μg ml^{-1}RNaseA and 200 μg ml^{-1}proteinase K, after that it was incubated at 40 °C for 1h. 10 µl of the loading buffer (0.25% bromophenol blue, 30% glycerol) with equal quantities of DNA were electrophoresed in 0.8% agarose gel containing 0.2 µg/ml of ethidium bromide. After electrophoresis, the gel was photographed by syngeneic gel documentation system.

Comet assay

DNA fragmentation is considered as a hallmark of apoptosis. The alkaline version of the single cell gel electrophoresis assay (comet assay) was used to evaluate DNA damage as previously described by Lima *et al.*[16]. DNA damage was quantified using CASP (Comet Assay Software Project) software. The images were used to estimate the DNA content of individual nuclei and to evaluate the degree of DNA damage in the comet tail.

Colony forming assay

B16F10 melanoma cells were seeded at a density of 300cells/ml in 6-well culture plates, treated with PE extract for 24 h, then washed with PBS and fresh medium was added. Cells were seeded in duplicate and allowed to grow for 10 d. Colonies were visualized by staining the cells with 0.5% crystal violet, and pictures were captured using a Nikon Coolpix L26 camera. The cell survival fraction was observed in treating cells to that of untreated control cells.

Wound healing assay

B16F10 cells were allowed to grow confluent in 6-well plates and after it had reached confluent wound was made scrapping with 200 µl pipette tips and washed with PBS. Cells were then incubated with fresh complete medium containing 100 ug/ml of PE extract. Photographs were taken at the wound at 0 and 24h of time interval.

Annexin V/PI apoptosis detection

An Annexin V/PI kit (Sigma, USA) was used according to the manufacturer's instructions. Briefly, 500 µl of binding buffer was added to the PE extract treated and control B16F10 cells followed by staining with an Annexin-V-FITC and PI solution for 30 min at room temperature in the dark. The images were taken in Nikon Eclipse Ti fluorescence microscope. Annexin V (Green fluorescence) uptake was observed in early apoptotic cells and red fluorescence was observed in necrotic cells or late apoptotic cells which uptake the PI dye.

Statistical analysis

All analysis was performed in triplicate, and the data were reported as means±SD. IC_{50}values were calculated by linear regression analysis using Microsoft excel 2010. Comparisons of the results were made using analysis of variance (ANOVA) by SPSS 16. Significant differences (*$p<0.05$) between the means of control and treated cells were analyzed by Tukey test.

Results

GC-MS analysis

The identified principles with their retention time (RT), molecular formula, molecular weight (MW) and concentration (peak area %) are presented in table S1 and fig. 1 which shows the presence of 43 compounds in the PE extract of *Bixa orellana*. Out of the 43 compound identified, Trans-Geranylgeraniol (19.69%)[17, 18] Squalene (3.14%)[19] and β-sitosterol (3.56%)[20, 21] have been reported for anticancer activity on the other cell lines.

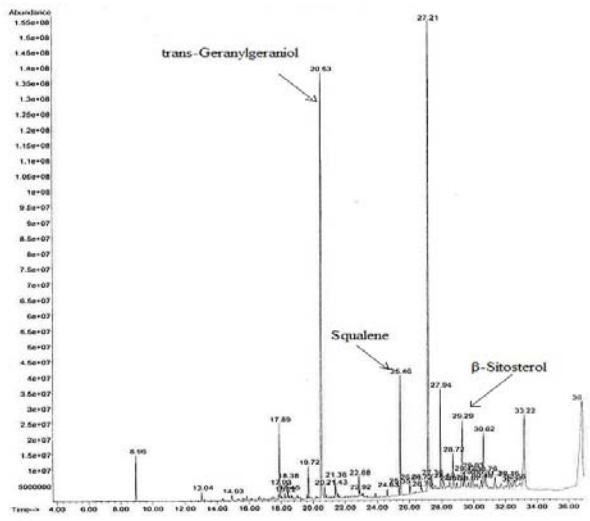

Fig. 1: GC-MS chromatogram for PE extract of Bixa Orellana L.

Cell viability by MTT assay

To determine the cell viability by PE extract, we performed an MTT assay. As the concentration of the drug increases the number of dead cell increases. PE extract treatment of B16F-10 cells significantly decreased the viability in a dose and time-dependent manner (Fig.2). The IC_{50} values for the B16F-10 melanoma cell was 145.98±7.49 µg/ml and 121.60±6.20 µg/ml for 24h and 48h respectively. Doxorubicin was used as a positive control, and IC_{50} values were found to be 7.91±0.98 and 6.18±0.62 µg/ml for 24h and 48h respectively.

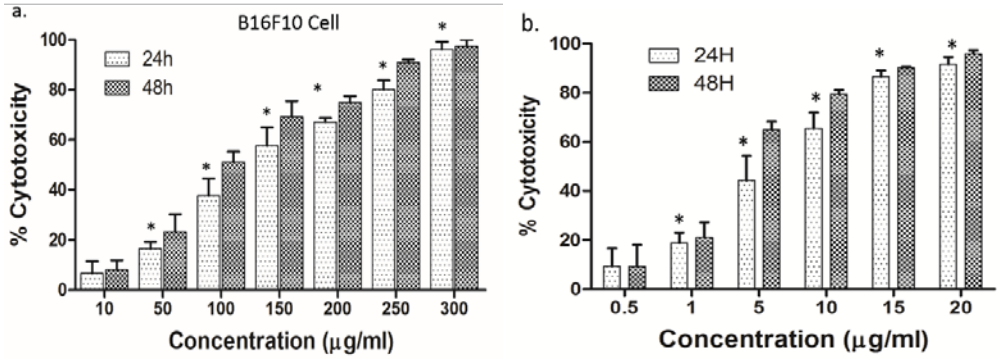

Fig. 2: Cytotoxic effect of (a) PE extract of Bixa Orellana seed and (b) doxorubicin against B16F-10melanoma cells after 24h and 48h of treatment. Cell proliferation was assessed by the MTT assay. The IC_{50} value for the PE extract was 145.98±7.49 µg/ml and 121.60±6.20 µg/ml, whereas for doxorubicin it was 7.91±0.98 µg/ml and 6.18±0.62µg/ml for 24h and 48h of treatment respectively. Data shown are mean of ±SD of four replicates. *P<0.05 from untreated cells

Acridine orange/ethidium bromide (AO/EB) staining

The results obtained with acridine orange & ethidium bromide double staining of control and treated B16F-10 cells are presented in the fig. 3. The untreated control cells fluoresced uniformly green and had normal features whereas cells treated with PE extract fluoresced orange-red that indicated the apoptotic features such as cell shrinkage, chromatin condensation, nuclear fragmentation and apoptotic body formation.

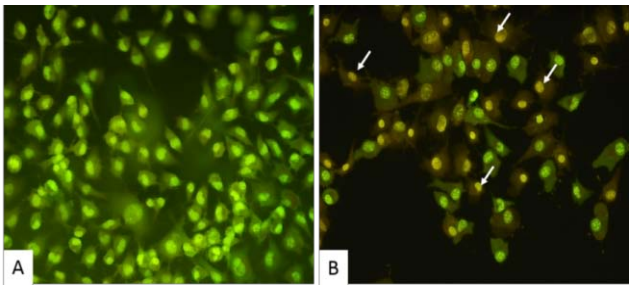

Fig. 3: Apoptosis of B16F-10 cells stained with AO/EB and viewed under a fluorescence microscope (magnification 200x). White arrow indicates cell undergoing morphological changes and apoptosis. A. Control, B. PE extracts treated (145 µg/ml) for 24h

DAPI staining

Programmed cell death plays an essential role for an effective cancer therapy [22]. The morphological changes of the nuclei DNA after PE extract treatment are shown in fig. 4. The result indicated that the PE extract induces nuclear morphological changes compared to control cells, and this may be indicative of apoptosis. Morphological changes observed in the treated cells included broken nuclei into discrete fragments.

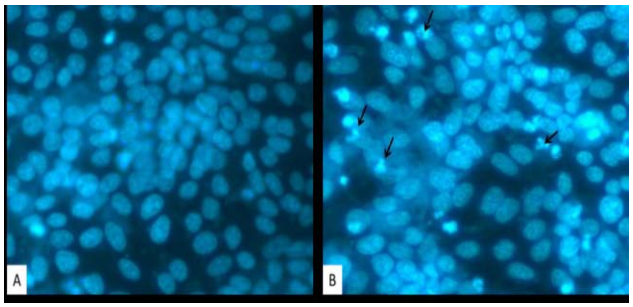

Fig. 4: Apoptosis in B16F-10 cells following 24h of PE extract exposure stained with nuclear dye DAPI. Arrows indicate the cell undergoing apoptosis and nuclear fragmentation. A. Control, B. 145 µg/ml of PE extract treated

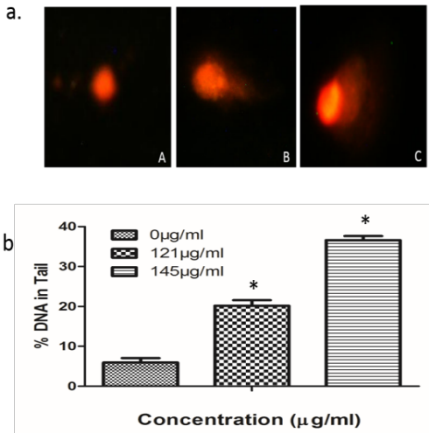

Fig. 5: DNA damage in PE extract-treated for 24h in B16F10 melanoma cancer cell as revealed in the comet assay. a. A. Control (DNA damage 5.96±1.07), B. 121 µg/ml (DNA damage 20.17±1.41) and C. 145 µg/ml (DNA damage 36.62±1.02). b.*P<0.05 from untreated cells

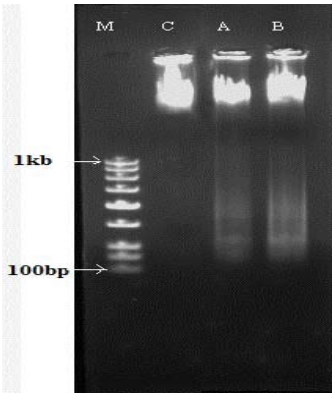

Fig. 6: DNA laddering of the genomic DNA extracted from B16F-10 cells after exposure to PE extract as assessed by 0.8% agarose gel electrophoresis, EtBr staining & UV-trans-illuminance (M=Marker, C=Control, A=100 µg/ml, B=145 µg/ml of PE extract)

DNA fragmentation and comet assay

The comet assay is a rapid and sensitive technique for measuring DNA damage at the level of a single cell. The control cells showed little or negligible DNA damage, but the treatments with PE extract

produced a high percentage of damaged cells. The treated cancer cell shows the 21.17 % and 37.35 % of DNA damage in tail after treatment of 121µg/ml and 145µg/ml PE extract for 24h respectively (fig. 5a&b). For cell undergoing apoptosis fragmentation of Genomic DNA into the characteristic ladder on agarose is an important feature. Treatment of B16F10 melanoma cell with 121 and 145 µg/ml PE extract produces a DNA ladder with a smear (fig. 6). The smear could be due to the some post-apoptotic cell necrosis. In comparison, of treated cells, control cells did not produce any ladder or smear.

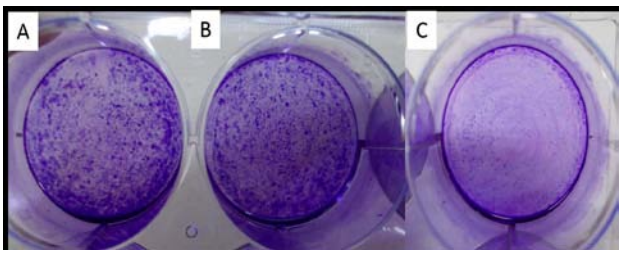

Fig. 7: PE extract suppresses the colony formation in B16F10 melanoma cell. B16F10 melanoma cell was incubated in the medium alone or the medium containing PE extract for 24h and the medium was replaced with fresh medium and incubated for 10 d. A. Control B. 50 µg/ml and C. 100 µg/ml

Colony forming and wound healing assay

The use of a colony formation assay further demonstrated that the treatment of PE extract resulted in decreased cell proliferation of B16F10 melanoma cell (Fig.7). As shown in Fig.8, treatment of B16F10 melanoma cell with 100 µg/ml of PE extract for 24h, reduced the migration of cell at the wound site. But in the control, major part of the wound site was covered by the cell. This shows the *in vitro* prevention of cell proliferation by PE extract.

Annexin V/PI apoptosis detection

Annexin V-FITC is a fluorescent probe, which binds to Phosphatidylserine in the presence of calcium. At the onset of apoptosis phosphatidylserine which is normally found on the internal part of the plasma membrane, becomes translocated to the external portion of the membrane, so it becomes available to Annexin V whereas PI detects necrotic cells and late apoptotic cell with permeabilized plasma membrane. Untreated cell did not show any green fluorescence but treated cell shows green fluorescence in early apoptotic cell and green with red or only red in late apoptotic and necrotic cells (fig. 9).

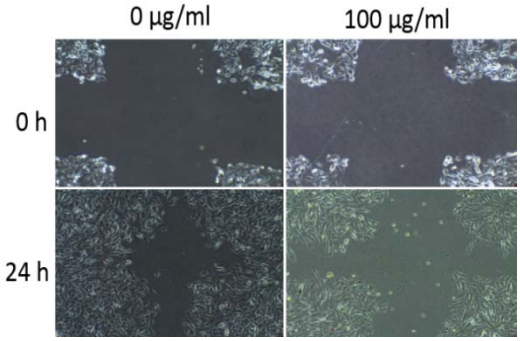

Fig. 8: Effect of PE extracts on B16F10 Melanoma Cell Migration. Wound healing assay was performed to assess cell migration. Cells were treated with 100 µg/ml of PE extract

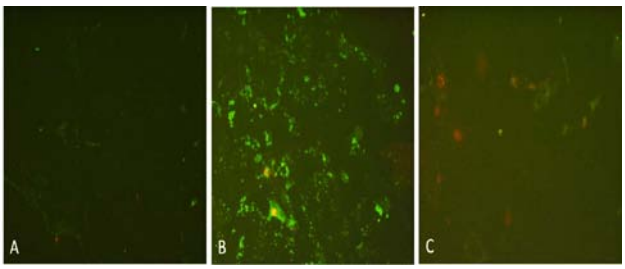

Fig. 9: Annexin V/PI dual staining. PE extract enhanced the apoptosis of B16F10 melanoma cell. Cells were incubated with medium alone (A) or with 50 µg/ml (B) and 145 µg/ml (C) of PE extract for 24h

Table S1: Identified compound by GC-MS analysis of seed extract of *Bixa orellana*

Peak no.	RT	Name of the compound	Molecular formula	Molecular mass	Peak area %
1	8.956	Naphthalene	$C_{10}H_8$	128.17	1.31
2	13.047	Alpha-Muurolene	$C_{15}H_{24}$	204.35	0.21
3	14.930	Ledene oxide-(II)	$C_{15}H_{24}O$	220.35	0.19
4	17.895	(E,E)-7,11,15-Trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene	$C_{20}H_{32}$	272.46	1.83
5	17.927	1,5,9-decatriene, 2,3,5,8-tetramethyl-	$C_{14}H_{24}$	192.34	0.28
6	18.219	(E,E,E)-3,7,11,15-Tetramethylhexadeca-1,3,6,10,14-pentaene	$C_{20}H_{32}$	272.46	0.15
7	18.385	Alpha-Farnesene	$C_{15}H_{24}$	204.35	0.47
8	18.448	1,5,9-cyclotetradecatriene, 1,5,9-trimethyl-12-(1-methylethenyl)-	$C_{20}H_{32}$	272.46	0.17
9	19.714	Androsta-3,5-dien-7-one	$C_{19}H_{26}O$	270.40	0.89
10	20.529	Trans-Geranylgeraniol	$C_{20}H_{34}O$	290.48	19.69
11	20.713	Hexadeca-2,6,10,14-tetraen-1-ol, 3,7,11,16-tetramethyl-	$C_{20}H_{34}O$	290.48	0.33
12	21.375	1,6,10,14-Hexadecatetraen-3-ol,3, 7,11,15-tetramethyl-(E,E)-	$C_{20}H_{34}O$	290.48	0.46
13	21.432	4-Cyclopropylmethylbenzonitrile	$C_{11}H_{11}N$	157.21	0.34
14	22.876	Butyl 9,12-octadecadienoate	$C_{22}H_{40}O_2$	336.55	0.56
15	22.921	Myrcenylacetat	$C_{12}H_{20}O_2$	196.28	0.18
16	24.626	Tetracosane	$C_{24}H_{50}$	338.65	0.25
17	25.351	Octacosane	$C_{28}H_{58}$	394.76	0.27
18	25.453	Squalene	$C_{30}H_{50}$	410.71	3.14
19	26.038	Octadecane	$C_{18}H_{38}$	254.49	0.37
20	26.713	Heneicosane	$C_{21}H_{44}$	296.57	0.32
21	26.763	7-Amino-4-methyl-1,8-naphthyridin-2-ol	$C_9H_9N_3O$	175	0.11
22	27.209	3,5,9-Undecatrien-2-one, 6, 10-dimethyl-(E,Z)-	$C_{13}H_{20}O$	192.29	25.77
23	27.381	Tricosane	$C_{23}H_{48}$	324.62	0.37
24	27.940	Benzenepropanenitrile, 3,4-dimethoxy-	$C_{11}H_{13}NO_2$	191.23	3.60
25	28.119	Eicosane	$C_{20}H_{42}$	282.54	0.40

26	28.500	Campesterol	$C_{28}H_{48}O$	400.68	0.37
27	28.723	Stigmasterol	$C_{29}H_{48}O$	412.69	1.59
28	28.787	2,6,10-Dodecatrien-1-ol, 3, 7, 11-trimethyl-	$C_{15}H_{26}O$	222.36	0.30
29	28.952	Octacosane	$C_{28}H_{58}$	394.76	0.23
30	29.296	β-sitosterol	$C_{29}H_{50}O$	414.70	3.56
31	29.423	Cholest-5-en-3-ol, 24-propylidene-,(3. Beta.)-	$C_{30}H_{50}O$	426.71	0.74
32	29.671	9,19-Cyclolanost-24-en-3-ol, (3. Beta.)-	$C_{30}H_{50}O$	426.71	0.27
33	29.862	2-Methyl-3-(3-methyl-but-2-enyl)-2-(4-methyl-pent-3-enyl)-oxetane	$C_{15}H_{26}O$	222.36	0.50
34	29.925	4,22-Stigmastadiene-3-one	$C_{29}H_{46}O$	410.67	0.84
35	30.485	1,3,3-Trimethyl-2-hydroxymethyl-3,3-dimethyl-4-(3-methylbut-2-enyl)-cyclohexane	$C_{15}H_{26}O$	222.37	0.54
36	30.625	Stigmast-4-en-3-one	$C_{29}H_{48}O$	412.69	2.75
37	30.759	Cycloprop[7,8]ergost-22-en-3-one,3',7-dihydro-,(5. alpha.,7. beta.,8. alpha.,22E)-	$C_{29}H_{46}O$	410.67	0.85
38	31.338	2,3-Dimethoxy-5-methyl-6-dekaisoprenyl-chion	$C_{59}H_{90}O_4$	863.34	0.80
39	32.158	1,6,10,14-Hexadecatetraen-3-ol, 7,11,15-tetramethyl-(E,E)-	$C_{20}H_{34}O$	290.48	0.87
40	32.368	Farnesol isomer a	$C_{15}H_{26}O$	222.37	0.75
41	32.559	2,6,10,14,18-pentamethyl-2,6,10,14,18-eicosapentaene	$C_{25}H_{42}$	342.60	1.16
42	33.221	Dimethyl(bis{[(2E,6E)-3,7,11-trimethyldodeca-2,6,10-trien-1-yl]oxy})silane	$C_{32}H_{56}O_2Si$	500.87	7.71
43	36.879	2,6,10,14-Hexadecatetraen-1-ol-3, 7,11,15-tetramethyl-,acetate, (E,E,E)-	$C_{22}H_{36}O_2$	332.52	14.53

DISCUSSION

Plants are directly or indirectly associated with human life and are an essential source of human well-being. This is a valuable source for medicine including cancer. They have contributed to fighting against numerous diseases. Many of the modern medicines are produced from the different pharmacological active ingredient of medicinal plants. The analysis and process of extraction of plant material play a crucial role in the development, modernization and quality control of herbal formulations. Studying about medicinal plants conjointly facilitates to understand plant toxicity and also helps to safeguard human and animals from natural toxins. Several anticancer studies have focused on the ability of pure compounds; however there have been reported that the total contents of a whole herb show a significantly better a synergistic effect than a single isolated active ingredient [23, 24]. The cytotoxicity of PE extract against B16F-10 melanoma cell was dose and time dependent manner. As the concentration of PE extract increased the number of cell death was more this was evident by MTT assay and morphological observations. In our study, the control cells showed nuclei with homogeneous chromatin distribution, whereas the chromatin amount was less in PE extract treated cell [25] and morphological modifications were clearly visible during apoptosis. Fluorescence microscopy analysis revealed that the PE extract induced apoptosis and caused morphological changes in cells. Some of the changes observed include chromatin condensation; plasma membrane blabbing and DNA fragmentation [26]. The characteristic of apoptosis was also further confirmed by DNA ladder which is a result of DNA fragmentation.

The presence of various bioactive compounds were detected using GC-MS analysis of the PE extract justifies the use of Bixa orellana seed for various elements by traditional practitioners. Oboh et al. have reported that non-polar fraction of Bixa orellana seed has a more protective of oxidative stress than the polar fraction [27]. Lopes et al. have reported gerangeraniol from Bixa orellana seed as a death inducer in Leishmania amazonensis [28]. trans-Geranylgeraniol [17], Squalene [19] and β-Sitosterol [21] have a report for anticancer activity on another cell line. This is the first time evaluation of PE extract of Bixa orellana seed on B16F-10 melanoma cell line. Medicinal plant forms the backbone of traditional medicine. In the last few decades, plants have been the subject for very intense pharmacological studies. This has been brought about by therapeutic value and as sources of lead compounds in drug development.

CONCLUSION

Considering the results of our *in-vitro* studies, it clearly shows a cytotoxicity and apoptosis inductive effect indicative of an anti-cancer activity. The presence of trans-Geranylgeraniol, Squalene and β-Sitosterol seems to be responsible for cytotoxicity and apoptotic-like characteristics. Further studies are being carried out on the expression of marker enzymes and protein expressed in apoptosis in order to provide more comprehensive data on the anticancer activity of the extract.

ACKNOWLEDGMENT

The author thanks the Bureau Veritas Consumer Products Services (I) Pvt. Ltd., Guindy, Chennai for carrying out GC-MS analysis of the sample. The author thanks the DBT IPLS, Pondicherry University for providing the infrastructural facility.

CONFLICT OF INTERESTS

Declared None

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