Methanol extraction revealed anticancer compounds Quinic Acid, 2(5H)-Furanone and Phytol in Andrographis paniculata

PAWAN KUMAR ANOOR1, A NICHITA YADAV1, KARTHIK RAJKUMAR1, RAMESH KANDE1, CHATURVEDULA TRIPURA2, K SRINIVAS NAIR3 and SANDEEPTA BURGULA1

1Department of Microbiology, University College of Science, Osmania University; 2CSIR-Centre for Cellular and Molecular Biology Annexe-II, Medical Biotechnology Campus, Annexe-II; 3Centre for Plant Molecular Biology, Osmania University, Hyderabad, Telangana 500007, India

Received January 30, 2022; Accepted June 29, 2022

DOI: 10.3892/mco.2022.2584

Abstract. Andrographis paniculata (Ap) has been a part of traditional medicine for its anti-inflammatory effects, treatment of snake bites and liver abnormalities. Several investigations have revealed its bioactive components to be andrographolides. The methanolic extracts of leaves from Ap were characterized, the non-andrographolides were identified and screened for anti-proliferative activity. The extracts showed significant toxicity against numerous cancer cells including HeLa, MCF7, BT549, 293 and A549 cells. Anti-proliferative activity and effect on cancer cell invasion (metastatic potential) and cell migration were examined. The extracts revealed significant inhibition of the ability of HeLa cells in repairing the gap created by a vertical wound made on a confluent cell monolayer. Similarly, a 40% reduction in cell migration was observed in presence of the extracts. Significant anti-angiogenic activity in terms of reduced blood capillary formation was observed on the Chorioallantoic membranes of embryonated hen eggs co-inoculated with HeLa cells and the extracts. Analysis of HeLa cells treated with the extracts using flow cytometry indicated the arrest of cell cycle progression at the G2/M phase. Variation in the Bax/Bcl-2 ratio and elevated caspase-3 levels by immunoblotting confirmed cell death induction via the apoptotic pathway. Investigation of the extracts by gas chromatography-mass spectrometry displayed the predominant components to be 2(5H)-Furanone (14.73%), Quinic acid (17.32%), and Phytol (11.43%). These components have been previously known to have anticancer activity, while being studied individually in other plants. This is the first study, to the best of our knowledge, on the anti-proliferative and anti-angiogenic activity of the non-andrographolide components from Ap.

Introduction

Phytomedicine has been used in chemoprotection and treatment of several types of cancer, due to the anti-proliferative activity of numerous plant-based compounds (1). Numerous chemotherapeutics are frequently obtained as derivatives of biological products used in traditional medicine (2) (Table SI). Hence, using medicinal plants with therapeutic properties is a valuable alternative and promising for pharmaceutical agents (3). Plants are abundant in a large collection of secondary metabolites and phytochemical elements such as alkaloids, flavonoids, tannins and terpenoids useful against various diseases (4-6). Phytomedicines are proving to be an inexpensive source for treatment and substantial precision than commercial chemotherapeutic agents (7). As the therapeutic options available for chronic and acute illnesses are significantly reduced, the search for new chemotherapeutic agents that may be applied as a treatment for these types of acute and chronic diseases increases (8,9). According to the reports from WHO, ~80% population from developing countries have been using herbal products for primary ailments. Numerous surveys also indicated that around 60% of patients with cancer have used some form of natural products for treatment (10-12). However, only a few medicinal plants applied have been scientifically assessed for their anticancer properties. The idea of deriving therapeutic drugs from natural products is therefore attractive for devising treatment strategies.

Andrographis paniculata (Ap) (Burm.f.) Nees or ‘King of Bitters, is a small, branched, annual plant belonging to the family Acanthaceae, has been used extensively in Unani, Siddha and Ayurveda (Table SI) (13). It is abundant in a broad range of phytochemical constituents such as flavonoids, diterpenes and lactones (12). In traditional medicine it is used in the treatment of fever, sore throat, upper respiratory tract and gastrointestinal diseases, hepatitis and a range of other chronic illnesses as well as infectious diseases (13,14). The herb and its components andrographolide, isoandrographolide and neoandrographolide have been reported to possess anti-inflammatory (15-17), hepatoprotective, antidiabetic (18),...
antimalarial (19), antimicrobial (20), anti-HIV, immuno-stimulatory (21) and anticancer activity (22,23).

Constant pursuit for natural compounds with the ability to target cancer cells is presently gathering much interest in the field of oncology (24). The andrographolide extracts have been revealed to suppress the development of cancer cells by inhibiting kinase pathways NF-kappaB, PI3K/AKT and induce apoptosis (25,26). The crude dichloromethane extract of the plant inhibits colon cancer cell proliferation and increased production of human peripheral blood lymphocytes at smaller concentrations (27). Ap extracts are known to have potential anti-metastatic and antitumor effect on esophageal cancer (28). Andrographolide, the key bioactive component of Ap has been shown to prevent the development of a diversity of cancer cells corresponding to various types of human cancer (29), including leukemia, melanoma, lung and breast cancer (30,31). Most of the aforementioned studies reported the activity of the andrographolides components of the AP extracts. In the present study, the anticancer activity of non-andrographolide components of Ap extracts was evaluated and the bioactive compounds were characterized. For the first time, to the best of our knowledge, a new set of compounds from the Ap extracts whose extracts exhibit anticancer activity was reported.

Materials and methods

Chemicals. MTT (cat. no. TC191) and dimethyl sulphoxide (DMSO; cat. no. MB058) were purchased from HiMedia Laboratories, LLC. RPMI-1640 medium and DMEM were procured from Hyclone; Cytiva. EZdetect PCR kit for Mycoplasma Detection (cat. no. CCK009) and LPS (Escherichia coli O55:B5) were obtained from Sigma-Aldrich; Merck KGaA. Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific, Inc. and polyclonal secondary antibodies were obtained from Thermo Fisher Scientific, Inc.

Collection of plant material. Ap is known in India as Nelavenu, Kalamegh, nilavempu, commonly known as King of Bitters and Green chiretta in English. Finely powdered plant leaves were purchased from Wonder herbals, Hyderabad, India (Batch number 10, GMP certified). The components were checked for the presence of any visible contaminants such as unsafe foreign material, sand, stones and poisonous chemical residues. The powder was entirely free of moulds or insects. As microbial contaminants can produce toxins, thin layer chromatography was performed to detect contamination from other plants using original plant leaf extract as control. The plant name has been checked with https://www.theplantlist.org.

Preparation of experimental plant extracts. A total of 10 g of pulverized leaf powder was macerated for 72 h in 100 ml of methanol at room temperature in orbital shaker (80 x g). In the present study, the method used to extract and identify bio-active compounds from Ap is somewhat different in terms of using the organic extraction method as this method could help in extraction of a new set of compounds previously unknown to be present in the plant. Filtrate was obtained by Whatman filtration and a crude extract was obtained after the evaporation of the methanol to complete dryness under reduced pressure following rotary evaporation at 55˚C, which yielded a dark green residue of ~5.75 g (26% w/w). The extract was preserved in sterile tubes under refrigerated conditions (2-8˚C) until further use. The final concentration of methanol was always <1% in the experimental solution.

Phytochemical screening. Phytochemical investigation of extracts was conducted to detect the occurrence of various chemical components. Air-dried plant extract was examined to check for existence of tannins, steroids, flavonoids, alkaloids, triterpenoids, saponins, cardioglycosides and anthraquinones, as previously described (32).

Gas chromatography-mass spectrometry (GC-MS) analysis. Gas chromatography is used to identify bioactive constituents like alkaloids, steroids, ester, short and long-chain hydrocarbons, acids, alcohols, amino and nitro compounds (33,34). Samples were analyzed using Gas Chromatograph Mass Spectrophotometer GC-2010, MS QP2010 (Shimadzu, Japan). Capillary column used for Chromatographic separation was from Restek Rxi-5MS (Shimadzu Corporation) was performed via Argon gas as carrier in a continuous flow rate mode at the rate of 2 ml/min. The GC oven temperature was progressively raised to 120°C at the rate of 5°C/min, then up to 150°C at the rate of 2°C/min and 10°C/min up to 240°C, with a total run time of 36 min. The ion source, injector, and interface were maintained at 230, 250 and 280°C, respectively. Detection was operated by selected ion monitoring mode and injection volume was kept at 1 µl.

Cell culture. Human cervical cancer cell line (HeLa-CCL-2; RRID: CVCL_0030), Human breast adenocarcinoma cell line (MCF7-HTB-22; RRID: CVCL_0030), Human alveolar basal epithelial cell line (A549-CCL-185; RRID: CVCL_0023), Human embryonic kidney 293 cell line (293-CRL-1573; RRID: CVCL_0045), Human invasive ductal carcinoma cell line (BT549-HTB-122; RRID: CVCL_1092) and a normal cell line human foreskin fibroblasts (HFF-1-SCRC-1041; RRID: CVCL_3285) were obtained from National Centre for Cell Science (NCCS) and were used to test anticancer potential of Ap extracts. All the cell lines used were cultured in DMEM with 10% FBS in an incubator containing 5% CO₂ at 37°C. The cells were washed and harvested using trypsin (0.4% Trypan Blue solution; Thermo Fisher Scientific, Inc.) staining method and seeded into six-well plates for further studies. PCR-based detection of Mycoplasma was performed for all the cell lines used in the study using EZdetect (Himedia Laboratories, LLC) as it is one of the most rapid, sensitive and convenient methods for detection of mycoplasma contamination. The primer set is specific to the highly conserved 16S rRNA coding region in the Mycoplasma genome. The cell lines were authenticated at NCCS using16 Short Tandem Repeat loci analysis as recommended by the American Type Culture Collection (ATCC).
Screening of Ap extracts for cytotoxicity against cancer cells. This bioactive component is extracted using methanol-based extraction method. In the present study, the leaf extract was prepared directly using methanol. To check for the cytotoxicity of the extracts on the different cancer cells while HFF-1 cells served as a non-cancer control cells, MTT assay was performed using the following formula: Inhibition (%)=(A570 of treated cells-blank)/(A570 of control cells-blank) x100-Eq: 1. Effect of Ap extracts on cytotoxicity against cancer cells was assessed by MTT assay, which is based on the reduction of MTT into formazan by the cellular mitochondrial dehydrogenase in viable cells which yields blue/purple formazan crystals upon cleaving the tetrazolium ring. Based on the amount of reduction, the level of cell metabolism can be studied. A-549, MCF-7, HEK, Hela and BT549 cancer cell lines were examined for viability after treatment with extracts using MTT assay. Briefly, 1x10^4 cells were seeded onto a 96-well plate and exposed to 2-100 µg/ml of Ap extract for 24 h, after which the cells were treated with plain DMEM medium containing MTT (0.5 mg/ml) and incubated in the dark for 4 h. The formazan crystals formed after treatment were dissolved with DMSO and the optical density of the supernatants was measured at 570 nm (Fig. 2).

Flow cytometry for cell cycle analysis. Propidium iodide (PI)-based measurement of the DNA content in cells by flow cytometry. Cell cycle analysis was performed using FlowJo software (version 10; Becton, Dickinson and Company). HeLa cells at a density of 1x10^6 cells per well were seeded onto six-well plates and treated with Ap extracts (25, 50 and 100 µg/ml) for 24 h. After treatment, the cells were detached using a cell scraper, washed with FACS buffer (PBS containing 2% FBS) and centrifuged at 801 x g for 10 min at room temperature. The cell pellet was resuspended and fixed in ice cold 70% ethanol dropwise and incubated at 4°C for 2 h. The fixed cells were centrifuged at 961 x g for 8-10 min at room temperature and the resultant pellet was washed twice with FACS buffer. Finally, the cell pellet was suspended in 500 µl PBS containing PI (1 mg/ml), RNase A (20 µg/ml) and NP-40 (0.1%) and incubated in the dark for an additional 30 min. The stained cells were analyzed using a Guava flow cytometer (MilliporeSigma) to detect changes in DNA content based on PI staining. A common gating strategy was followed, wherein cells are distinguished by bivariate analyses of side scatter vs. forward scatters in terms of height and area of the scatter, respectively.

Immunoblotting. HeLa cells (2x10^5) were seeded per well in a six-well plate and treated with Ap extracts (25, 50 and 100 µg/ml) for 24 h. Treated cells were washed with PBS and lysed in RIPA buffer (Sigma-Aldrich; Merck KGaA) and the protein concentration was determined using Bradford's method and ~10 µg of protein was loaded into each well of a 12% gel and separated. Samples were immunoblotted onto a nitrocellulose membrane (Bio-Rad Laboratories, Inc.) via the Criterion blotting system (Bio-Rad Laboratories, Inc.). Membranes were blocked at 25°C using 5% skimmed milk in TBST (20 mM Tris, 150 mM NaCl and 0.1% Tween 20) for 1 h and were subsequently incubated overnight at 4°C with appropriate antibodies diluted (1:1,000) according to the manufacturer's protocol. The membranes were washed using TBST and incubated for 1 h at room temperature with appropriate HRP-conjugated secondary antibody (1:2,000). Bound secondary antibodies were detected using the enhanced chemiluminescence system (ECL; Bio-Rad Laboratories, Inc.). Images were acquired using LAS500 gel documentation System (Bio-Rad Laboratories, Inc.) and analyzed using ImageJ software (version 1.53a; National Institutes of Health). The relative intensity of the bands was calculated and expressed as ratio of treated vs. control. An average of independent experiments was used for representation.

In vitro cell migration assay. Scratch assay was performed in six-well plates as previously described (35-37). HeLa cells were cultured in DMEM medium containing 10% FBS until a confluent monolayer was formed. The monolayer was scratched with a sterile 10-µl micropipette tip to create a wound/gap of ~1 mm wide. Cells were washed twice with PBS to remove detached and loosely bound cells and the wells were replaced with fresh complete medium containing 25, 50 and 100 µg/ml of the plant extracts and the cells were cultured for 24 h. Images of the wounds were captured at 0 and 24 h under a microscope using EVOS XL Core Cell Imaging System. ImageJ software was used to measure the width of the scratch and cell migration was assessed. The results are presented as a mean percentage of inhibition of migration.

Chorioallantoic membrane (CAM) assay. Ap extracts were tested for anti-angiogenic activity using CAM of 9-day old fertilized hen eggs (n=64) (Gallus gallus) (Venkatashwara hatcheries, Hyderabad, Telangana, India). The shells of fertilized eggs were disinfected and incubated for 2 days at 37°C with a relative humidity of 70-75%. The CAM layers were inoculated with 1x10^6 (100 µl) of HeLa cells in absence and presence of plant extracts at concentrations 25, 50 and 100 µg/ml, (n=4x8 groups) with PBS was used as a Placebo and the eggs were transferred to the incubator for 72 h with eggs turned three to five times each over a 24-h period. After incubation, the embryos were euthanized on the 14th day in a humane method by placing the eggs in a gas chamber and filling the chamber with 100% CO₂ and maintaining it for 10 min (38), after which the embryos were inspected by candling to check for any movement. Later the shells were gradually broken and the embryo and yolk were removed. The developing vasculature on CAM was imaged and analyzed followed by manually counting the vessels. The vessels were counted by the experimenter and two unbiased individuals to authenticate the data. Changes in angiogenesis were evaluated by observing the secondary and tertiary blood capillaries. The secondary blood vessels are the fine blood vessels arising from the larger blood vessels while the tertiary vessels arise from the secondary vessels. For measurement of the area covered by different veins, capillary branching points were used as initiation and termination markers. All analyses were made in triplicates and any dead embryos were excluded from the present study. % Angiogenesis=(total number of Secondary blood vessels in treated-total number secondary blood vessels in untreated)/(total number of secondary blood vessels in vehicle control-total number secondary blood vessels in untreated) x100-Eq:2.
Cell invasion assay. Invasiveness of HeLa cells was tested in presence of Ap extracts by Transwell migration assay using Matrigel (Thermo Fisher Scientific, Inc.). Matrigel (20-30 µl) was added to each insert in a 24-well plate which was incubated in a CO₂ incubator at 37°C for 30 min. After incubation, DMEM with 1x10⁶ HeLa cells in 1% FBS along with Ap extracts was added to each of the inserts. To the lower chamber, 600 µl of complete medium (containing 12% FBS) was added as a chemoattractant. These wells were incubated in an incubator with 5% CO₂ at 37°C for 24 h. The medium was removed carefully from the insert and the insert was fixed using 70% ethanol for 10 min at room temperature. After incubation, ethanol was aspirated carefully and the inserts were allowed to dry at room temperature. Cells were stained with 0.2% crystal violet for 2 min at room temperature followed by rinsing with 70% ethanol to remove the excess stain. Images were captured using an inverted light microscope at x40 magnification (Nikon Corporation).

Statistical analysis. All analyses were performed in triplicates and these values were presented as the mean ± SD and plotted using GraphPad Prism 7.0 software (GraphPad Software, Inc.). Statistical comparisons were conducted using analysis of variance (One way-ANOVA and two way ANOVA) followed by Tukey’s post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Chemical composition of methanolic extract of Ap. Methanol was used to prepare Ap extracts from dried and powdered leaves of Ap. The qualitative tests to show the presence of alkaloids, flavonoids, proteins, terpenoids, cardioglycosides and steroids were performed, results of which have been presented in Table I. In the present study, a method was used to extract and identify bio-active compounds from Ap which is somewhat different in terms of using the organic extraction method. This method could help in extraction of a new set of compounds previously unknown to be present in the plant. Previous studies have reported biological activity due to Andrographolides which are terpenoids, 5,7,2',3'-tetramethoxyflavone, an alkaloid and arabinogalactan, a protein, as certain of the major biologically active components in the plant. These have been widely studied in their applications in disease such as fever, infection and even cancers (39). To explore the presence and identification of more bioactive components, GC-MS was carried out.

Identification of components by GC-MS. Ap extracts displayed 21 peaks in the GC-MS chromatogram showing the presence of 21 phytochemical constituents (Fig. 1). The GC-MS data was interpreted using National Institute Standard and Techniques (NIST) database which comprises more than 62,000 patterns. By comparing the average peak area to the total area the relative percentage of each component in the mixture was estimated. By comparing the spectrum of the unknown component with that of a known component found in the NIST library, the molecular weights, structure and names of the components in the test materials were determined. Among all the compounds, 2(5H)-Furanone (14.73%), Quinic acid (QA; 17.32%) and Phenol (11.43%) were found to be the major phytochemical constituents, the retention time, molecular formula, molecular weight and concentrations of the compounds are presented in Table II. Apart from the three aforementioned components, 5-Hydroxymethylfurural (4.75%) was also observed in some abundance, followed by Dimethyl sulfone (3.93%), 3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one (3.15%), 1,2-Cyclopentanedione (2.33%), 1-Ascorbic acid 2,6-dihexadecanoate (2.30%), 2-Propanoic acid (1.76%), Benzofuran, 2,3-dihydro-Coumaran (1.74%), 3,5-Dimethylanisole (1.23%), Hexadecanoic acid (1.17%) and 3-Furannmethanol (1.03%), 8,11,14-Docosatrienoic acid (0.89%), Benzenepropanoic acid (0.89%), Dibutyl phthalate (0.87%), 2(4H)-Benzo[4,5]furanone (0.59%), 9-Heptadecanone (0.57%), 9,12-Octadecadienoic acid (0.57%), 2-Pentadecanone (0.52%), presented in Fig. S1 and Table SII.

Methanol extracts of Ap show cytotoxicity against multiple cancer cell lines. The cytotoxicity of the extracts was compared between different cancer cell lines in the concentration range of 2-100 µg/ml and expressed as percent viability (Fig. 2). A range of 25-100 µg/ml showed effective inhibition against cancer cell lines, wherein, a dose dependent increase in cytotoxicity was observed. However, at 25 µg/ml cell viability against MCF-7, Hela, and BT-549 cancer cell lines was observed to be less than 50%. The extracts demonstrated strong activity against cancer cell lines in vitro with IC₅₀ <100 µg/ml. An IC₅₀ of 50.347±1.975, 36.237±1.729, 43.779±5.516, 33.136±2.370, 33.514±2.261 µg/ml was recorded against A-549, MCF-7, HEK, HeLa and BT-549 cell lines, respectively. Inhibitory effects were observed from 5 µg/ml itself in MCF-7, HEK, HeLa and BT-549 cells whereas in A-549 cells, inhibition was observed from 25 µg/ml. Cell viability decreased significantly with increasing concentrations and at 25 µg/ml less than 50% viability was observed, i.e., 42.1, 45, and 43.8% cell viability in MCF-7, Hela and BT-549 cells respectively with no cytotoxicity against HFF cells.
Ap extracts inhibit angiogenesis in CAM layer of eggs co-inoculated with HeLa cells. In order to assess the effect of the Ap extracts on the angiogenesis and cell migration, HeLa cell line was chosen for all the further experiments as Ap extracts appeared to be exhibiting highest inhibition against HeLa as compared with the other cell lines examined. Ap extracts were able to reduce the in vivo angiogenesis induced by HeLa cancer cells on CAM of embryonated eggs (Fig. 3). In the CAM of untreated/placebo controls, secondary vessel number in the area covered did not show much change after 72 h, while in the Hela injected CAMs, (treated control) number of secondary vessels were higher after 72 h. In the CAMs injected with Hela and treated with the Ap extract, anti-angiogenic activity was observed to be dose-dependent showing a decrease in fresh capillary formation with increasing concentrations. Reduction in the secondary blood vessel formation was observed as 38.71, 56.46 and 67.75% with 25, 50 and 100 µg/ml respectively, as compared with CAM of the embryos treated with only HeLa which showed increased number of capillaries, unlike untreated CAMs which did not show significant change. These results supported the anti-angiogenic effects of Ap.

Inhibition of in vitro cell migration by Ap. Ap extracts were further examined for their ability to inhibit the migration of HeLa cells, which was tested by performing an in vitro scratch assay. The doubling time required for HeLa is 33-35 h, since the experiment was performed for a period of 24 h which limited the possibility of cells proliferating into the wound. Furthermore, flow cytometric analysis revealed that the cells treated with the extract were arrested in the G2/M phase lowering the possibility that the cells proliferated into the wound rather than migrating into it (Fig. 4A). After incubation for 24 h with the extracts, the gap created by the scratch remained fairly intact and minimum number of cells were observed to fill the wound in the treated

Table II. Major compounds identified in the crude extract of Ap 2(5H)-Furanone (14.73%), Quinic acid (17.32%) and Phytol (11.43%) using gas chromatography-mass spectrometry analysis with its retention time, peak area and reported biological activities.

| S. No. | Compounds     | Molecular weight (g/mol) | Retention time | Area% | Biological activities                                                                 | (Refs.)          |
|--------|---------------|--------------------------|----------------|-------|--------------------------------------------------------------------------------------|------------------|
| 1.     | 2(5H)-Furanone| C₅H₄O₂                   | 84             | 4.591 | Anti-inflammatory, anticancer, antimicrobial, and antifungal activity                  | (46-48)          |
| 2.     | Quinic acid   | C₇H₁₂O₆                  | 192            | 13.21 | Antioxidant activity, anti-inflammatory                                                | (53,55)          |
| 3.     | Phytol        | C₂₂H₄₂O₂                  | 338            | 14.91 | Antimicrobial, anticancer, anti-inflammatory and diuretic                               | (56,57,62)       |

Figure 1. A list of components identified by gas chromatography performed for crude methanolic extracts of Andrographis paniculata showing the major bioactive components.
groups (25, 50, and 100 µg/ml) compared with the untreated group. Percentage of cell migration was significantly reduced with increasing concentrations of the Ap extract indicating inhibitory effect of the Ap extract on HeLa cell migration (Fig. 4B).

**Ap extracts inhibit the invasiveness and migration of cervical cancer.** Metastasis involves a sequence of steps linking cancer cell invasion followed by migration. In order to investigate if Ap extract has any anti-invasion properties, Matrigel-coated Transwell chambers were used in the presence of different concentrations (25, 50 and 100 µg/ml) of Ap extracts. The total number of cells passing through the Matrigel-coated filter reduced significantly in presence of the extracts (Fig. 4C). Compared with the control group, the inhibition percentage of invasion was 21.37, 28.2 and 38.47% for 25, 50 and 100 µg/ml of extracts, respectively (Fig. 4D). Thus, in addition to its

---

**Figure 2.** Effect of Ap extracts on cell toxicity. Cytotoxicity of Ap extracts is expressed as percentage of viability of cells. Statistical analyses were performed by One-way ANOVA, followed by Tukey’s post-hoc test. Data obtained from three individual experiments was compared between each of the treatments with their respective vehicle control (methanol) expressed as the mean ± SD. **P<0.01, ***P<0.001 and ****P<0.0001 vs. vehicle control. Ap, Andrographis paniculata; ns, non-significant.

**Figure 3.** Analysis of neo-vascularization of CAM layer after treatment with Ap extracts. CAM layer of embryonated chick egg was injected with (A) PBS (control) and (B) HeLa cells; (C-E) 25, 50 and 100 µg of the Ap extract along with HeLa cells. The capillaries arising from the central blood vessel were enumerated and represented as % angiogenesis. (F) Graph showing percentage of angiogenesis. Statistical analyses were performed by One-way ANOVA followed by Tukey’s post hoc test. Data from three individual experiments vs. the control group are expressed as the mean ± SD. **"P<0.001 and ‘’P<0.0001 vs. A (untreated). CAM, chorioallantoic membrane; Ap, Andrographis paniculata.**
inhibitory effect on cell viability, Ap extracts also inhibited the cell invasion potential of HeLa cells in vitro. This effect of the extracts could be due to cytotoxicity leading to cell death, thereby, preventing cell migration and invasion by tumor cells.

**Ap extract arrests cell cycle progression in cancer cells.** To investigate the mechanism behind growth inhibition, the effect of the Ap extracts on cell cycle distribution of HeLa cells was analyzed after 24 h of treatment by flow cytometry. An alteration in the distribution of different phases of the cell cycle was clearly evident (Fig. 5A-D). Paclitaxel was used as a positive control for cell cycle arrest. In the treated cells, increase in cell population in the G2/M phase was observed. A surge in the percentage of cells accumulating in the G2/M phase with increasing concentrations of the extract (Fig. 5E) was identified. While the untreated cells showed lower percentage at 16.18% in the G2/M phase, Ap extract-treated cells showed a considerable increase in percentage of accumulated cells at 41.93% at a concentration of 100 µg/ml. No significant change in all other phases was observed. These findings suggested induction of apoptotic cell death in HeLa cells upon treatment with Ap extracts via G2/M phase arrest of cell cycle progression.

**Ap extracts induce apoptosis in cancer cells.** Cell death has been observed in human cervical carcinoma (HeLa) induced by treatment with methanolic extract prepared from Ap. To validate aforementioned data from flow cytometry, an attempt was made to understand the mechanism leading to apoptotic cell death. Cells treated with the extracts showed significant increase of ~20 fold in the activity of caspase-3, a crucial mediator of apoptosis with increasing concentrations when compared with the untreated cells (Fig. 6B). The extracts were also able to increase the levels of Bax, a pro-apoptotic marker, by 15 and 20 fold when exposed to 25 and 50 µg/ml of the extract while a maximum of 30 fold increase was observed when exposed to 100 µg/ml (Fig. 6D). By contrast, a simultaneous downregulation by 8 fold in the expression of the anti-apopotic marker Bcl-2 was observed for 25 and 50 µg/ml which was completely inhibited when treated with 100 µg/ml of the extract (Fig. 6C), confirming induction of apoptotic cell death in Ap-treated HeLa cells (40).

**Discussion**

Medicinal plants and their activities are of immense interest due to their unique properties including significant anticancer activity and milder or negligible side effects. Recent investigations have involved the identification and separation of new and beneficial compounds of therapeutic importance from higher plants for specific diseases. As per the WHO report published in the year 2002, 60% of medicinal drugs including anticancer drugs are derived from natural sources (41). HeLa cells are highly invasive
ANOOR et al.: METHANOL EXTRACTS OF Andrographis paniculata HAVE ANTICANCER ACTIVITY

and robust cells with shorter multiplication time and simple growth requirements and hence were chosen for the present study. After complete analysis of the Ap methanolic extracts, it was estimated that the anti-proliferative activity of the extracts could be due to the presence of 2(5H)-Furanone, QA and Phytol which share a major percentage of the components even when concentrations as low as 25 µg/ml were used in the assay thus suggesting that the activity could be due to the major components of the extract, while the remaining components accounted for a markedly lower share. Notably, these compounds have not

Figure 5. Ap extracts arrest cell cycle progression in HeLa cells. Ap induced cell cycle arrest at G2/M phase in HeLa cells. Cells were stained with PI after treatment with Ap extracts for 24 h (A) Control, (B) 50 µg/ml (C) 100 µg/ml and (D) Paclitaxel. (E) Bar diagram showing groups B and C had significantly higher percentages of cells at G2/M phases than the other phases. Statistical analyses were performed by One-way ANOVA followed by Tukey's post hoc test. Data are expressed as the mean ± SD from three individual experiments against the control group. *P<0.05 vs. A (untreated cells). Ap, Andrographis paniculata.
been previously reported in Ap. Most of the previous studies used dichloromethane with methanol (1:1) while fractionation by cold maceration method was used for extraction of bioactive components (27,42-44). In the present study, only methanol was used during extraction and it could be an important factor in extraction of tough to isolate ingredients from the extracts. Methanol is suitable for extraction of primary and secondary metabolites since it has a higher dielectric constant and can strongly penetrate the cell to extract even polar contents. Due to its high polarity, methanol has higher extraction yields and can extract both hydrophilic and lipophilic molecules from plant parts. After extraction, methanol can be removed by distillation at lower temperatures as methanol is highly volatile. Moreover, the isolation of andrographolides which form the major component in Ap, involves extraction of the leaf powder by cold maceration in a 1:1 mixture of dichloromethane and methanol and recrystallisation of andrographolide directly from the resulting extract (42). The presented method of extraction with methanol did not help to isolate the main andrographolides and other bioactive components previously reported by other groups. Hence, it can be suggested as an adjunct to other methods for maximum extraction of bioactive components from the plant, as it was mainly possible to isolate the active non-andrographolide components using methanol-based extraction.

2(5H)-Furanone, also known as γ-crotonolactone, is a part of a large number of biologically active compounds, comprising natural products and pharmaceutical agents. It belongs to the group of α, β-unsaturated lactones, which are observed in numerous natural products. 2(5H)-furanone derivatives are known to have good pharmacological activities; particularly 2(5H)-furanone skeleton are known for their extensive biological activity, together with antitumor (45), antibacterial (46), antifungal (44), antiviral (48), anti-inflammatory (38,49) and antioxidant effects. QA is an important natural cyclitol found in tea, coffee, fruits and vegetables (50,51). This bioavailable, non-toxic, natural polyol was found to be exhibiting potent antioxidant, anti-inflammatory (52) and anti-mutagenic activities (38) and was also revealed to chelate transition metals (53). Hence this versatile parent chiral compound is used in the synthesis of new drugs (54). QA has also been studied as a potent drug for treatment of prostate cancer (55).

Phytol, is a chlorophyll-derived acyclic diterpene alcohol and a scented ingredient used in numerous fragrant compounds abundantly present in nature which is utilized in cosmetic and
non-cosmetic products (52). It exhibits various pharmacological activities such as antimicrobial (55), anticancer (56), immunoadjuvant, antihoodemic and lipid lowering (57), anxiolytic (58), antinoceptive (59), anti-teratogenic (60) antioxidant, anti-inflammatory (61) as well as anti-allergic effects (40,62). Phytol also functions as immuno-stimulant and is superior to several commercial adjuvants in terms of immunological memory induction over a longer-term and activation of innate and acquired immunity (61). Phytol and its byproducts have no collective inflammatory or toxic effects even in immuno-compromised mice (63). It is also known to inhibit the growth of pathogens such as Mycobacterium tuberculosis (62,64) and Staphylococcus aureus (65). Apart from the aforementioned effects, phytol also showed anti-angiogenic activity by inducing apoptosis in A549 cells by depolarizing the mitochondrial membrane potential (66).

In addition, certain minor components in the extract such as 5-Hydroxyethylfurural are known to exhibit anti-inflammatory and antitumor effects (67). For example, dimethyl sulfone has antimicrobial, anti-inflammatory and anticancer properties (68), while 3,5-Dihydroxy-6-methyl-2,3-dihydro-4H-pyran-4-one is considered to exhibit free radical scavenging activity (69). Similarly, 1,2-cyclopentanedione was reported to be effective in prevention of gastrointestinal tumor growth (70) and 1-Ascorbic acid 2,6-dihexadecanoate was found to have antiproliferative efficacy (71). 2-Propenoic acid is said to have antibacterial properties (72) and 2,3-dihydro-Coumaran was reported to express anti-inflammatory effects (73). 3,5-Dimethylanisole has antimicrobial, anti-inflammatory and antioxidant effects (74). Unlike other compounds, Hexadecanoic acid has potent antimicrobial activity along with antifungal, antitumor, antioxidant, chemo-preventive, antioxidant and immuno-stimulant effects (75). 3-Furanmethanol is known to possess anticancer, anti-inflammatory and antimicrobial activities (76); 8,11,14-Docosatrienoic acid is cardioprotective (77) and benzenepranoic acid was reported to possess antifungal, antioxidant activities (78); Diethyl phthalate was found to express antifungal and antitumor (79) properties. Furthermore, 2(5H)-Benzofuranone has been revealed to possess analgesic, anti-diabetic, antibacterial and antifungal properties (72) whereas 9-Heptadecanone expressed antibacterial (80) activity. Similarly, 9,12-Octadecadienoicacid was found to have anticancer properties (81) while 2-Pentadecanone (0.52%) was reported for hypocholesterolemic, antioxidant effects (82). It is definitely possible that all the aforementioned compounds, though present in minor proportions, act along with phytol, QA and (5H)-furanone. However, it has been revealed that the 3 major aforementioned compounds showed activity at very low concentrations of 50 and 100 μg.

In such low concentrations it is expected that the predominant components can exhibit apparent biological activity.

Cell division is an intricate phenomenon regulated by various protein kinases which oversee the gene transcription necessary for progression into subsequent phases of the cell cycle. The mitogen-activated protein kinases play a crucial role in the initiation, advancement and coupling of these phases. Ap extracts screened for effect on cell cycle progression were observed to cause buildup of cells in the G2/M phase, reflecting increase in apoptotic cell death. This outcome was also dose-dependent, indicating that Ap extracts work by arresting the cell cycle at G2/M phase. Evasion of malignant cells from apoptotic cell death is crucial in tumor formation. Most anticancer therapies currently in use such as chemotherapy and γ-irradiation and immunotherapy involve killing tumor cells by activation of the intrinsic and/or extrinsic apoptotic pathways in cancer cells. Bcl-2 and Bax are an important signaling proteins in the mitochondria-mediated apoptotic pathways that regulate the release of cytochrome c, DNA fragmentation and activation of caspases. The ratio of Bcl-2 and Bax determines the sensitivity of cancer cells to death signals. Bcl-2, is an anti-apoptotic protein which promotes cancer cell growth and is a member of the Bcl-2 family while Bax, another component of the Bcl-2 family, induces apoptosis (83). Expression of pro-apoptotic Bax is required to induce apoptosis and to correct uncontrolled cell proliferation (84). Caspases are proteases that are essential mediators of apoptosis and inflammation. HeLa cells exposed to Ap extracts showed increased Caspase-3 and Bax and lowered Bcl-2 levels, indicating activation of intrinsic apoptotic pathway. This indicated that Ap extracts can induce the intrinsic apoptotic pathway in HeLa cells.

The anti-angiogenesis concept has been an important component in cancer treatment and it is considered that blocking angiogenesis could be a strategy to arrest tumor growth and metastasis (85). Angiogenesis leads to development of tumors leading to invasion and metastasis. Anti-angiogenesis is a strategy that prevents tumor progression and can be contemplated as one of the targets for anticancer therapy, since the tumor growth can be controlled if oxygen and nutrient supply to a tumor could be reduced. New blood vessel formation (angiogenesis) involves a multistep process involving cell proliferation, migration and remodeling. Suppression of any step may result in inhibition of new blood vessel formation. Using the chick embryo CAM model, it could be demonstrated that Ap extracts inhibit angiogenesis in CAM even at minimum concentrations, indicating potent anti-angiogenic activity. Numerous flavonoids, terpenoids and polyphenols, are known to inhibit carcigenogenesis and tumorigenesis in vivo and in vitro. Among the identified compounds 2(5H)-Furanone (86-89) along with phytol (90,91) and QA (92,93) are used in the treatment of several types of cancer. Since it is the predominant component identified by GC-MS, it could be the predominant anticancer and anti-inflammatory component of the extracts. With the existence of other bioactive components in the extract, it is also possible that the antiangiogenic activity of the extracts is due to the synergistic effect of the predominant compounds; 2(5H)-furanone, phytol and QA augmenting the antiangiogenic effect. Anti-migratory activity, motility and invasion of cervical cancer cells after Ap treatment were evaluated by scratch wound and Transwell invasion assays. Our results suggested that Ap significantly inhibited the motility and invasiveness of cervical cancer cells. Cervical cancer accounts for 6-29% of cancers in India and is one of the most common forms of cancer in women worldwide. Therefore, the present study concentrated on utilization of HeLa cells for establishing the mechanism of action of the extracts. However, it is recommended that testing multiple cell lines derived from cervical cancer or other cancers will be extremely useful in establishing the mechanism. It is a limitation to the present study that the findings could not be confirmed in other cell
lines, but since the main goal was to characterize the mechanism of action of Ap extracts, it could be stated that some new previously unreported components from Ap which have strong anti-proliferative activity were identified by mainly suppressing cell cycle progression and promoting intrinsic apoptotic pathway. In conclusion, the non-andrographolide components of Ap appear to have significant anticancer and anti-angiogenic activity. While the anticancer properties of andrographolides are well established, to the best of our knowledge, this is the first investigation reporting the anti-cancer effects of the non-andrographolide components of Ap. Though in the present study the activity of the total methanol extract, 2(5H)-furanone, phytol and QA were revealed to be the major components, the anticancer properties could not be attributed to these compounds, 2(5H)-furanone, phytol and QA are known separately for having anti-inflammatory and antitumor activity and these compounds appear to target cell cycle arrest and induce the intrinsic apoptotic pathways. It is possible that their combinations in crude extract have synergistic activity causing significant reduction in invasiveness and metastatic properties of malignant cells. Further studies using purified compounds would help in specifically identifying the role of each of these components.

Acknowledgements

The authors would like to thank Sreeram Kaveti, (Senior Research Fellow, Applied Biology, CSIR-IICT, Hyderabad, Telangana 500007) for helping with experimentation and Dr Gopal Reddy, (Professor (Retd) Department of Microbiology, Osmania University, Hyderabad, Telangana 500007) for providing logistic support during the study.

Funding

The present study was supported by UGC-BSR Fellowship (grant nos. F4-1/2006(BSR)/7-369/2012(BSR)/2013-2014/4 and 122/C/BB/MICRO/HOD/2015). The APC was supported by Rashtriya Uchchatar Shiksha Abhiyan.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

PKA conceptualized the study, provided methodology and software, validated the data and prepared the original draft. ANY performed investigation, data curation and contributed to preparation of the original draft. KR conducted investigation, software and formal analysis. RK performed investigation and formal analysis. CT conducted investigation, validation, wrote, reviewed and edited the manuscript. KSN performed validation, formal analysis, wrote, reviewed and edited the manuscript. SB conceptualized and supervised the study, provided resources, performed project administration, prepared the original draft, wrote, reviewed and edited the manuscript. All authors read and approved the final version of the manuscript. PKA and SB confirm the authenticity of all the raw data provided in the present article.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Ohtitit A, Gaur RL, Abdraboh M, Ireland SK, Rao PN, Raj SG, Al-Ryami H, Shammasanath S, Gupta L, Murthy SN, et al: Simultaneous inhibition of cell-cycle, proliferation, survival, metastatic pathways and induction of apoptosis in breast cancer cells by a phytochemical super-cocktail: Genes that underpin its mode of action. J Cancer 4: 703-715, 2013.
2. Sukanya SL, Sudisha J, Hariprasad P, Niranjana SR, Prakash HS and Fathima SK: Antimicrobial activity of leaf extracts of Indian medicinal plants against clinical and pathogenic bacteria. Afr J Biotechnol 8: 6677-6682, 2009.
3. Abutbul S, Golan-Goldhirsh A, Barazani O, Ofir R and Zilberg D: Screening of desert plants for use against bacterial pathogens in fish. Isr J Aquacult-Bamid 57: 71-80, 2005.
4. Pandey G and Madhuri S: Significance of fruits and vegetables in malnutrition cancer. Plant Arch 10: 517-22, 2010.
5. Pandey G, Madhuri S and Mandloi AK: Medicinal plants useful in fish diseases. Plant Arch 2: 1-4, 2012.
6. Ravikumar S, Selvan GP and Gracelin AA: Antimicrobial activity of medicinal plants along Kanyakumari coast, Tamil Nadu, India. Afr J Basic Appl Sci 2: 153-157, 2010.
7. Umainaheswari S and Mainzen Prince PS: Antihyperglycaemic effect of ‘ilogen-excel’, an ayurvedic herbal formulation in streptozotocin-induced diabetes mellitus. Acta Pol Pharm 64: 53-61, 2007.
8. Misra P, Pal NL, Guru PY, Katiyar JC, Srivastava V and Tandon JS: Antimalarial activity of Andrographis paniculata (Kalmegh) against plasmodium berghei NK 65 in mastomys natalensis. Int J Pharmacogn 30: 263-274, 1992.
9. Singha PK, Roy S and Dey S: Antimicrobial activity of Andrographis paniculata. Fitoterapia 74: 692-694, 2003.
10. Ernst E and Cassileth BR: The prevalence of complementary/alternative medicine in cancer: A systematic review. Cancer 83: 777-782, 1998.
11. Newman DJ and Cragg GM: Natural products as sources of new drugs from 1981 to 2014. J Nat Prod 79: 629-661, 2016.
12. Newman DJ and Cragg GM: Natural products as sources of new drugs over the nearly four decades from 01/1981 to 09/2019. J Nat Prod 83: 770-803, 20/20.
13. See D, Mason S and Roshan R: Increased tumor necrosis factor alpha (TNF-alpha) and natural killer cell (NK) function using an integrative approach in late stage cancers. Immuno Invest 31: 137-153, 2002.
14. Ahilan B, Nithiyapriyatharshini A and Ravaneswaran K: Influence of certain herbal additives on the growth, survival and disease resistance of goldfish, Carassius auratus (Linnaeus). Tamilnadu J Vet Ani Sci 6: 5-11, 2010.
15. Zilberberg MD, Short AF, Micek ST, Vazquez-Guillamet C and Kollef MH: Multi-drug resistance, inappropriate initial antibiotic therapy and mortality in Gram-negative sepsis and septic shock: A retrospective cohort study. Crit Care 18: 596, 2014.
16. Runyoro DK, Matee MI, Ngassapa OD, Joseph CC and Mbwambo ZH: Screening of Tanzanian medicinal plants for anti-Candida activity. BMC Complement Altern Med 6: 11, 2006.
17. Shukla B, Visen PK, Patnail GK, and Dhawan BN: Choleric effect of andrographolide in rats and guinea pigs. Planta Med 58: 146-149, 1992.
18. Srinivas Reddy P, Jamil K, Madhusudhan P, Anjani G and Das B: Antibacterial activity of isolates from piper longum and taxus baccata. Pharm Biol 39: 236-238, 2001.

19. Prajapati ND, Purohit SS, Sharma AK and Kumar T: A handbook of medicinal plants: A complete source book. Agrobios, Rajasthan, pp54-2003.

20. Chang HM and But PP: Pharmacology and Applications of Chinese Materia Medica. Vol 1. World Scientific, Singapore, 19986.

21. Liu J, Wang ZT and Ji LL: In vivo and in vitro anti-inflammatory activities of neandrographolide. Am J Chin Med 35: 317-328, 2007.

22. Islam MT, Ali ES, Üdдин SJ, Shaw S, Islam MA, Ahmed MI, Chandra Shil M, Karmarkar UK, Yarla NS, Khan IN, et al: Phyto: A review of biomedical investigations. Food Chem Toxicol 121: 342-399, 2018.

23. Suriyo T, Pholphana N, Rangkadikol N, Thianantanawat A, Watcharisat P and Satayavivad J: Andrographis paniculata extracts and major constituent diterpenoids inhibit growth of intrahepatic cholangiocarcinoma cells by inducing cell cycle arrest and apoptosis. Planta Med 80: 533-543, 2014.

24. Fulda S: Modulation of apoptosis by natural products for cancer therapy. Planta Med 76: 1075-1079, 2010.

25. Mishra T: Ethno-botanical and therapeutic importance of sacred plants of Terai region of Gorakhpur division. Eur J Pharm Res 3: 534-540, 2016.

26. Plubrukarn A, Pinsuwan S, Ingkatawornwong S and Supavita T: Planta Med 79: 1001-1004, 2010.

27. Rajagopalan R: Andrographolide, a potential cancer therapeutic in metastatic esophageal cancer treatment—from preclinical perspectives. Sci Rep 7: 854, 2017.

28. Chiu PW: The adjuvant value of Andrographis paniculata extracts and major constituent diterpenoids inhibit growth of cancer cells by inducing cell cycle arrest and apoptosis. Planta Med 80: 533-543, 2014.

29. Fulda S: Modulation of apoptosis by natural products for cancer therapy. Planta Med 76: 1075-1079, 2010.

30. Mishra T: Ethno-botanical and therapeutic importance of sacred plants of Terai region of Gorakhpur division. Eur J Pharm Res 3: 534-540, 2016.

31. Plubrukarn A, Pinsuwan S, Ingkatawornwong S and Supavita T: Stability of andrographolide in powdered Andrographis herb under accelerated conditions. Planta Med 72: 954-956, 2006.

32. Kumar RA, Sridevi K, Kumar NV, Nanduri S and Rajagopal S: Andrographolide, a potential cancer therapeutic agent isolated from Andrographis paniculata. J Ethnopharmacol 107: 291-295, 2006.

33. Li L, Yue GG, Lee JK, Wong EC, Fung KP, Yu J, Lau CB and Chiu PW: The adjuvant value of Andrographis paniculata in metastatic esophageal cancer treatment—from preclinical perspectives. Sci Rep 7: 854, 2017.

34. Chiu PW, Chen CF and Lin J: Mechanisms of suppression of inducible nitric oxide synthase (iNOS) expression in RAW 264.7 macrophages by andrographolide, andrographisolate and andrographimide. J Ethnopharmacol 107: 291-295, 2006.

35. Tsai HC, Li L, Yue GG, Lee JK, Wong EC, Fung KP, Yu J, Lau CB and Chiu PW: The adjuvant value of Andrographis paniculata in metastatic esophageal cancer treatment—from preclinical perspectives. Sci Rep 7: 854, 2017.

36. Ganguly A, Yang H, Sharma R, Patel KD and Cabral F: The role of microtubules and their dynamics in cell migration. J Biol Chem 287: 43359-43369, 2012.

37. Nanduri S, Nyavanandi VK, Thunuguntla SS, Kasu S, Palarla MK, Ram PS, Rajagopal S, Kumar RA, Ramanujam R, Chandra Shill M, Karmakar UK, Yarla NS, Khan IN, et al: Phyto: A review of biomedical investigations. Food Chem Toxicol 121: 342-399, 2018.

38. ANOOR et al: METHANOL EXTRACTS OF Andrographis paniculata HAVE ANTICANCER ACTIVITY

39. Khandelwal KR: Practical Pharmacognosy. Pragati Books Pvt. Ltd., Pune, 2008.

40. Özőcölk B, Kartal M and Orhan I: Cytotoxicity, antiviral and antimicrobial activities of alkaldoids, flavonoids, and phenolic acids. Pharm Biol 49: 396-402, 2011.

41. Pejin B, Savic A, Sokovic M, Tesevic V, Savic A, Radotic K and Mojovic M: Further investigation on the toxicity, and antimicrobial activities of phytol. Nat Prod Res 28: 372-376, 2014.

42. Sareer O, Ahmad S and Umar S: Andrographis paniculata: A critical appraisal of extraction, isolation and quantification of andrographolide and other active constituents. Nat Prod Res 28: 3-20, 2014.

43. Rajani M, Shrivastava N and Ravishankara MN: A rapid method for isolation of andrographolide from Andrographis paniculata nees (Kalmeg). Pharm Biol 38: 204-209, 2000.

44. Redza-Dutoirdor M and Averill-Bates DA: Activation of apoptosis signaling pathways by reactive oxygen species. Biochim Biophys Acta 1863: 2977-2992, 2016.

45. Wang CZ, Calway T and Yuan CS: Herbal medicines as adjuvants for cancer therapies. Am J Chin Med 40: 657-669, 2012.

46. Wu YC, Luo SH, Mei WJ, Cao L, Wu HQ and Wang ZY: Synthesis and biological evaluation of 4-biphenylamino-S-hy (1H)-furanones as potential anticancer agents. Eur J Med Chem 139: 84-94, 2017.

47. Lattmann E, Sattayayasi S, Schwalbe CS, Niamsanit S, Billington DC, Lattmann P, Langley CA, Singh H and Dunns S: Novel anti‑bacterial agents against MRSA: Synthesis of focussed compound libraries of tri‑substituted 2(5H)‑furanones. Curr Drug Discov Technol 3: 125-134, 2006.

48. Zapa S, Anke T and Sterner O: Incrustoporin, a new antibiotic from Incrustoporaria carneola (Bres.) Bryv. (Basidiomycetes). Acta Chem Scand (Cph) 49: 233-234, 1995.

49. Haaland M, Tranmo KE and Abou‑Elmaged WS: Conversion of some 2(3H)‑furanones bearing a pyrazolyl group into other heterocyclic systems with a study of their antioxidant activity. Eur J Med Chem 42: 934-939, 2007.

50. Weber V, Couderp F, Rabut C, Duroux E, Vallée‑Goyet D, Guérin‑D'Elia D, Brin M, Albusseau E, Leval F, Gramain JC, et al: Novel 4,5‑diaryl‑3‑hydroxy‑2(5H)‑furanones as anti‑oxidants and anti‑inflammatory agents. Bioorg Med Chem 10: 1647-1658, 2002.

51. Prasad PV, Srinivasa Reddy P, Jamil K, Madhusudhan P and Khamun F: Antioxidant, biomolecule oxidation protective activities of Nandostachys jatamansi DC and its phytochemical analysis by RP‑HPLC and GC‑MS. Antioxidants (Basel) 4: 185‑203, 2015.

52. Koo HJ and Kang SC: Quinic acid inhibits vascular inflammation in TNF‑α‑stimulated vascular smooth muscle cells. Biomed Pharmacother 69: 371-376, 2014.

53. Khandelwal KR: Practical Pharmacognosy. Pragati Books Pvt. Ltd., Pune, 2008.
62. Santos CC, Salvadori MS, Mota VG, Costa LM, de Almeida AA, de Oliveira GA, Costa JP, de Sousa DP, de Freitas RM and de Almeida RMN: Antineoplastic and antioxidant activities of phytol in vivo and in vitro models. Neurosci J 2013; 94:9452, 2013.
63. Arnhold T, Elmarz MM and Nau H: Prevention of vitamin deficiency. Toxicol Sci 66: 274-282, 2002.
64. Roy RN, Laskar S and Sen SK: Dibutyl phthalate, the bioactive compound produced by Streptomyces albidoflavus 321.2. Microbiol Res 161: 121-126, 2006.
65. Rajab MS, Cantrell CL, Franzblau SG and Fischer NH: Antimycobacterial activity of (E)-phytol and derivatives: A preliminary structure-activity study. Planta Med 64: 2-4, 1998.
66. Chowdhury RR and Ghosh SK: Phytol-derived novel isoprenoid behavior. J Pharmacogn Phytochem 3: 208-512, 2010.
67. Saikia D, Parihar S, Chanda D, Ojha S, Kumar JK, Chanotiya CS, Shanker K and Negi AS: Antitubercular potential of some semi-synthetic analogues of phytol. Bioorg Med Chem Lett 20: 508-512, 2010.
68. Inoue Y, Hada T, Shiraishi A, Hasegawa JH: Saffron chemoprevention in biology and medicine: A review. Cancer Biother 10: 2415-2419, 2003.
69. Abdullah BM, Mehdi MAH, Khan AR and Pathan JM: Gas chromatography-mass spectrometry (GC-MS) analysis of awajin (Trachyspermum ammi) seed extract. Int J Pharm Qual Assur 8: 528-535, 2015.
70. Tian Z, Shen J, Moseman AP, Yang Q, Yang J, Xiao P, Wu E and Kohane IS: Duxalthone A induces cell cycle arrest and apoptosis via up-regulation of p53 through mitochondrial pathway in HepG2 cells. Int J Cancer 122: 31-38, 2008.
71. Yue GG, Lee JK, Kwok HF, Cheng L, Wong EC, Jiang L, Yu H, Leung HW, Wong YL, Leung PC, et al: Novel PI3K/AKT targeting anti-angiogenic activities of 4-vinylphenol, a new therapeutic potential of a well-known styrene metabolite. Sci Rep 5: 11149, 2015.
72. Roy RN, Laskar S and Sen SK: Dibutyl phthalate, the bioactive compound produced by Streptomyces albidoflavus 321.2. Microbiol Res 161: 121-126, 2006.
73. Sunita A, Ganesh K and Sonam M: Screening and evaluation of bioactive components of Cenchrus ciliaris L. by GC-MS analysis. Int J Pharm 8: 69-76, 2017.
74. Yu FR, Lian XZ, Guo HY, McGuire PM, Li RD, Wang R and Yu FH: Isolation and characterization of methyl esters and derivatives from euphorbia kansui (euphorbiaceae) and their inhibitory effects on the human SGC-7901 cells. J Pharm Pharm Sci 8: 528-535, 2005.
75. Siyumbwa SN, Ekeuko SO, Amini F, Emerald NM, Sharma D and Okechukwu PN: Wound healing and antibacterial activities of 2-Pentadecaneone in streptozotocin-induced Type 2 diabetic rats. Pharmacogn Mag 15: 71-77, 2019.
76. Gupta S, Afaf F and Mukhtar H: Involvement of nuclear factor-kappa B, Bax and Bcl-2 in induction of cell cycle arrest and apoptosis by apigenin in human prostate carcinoma cells. Oncogene 21: 3727-3738, 2002.
77. Zhang Q, Liu J, Tian Z, Shen J, Moseman AP, Yang Q, Yang J, Xiao P, Wu E and Kohane IS: Duxalthone A induces cell cycle arrest and apoptosis via up-regulation of p53 through mitochondrial pathway in HepG2 cells. Int J Cancer 122: 31-38, 2008.
78. Yue GG, Lee JK, Kwok HF, Cheng L, Wong EC, Jiang L, Yu H, Leung HW, Wong YL, Leung PC, et al: Novel PI3K/AKT targeting anti-angiogenic activities of 4-vinylphenol, a new therapeutic potential of a well-known styrene metabolite. Sci Rep 5: 11149, 2015.
79. Nair SC, Kurunnoor SK and Hasegawa JH: Saffron chemoprevention in biology and medicine: A review. Cancer Biother 10: 2415-2419, 2003.
80. Huang CB and Ebersole JL: A novel bioactivity of omega-3 polyunsaturated fatty acids and their ester derivatives. Mol Oral Microbiol 25: 75-80, 2010.
81. Ahmad B, Khan I, Bashir S and Azam S: Chemical composition and antifungal, phytotoxic, brine shrimp cytotoxicity, insecticidal and antibacterial activities of the essential oils of Acacia modesta. J Med Plants Res 6: 4653-4659, 2012.
82. Roy RN, Laskar S and Sen SK: Dibutyl phthalate, the bioactive compound produced by Streptomyces albidoflavus 321.2. Microbiol Res 161: 121-126, 2006.
83. Sunita A, Ganesh K and Sonam M: Screening and evaluation of bioactive components of Cenchrus ciliaris L. by GC-MS analysis. Int J Pharm 8: 69-76, 2017.
84. Yu FR, Lian XZ, Guo HY, McGuire PM, Li RD, Wang R and Yu FH: Isolation and characterization of methyl esters and derivatives from euphorbia kansui (euphorbiaceae) and their inhibitory effects on the human SGC-7901 cells. J Pharm Pharm Sci 8: 528-535, 2005.
85. SIYUMBWA SN, EKEUKO SO, AMINI F, EMERALD NM, SHARMA D and OKECHUKWU PN: WOUND HEALING AND ANTIBACTERIAL ACTIVITIES OF 2-PENTADECANONE IN STREPTOZOTOCIN-INDUCED TYPE 2 DIABETIC RATS. PHARMACOGN MAG 15: 71-77, 2019.
86. GUPTA S, AFAF F AND MUKHTAR H: INVOLVEMENT OF NUCLEAR FACTOR-KAPPA B, BAX AND BCL-2 IN INDUCTION OF CELL CYCLE ARREST AND APOPTOSIS BY APIGENIN IN HUMAN PROSTATE CARCINOMA CELLS. ONCOGENE 21: 3727-3738, 2002.
87. TIAN Z, SHEN J, MOSEMAN AP, YANG Q, YANG J, XIAO P, WU E AND KOHANE IS: DUXALTHONE A INDUCES CELL CYCLE ARREST AND APOPTOSIS VIA UP-REGULATION OF P53 THROUGH MITOCHONDRIAL PATHWAY IN HEPG2 CELLS. INT J CANCER 122: 31-38, 2008.
88. YUE GG, LEE JK, KWOK HF, CHENG L, WONG EC, JIANG L, YU H, LEUNG HW, WONG YL, LEUNG PC, ET AL: NOVEL PI3K/AKT TARGETING ANTI-ANGIOGENIC ACTIVITIES OF 4-VINYLPHENOL, A NEW THERAPEUTIC POTENTIAL OF A WELL-KNOWN STYRENE METABOLITE. SCI REP 5: 11149, 2015.
89. CIMMINO A, SCAFATO P, MATHIEU V, INGELS A, D’AMICO W, PISANI L, MADDAU L, SUPERCHI S, KISS R AND EVIDENTE A: NATURAL AND SYNTHETIC FURANONES WITH ANTICANCER ACTIVITY. NAT PROT COMMUN 11: 1471-1474, 2016.
90. MA F, ZHANG J, LI M, YU J, LUO W, LI X AND WEI M: SYNTHESIS OF NOVEL MATRINE DERIVATIVES CONTAINING FURANOONE SKELETON AND PRELIMINARY EVALUATION OF THEIR ANTICANCER ACTIVITY IN VITRO. CHIN J ORG CHEM 38: 2633-2638, 2018.
91. DE ALENCAR MVOB, IBRAHIM MT, DE LIMA RMT, PIZZI MFCJ, DOS REIS AC, DA MATA AMOF, FELIPE WJGO, CERCESINA QS, PEREIRA PMF, E SOUSA JMC, ET AL: PHYTOL AS AN ANTICARCINOGENIC TARGETING ANTI-ANGIOGENIC ACTIVITIES OF 4-VINYLPHENOL, A NEW THERAPEUTIC POTENTIAL OF A WELL-KNOWN STYRENE METABOLITE. SCI REP 5: 11149, 2015.
92. Murugesan A, Holmstedt S, Brown KC, Koivuporras A, Siyumbwa SN, Ekeuko SO, Amini F, Emerald NM, Sharma D and Okechukwu PN: Wound healing and antibacterial activities of 2-Pentadecanone in streptozotocin-induced Type 2 diabetic rats. Pharmacogn Mag 15: 71-77, 2019.
93. Gupta S, Afaf F and Mukhtar H: Involvement of nuclear factor-kappa B, Bax and Bcl-2 in induction of cell cycle arrest and apoptosis by apigenin in human prostate carcinoma cells. Oncogene 21: 3727-3738, 2002.
94. Tian Z, Shen J, Moseman AP, Yang Q, Yang J, Xiao P, Wu E and Kohane IS: Duxalthone A induces cell cycle arrest and apoptosis via up-regulation of p53 through mitochondrial pathway in HepG2 cells. Int J Cancer 122: 31-38, 2008.