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ORIGINAL ARTICLE

Selective induction of DNA damage, G2 abrogation, and mitochondrial apoptosis by leaf extract of traditional medicinal plant *Wrightia arborea* in K562 cells

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Abstract Plants have proved to be an important source of anti-cancer drugs. *Wrightia arborea*, an Indian Ayurvedic medicinal plant, is used traditionally to treat a variety of ailments. This study evaluates the antiproliferative/apoptotic potential of *Wrightia arborea* leaf extracts, prepared in different organic solvents, on cancer cell lines. MTT assay, light and fluorescence microscopy, flow cytometry, DNA laddering, alkaline comet assay, and western blotting were some of the techniques used for evaluation. Combinations of camptothecin, either with CHK1 inhibitor-PD407824 or with *W. arborea* leaf extract, were deployed to determine the G2 abrogating potential of the extract. The chloroform extract (WAC) selectively killed K562 cells, without affecting cancerous MCF-7, Hep G2 cells, and normal human peripheral blood lymphocytes. Cell death was characterized by observation of apoptotic bodies, increased Ca2+ and ROS, phosphatidyl serine externalization, mitochondrial membrane depolarization, DNA laddering, increased sub-G1 population, and altered expression of caspase 3, −9, and PARP. WAC also induced DNA damage, alterations in key G2/M phase protein expression, cell cycle perturbation, and potent G2 abrogation. The present study showed that *W. arborea* leaf extract, WAC, is capable of selectively killing leukemic cancer cells leaving normal lymphocytes unaffected. Our results indicate that this is effectuated through DNA damage and G2 abrogation leading to mitochondrial apoptosis. Taken together, this report contributes toward a better understanding of the anticancer properties of this traditional medicinal plant extract possessing valuable bioactive constituents which can serve as a bioresource for promising complimentary/alternative/chemopreventive therapeutics.

Keywords Apoptosis · G2 abrogation · Wrightia arborea

Introduction

Plants have served as a bioresource for natural products and have significantly contributed in drug development. Over 60% of anticancer drugs of yesteryears and those in current use are plant-based (Jain et al. 2011; Cragg and Newman 2013). The understanding that 85–90% of human cancers are rooted in preventable causes such as environmental factors and life style has ushered in the concept of cancer chemoprevention. This paradigm shift emanated from research findings within the past decade which demonstrated that phytochemicals can potentially block, interfere, or even reverse many processes underlying tumor initiation, promotion, and progression (Anand et al. 2008; McCormack and Boffetta 2010; Steward and Brown 2013).

The therapeutic value of medicinal plants is not yet fully explored and remains underexploited (Biswas et al. 2015). Despite intensive investigations on terrestrial plant community, it is estimated that only 6% of the approximately 300,000–500,000 species of higher plants have been systematically investigated pharmacologically

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and only about 15% phytochemically (Ginsburg and Deharo 2011; Patwardhan 2012). The knowledge base of various traditional systems of medicine worldwide deserve better consideration as they provide valuable clues and guidance useful for development of new therapeutic drugs. These systems also demonstrate the rationale of synergism exhibited by the concerted action of a mixture of bioactive compounds as compared to the effect of single constituents (Wagner 2011).

W. arborea is a small tree (Family Apocynaceae), which features in the Indian traditional systems of medicine such as Ayurveda and Siddha (Sivarajan and Balachandran 1994) as a cure for a multitude of ailments. It inhabits deciduous forests of Deccan - Carnatic region in India and has also been reported from the neighboring countries like Sri Lanka, Burma, and Thailand. The tree bark, endowed with antipyretic and antibacterial properties (Khayde and Vaikos 2011), is also used for curing menstrual and renal complaints (Chopra et al. 1956). The latex has found use as an antidote for snake bites and scorpion-stings (Sri and Reddi 2011). The leaves are used as a diaphoretic, expectorant (Nahar et al. 2013), and to treat dysentery, toothache, and diarrhea (Juyal and Ghildiyal 2013). Alcoholic extracts of the leaves possess remarkable analgesic, anti-inflammatory, and wound healing activities (Nahar et al. 2013; Devi and Divakar 2012). Notwithstanding the broad spectrum of pharmacological action of *W. arborea*, the plant remains virtually unexplored with respect to its anticancer potential barring a single study on in vivo antitumour activity of methanolic extracts of its leaves (Zahan et al. 2013). However, the mechanism(s) underlying tumor growth reduction have remained unclear and needs to be ascertained. Hence, in the present study, a cell and molecular biological approach has been used to evaluate the antiproliferative and apoptosis inducing potential of crude organic solvent extracts prepared from *W. arborea* leaves.

Materials and methods

Chemicals and reagents

5-Bromo- 4- chloro-3- indolyl phosphate/nitro blue tetrazolium (BCIP/NBT), dimethyl sulfoxide (DMSO), camptothecin, dichloro-dihydro-flourescien diacetate (DCFH-DA), rhodamine-123, Fluo-3 AM, annexin V-FITC apoptosis detection kit, propidium iodide, anti-cyclin B1, anti-ϒ H_2AX, anti-PSTAIR, and anti-CHK1 antibodies were purchased from Sigma-Aldrich (USA). Anti-caspase −3, −9, anti-PARP, and anti-actin were purchased from Cell Signaling Technology, USA. Tris base, glycine, acridine orange (AO), ethidium bromide, and Triton X-100 were purchased from SRL Pvt. Ltd., Mumbai, India. Goat anti-rabbit IgG-alkaline phosphatase (AP)-conjugate, goat anti-mouse IgG-AP—conjugate, and agarose were purchased from Bangalore Genei (Merck Life Sciences, Mumbai, India). Bovine serum albumin (BSA), Fetal bovine serum (FBS), Roswell Park Memorial Institute medium (RPMI-1640), Dulbecco's Modified Eagle's Medium (DMEM), HiKaryo XL™ RPMI medium, and MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) were purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, India. 9-hydroxy-4-phenyl-6 h-pyrrolo [3,4-c]carbazole-1,3-dione (PD-407824) was purchased from Calbiochem (Merck, Germany). All other chemicals and reagents used were of analytical grade.

Plant material

Leaves of *W. arborea* were collected from the botanical garden of Calicut University in July 2013. The plant was authenticated at the Department of Botany, University of Calicut. A voucher specimen (Voucher No 6833) was deposited in the Calicut University Herbarium.

Preparation of *W. arborea* leaf extracts

Shade-dried leaves were coarsely powdered (20 g) and subjected to defatting by refluxing with 100 ml of petroleum ether at 60–80 °C for 12 h. This was followed by successive solvent extractions by a process of continuous soxhlation. The extractions were done with individual solvents (100 ml) possessing increasing polarities such as chloroform, acetone, ethyl acetate, and methanol. The crude extracts, obtained were filtered through Whatman No.1 filter paper. Following evaporation of the solvents, the resultant residues were dissolved in DMSO to get a stock solution of 20 mg/ml. The extracts were designated as WAA, WAC, WAE, and WAM with the first two letters denoting *W. arborea* and the third denoting the solvents—acetone, chloroform, ethyl acetate, and methanol, respectively.

Cell culture

Three human cancer cell lines—chronic myelogenous leukemic K562 cells, breast cancer MCF-7 cells, and hepatic carcinoma Hep G2 cells were employed in this study. K562 cells were cultured in RPMI-1640 medium while MCF-7 and Hep G2 cells were cultured in DMEM. Both media were supplemented with FBS (10% *v*/v), streptomycin (100 μg/ml), and penicillin (100 U/ml) and maintained in an incubator at 37 °C in a humidified, 5% CO_2 atmosphere. Human peripheral blood lymphocytes (hPBL) were isolated from healthy donors to serve as

Table 1 IC 50 concentrations of WAC on human cancer cell lines

Cell lines tested	IC 50 obtained following WAC treatment for different time periods		
	24 h	48 h	72 h
K562	40 ± 3 μg/ml	26 ± 2 μg/ml	5 μg/ml
MCF 7	96 ± 3 μg/ml	99 ± 1 μg/ml	105 ± 3 μg/ml
Hep G2	>105 ± 3 μg/ml	103 ± 1 μg/ml	100 ± 3 μg/ml

normal control cells. hPBL cells were separated from whole blood collected from healthy donors and cultured in HiKaryo XL™ RPMI media according to manufacturer's protocol (Himedia Laboratories, Mumbai).

Evaluation of *W. arborea* extract cytotoxicity by MTT assay and trypan blue dye exclusion method

The cytotoxicities of the different leaf extracts were evaluated by the MTT assay. For this, cells were cultured in 96-well microtitre plates with 2×10^4 K 562 and 7000 each of MCF and Hep G2 cells per well. hPBL (10^5 cells/ml) were added to 5 ml HiKaryo XL™ RPMI medium and incubated at 37 °C for 48 h. To determine the IC50 concentration, cell line cultures were initially exposed to varying concentrations (20–100 μg/ml) of the different leaf extracts—WAA, WAC, WAE, and WAM for a period of 24, 48, and 72 h. After the extract treatment, MTT (500 μg/ml) was added to each well, and the cells were then incubated for 3 h at 37 °C. The medium was discarded and an equal amount of DMSO (150 μl) was added to each well. After 10 min of shaking at room temperature to dissolve the formazan crystals, absorbance was measured at 570 nm on a plate reader (Multiscan EX, Thermo scientific, USA). Cultures of hPBLs, taken as positive controls, were treated with concentrations—lower, equal to, and higher than the IC50 values obtained for the extracts. Untreated and vehicle (DMSO)-treated cells served as negative controls. The difference of absorbance between the treated and untreated control groups was used to determine cell viability (Mosmann 1983).

Cell viability was determined by culturing 2×10^4 cells per well in a 96-well plate followed by extract treatment for 24 h. The cells were then stained with trypan blue dye and counted using a hemocytometer. The result was expressed as a percentage of the stained dead cells relative to the control (Feng et al. 2011).

Microscopic evaluation of cell morphology

The control and the extract-treated cells were harvested and washed with ice-cold PBS, and the morphological changes induced by the extract treatment were observed under a phase-contrast inverted microscope. Separate aliquots of cells were also subjected to staining with Hoechst 33258 as well as differential staining with acridine orange/ethidium bromide to detect any cellular changes associated with apoptosis induction using a fluorescence microscope (Kasibhatla et al. 2006).

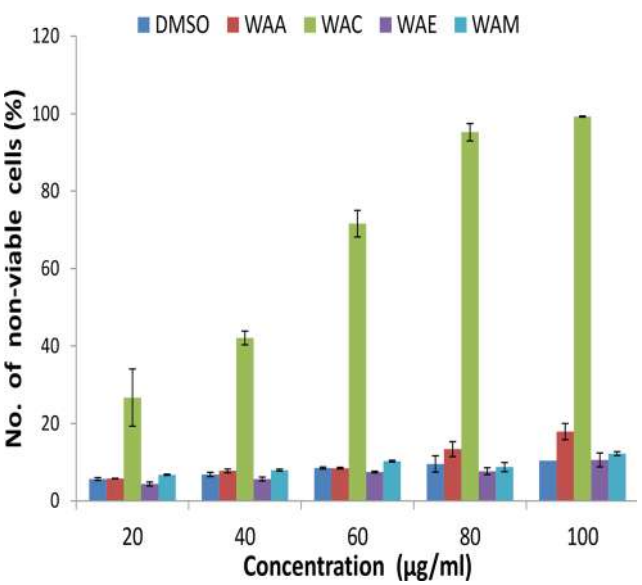

Fig. 1 Effect of WAC on viability of K562 cells. The percentage of non-viable cells was evaluated by trypan blue dye exclusion method

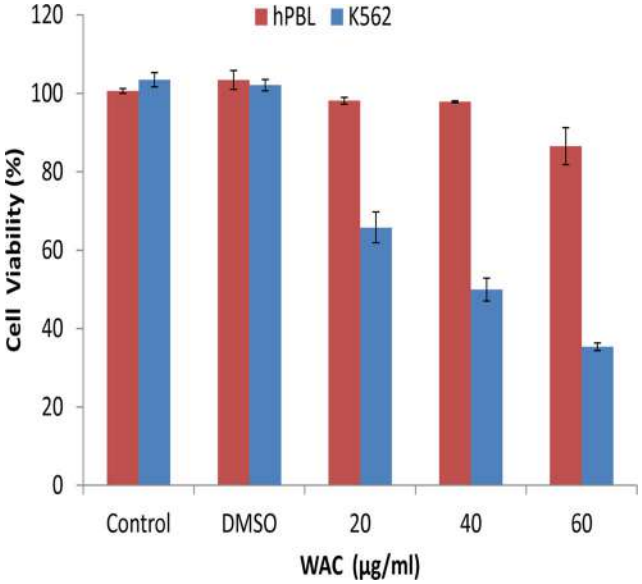

Fig. 2 Effect of WAC on hPBLs. Cell viability of normal hPBLs was assessed by MTT method after 24 h of treatment

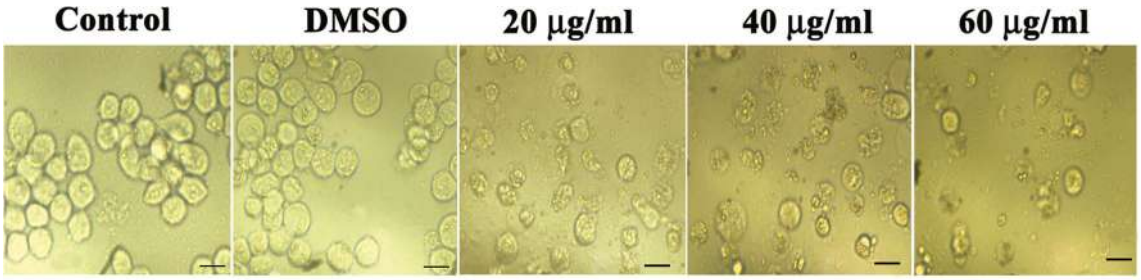

Fig. 3 Light microscopic images of K562 cells after 24 h treatment with different concentrations of WAC. Untreated control and vehicle (DMSO) treated cells have been included in all figures. *Scale bars* represent 15.5 μm in all figures

Genotoxicity evaluation by comet assay

Alkaline gel electrophoresis was carried out for genotoxic evaluation of the extract. The extract-treated and control cells were subjected to comet assay essentially as described by Singh et al. (1988). The slides were stained with ethidium bromide (10 μg/ml) for 30 min and observed under a fluorescence microscope.

Cell cycle analysis and assessment of G2 abrogation

Flow cytometry of the control and extract-treated cell populations were carried out to determine cellular distribution in the different phases of cell cycle. For this, cells were stained with propidium iodide (PI), and the percentage sub-population of cells in sub-G1, G0/G1, S, and G2/M phases were quantified using the BD FACS Diva software version 5 on a BD Biosciences FACS SORP cytometer.

To determine G2 checkpoint abrogation, p53-null, G1-compromised cells were exposed to a sublethal concentration (IC10) of camptothecin at 100 nM for 24 h for G2 checkpoint activation leading to G2 arrest. The cells were then washed with PBS, resuspended in fresh growth medium, and incubated separately in the extract/CHK 1 inhibitor (PD 407824) at IC50 concentrations for a further period of 12 and 24 h. The inhibitor was added to effectuate G2 abrogation in cells to serve as positive control. The control and treated cells were then subjected to FACS analysis (Astuti et al. 2012). Cells were also stained with Hoechst 33258 to check for signs of abortive/catastrophic mitosis.

Assessment of intracellular reactive oxygen species (ROS), Ca^{2+} concentration, phosphatidylserine externalization, and mitochondrial membrane potential

DCFH-DA-based assay was employed for measuring the intracellular ROS generation. Following incubation with 10 μM DCFH-DA at 37 °C for 30 min, cells were washed twice with phosphate buffered saline (PBS) and subjected to ROS assessment (Kim et al. 2012). Mitochondrial membrane potential was determined by incubating cells with lipophilic cationic dye Rhodamine 123 (10 μg/ml) for 30 min followed by a wash with PBS (Zhen et al. 2011). Alterations in levels of intracellular Ca^{2+} content were determined by employing a fluorescent dye, Fluo-3 AM. Cells were washed twice with PBS following incubation with 5 μM Fluo-3 AM at 37 °C for 30 min and washed with PBS (Liu et al. 2005). Externalization of phosphatidylserine (PS) was evaluated by using annexin V/FITC apoptosis detection kit as per the manufacturer's instructions (APOAF- Annexin V- FITC detection kit, Sigma Aldrich, USA). All assessments mentioned above were carried out by fluorescence microscopy, and flow cytometric analyses were performed on a BD Bioscience FACS ARIA II cytometer.

DNA fragmentation assay

DNA isolated from control and extract-treated cells were analyzed on 1.0% agarose gels to check for DNA laddering arising due to internucleosomal cleavage considered to be a hallmark of apoptosis induction (Kim et al. 2001). DNA from curcumin-treated (25 μg/ml) cells was taken as positive control.

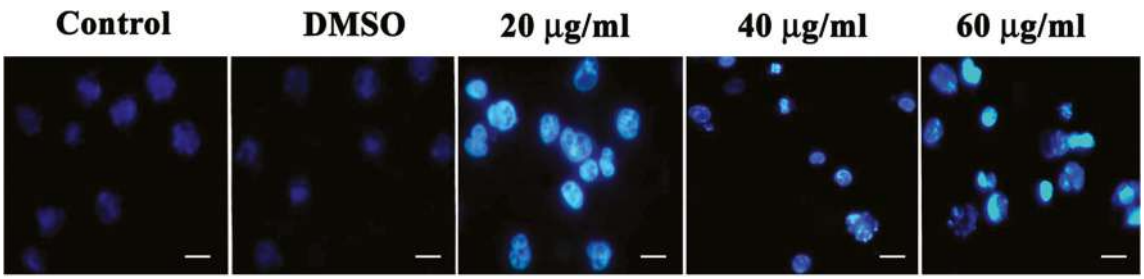

Fig. 4 Fluorescent microscopic images of Hoechst 33258 stained K562 cells after 24 h treatment with WAC

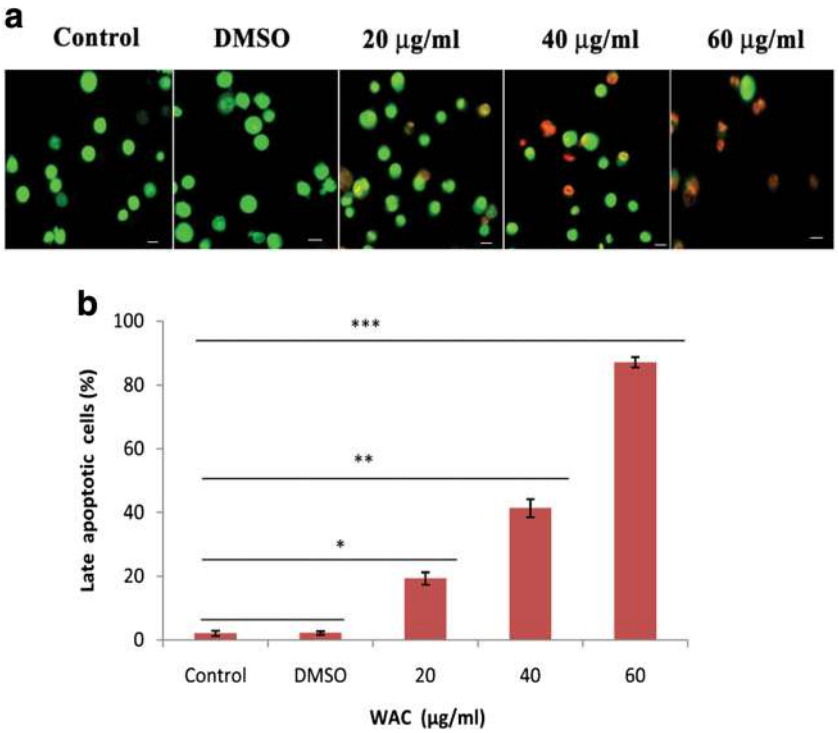

Fig. 5 Effect of WAC on K562 cells after 24 h treatment. **a** Fluorescence microscopic images of WAC-treated cells stained with acridine orange/ethidium bromide. **b** Quantitative analysis of late apoptotic cells. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$

Western blot analysis

SDS PAGE and western blotting were performed essentially as described by Ausubel et al. (1992) to detect apoptosis and cell cycle related proteins. For this, the control and extract-treated cells were first washed with cold phosphate buffered saline (PBS) and lysed in RIPA (radioimmunoprecipitation assay) buffer containing protease/phosphatase inhibitors. The protein concentration was measured by the Bradford method.

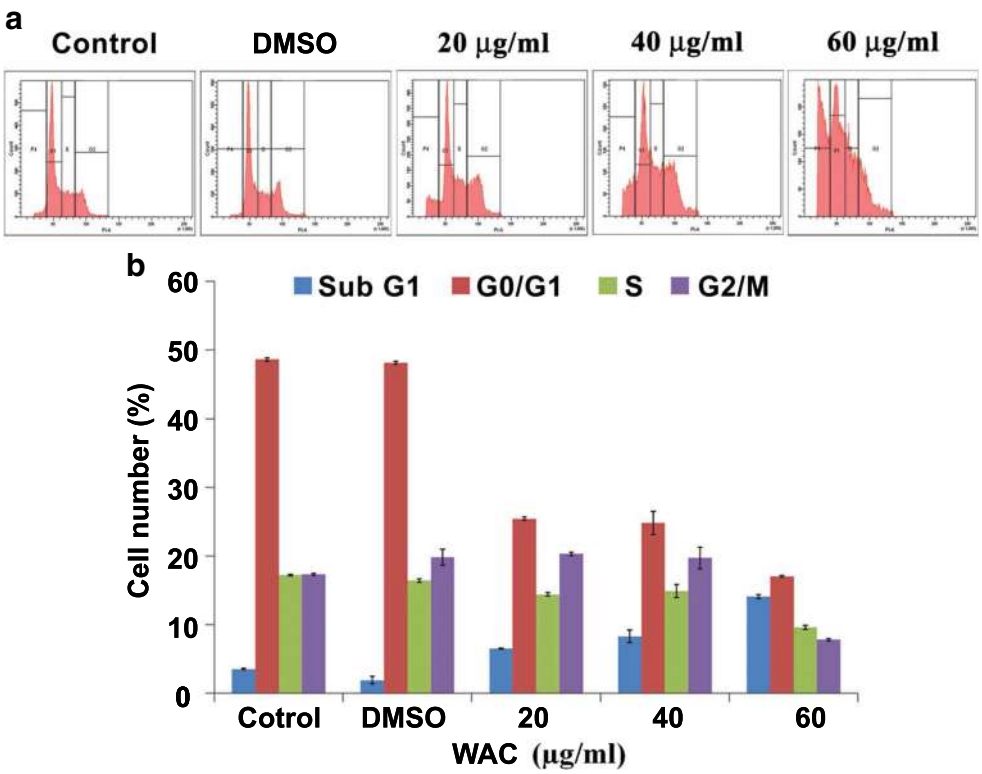

Fig. 6 Effect of WAC on cell cycle distribution of K562 cells. **a** Flow cytometric data. **b** Quantification of cells in various cell cycle phases including sub-G1. Fluorescence data from control and vehicle (DMSO)–treated cells has been included in all figures representing FACS analysis

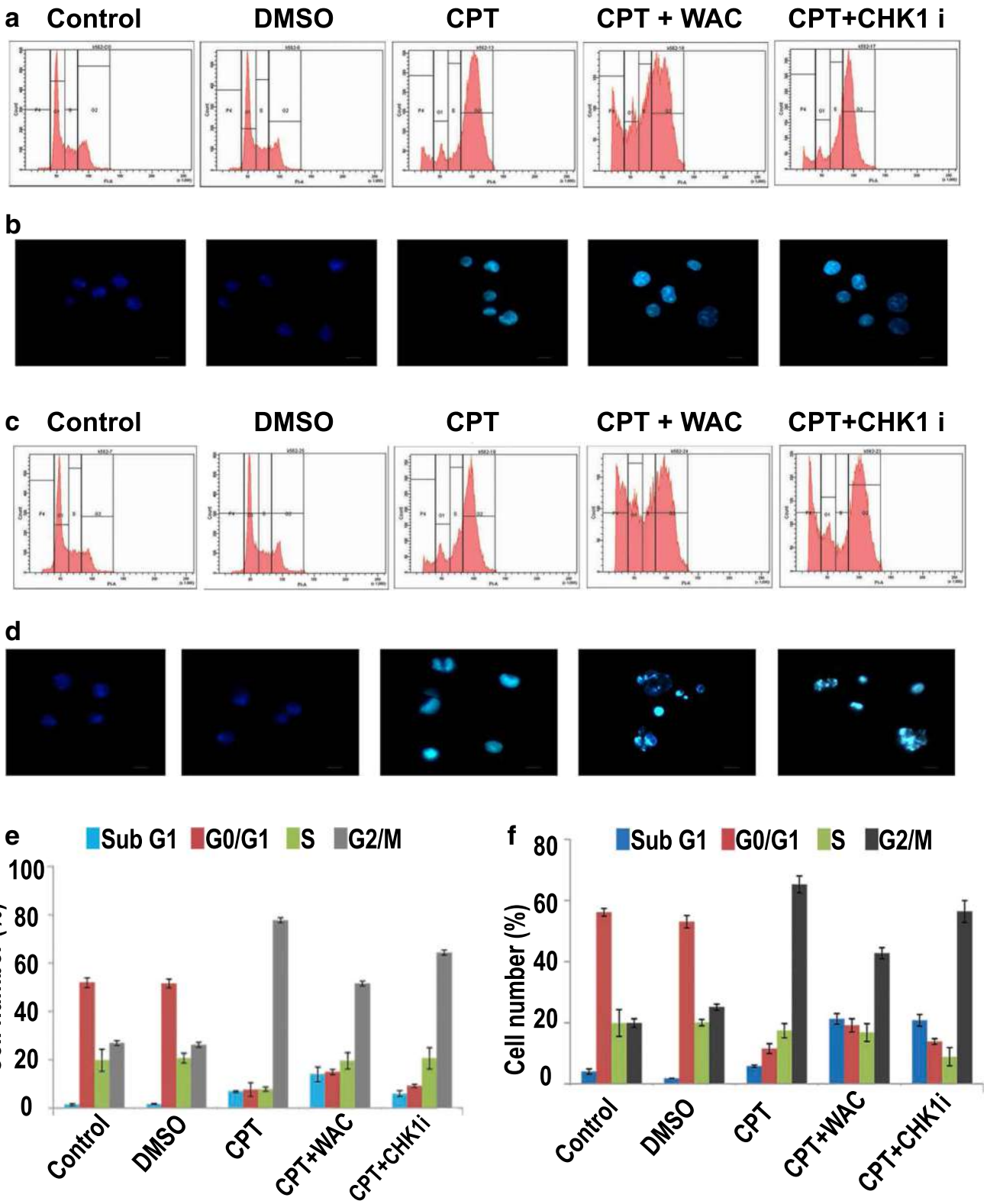

Fig. 7 Effect of WAC/CHK1i (PD407824) on camptothecin-induced G2-arrested K562 cells. Flow cytometric data from cells treated with WAC for 12 h (**a**), 24 h (**c**), Hoechst 33258 stained cells showing WAC-induced abortive mitosis after 12 h (**b**), 24 h (**d**), and quantification of cells in various cell cycle phases including sub-G1 (**e**, **f**)

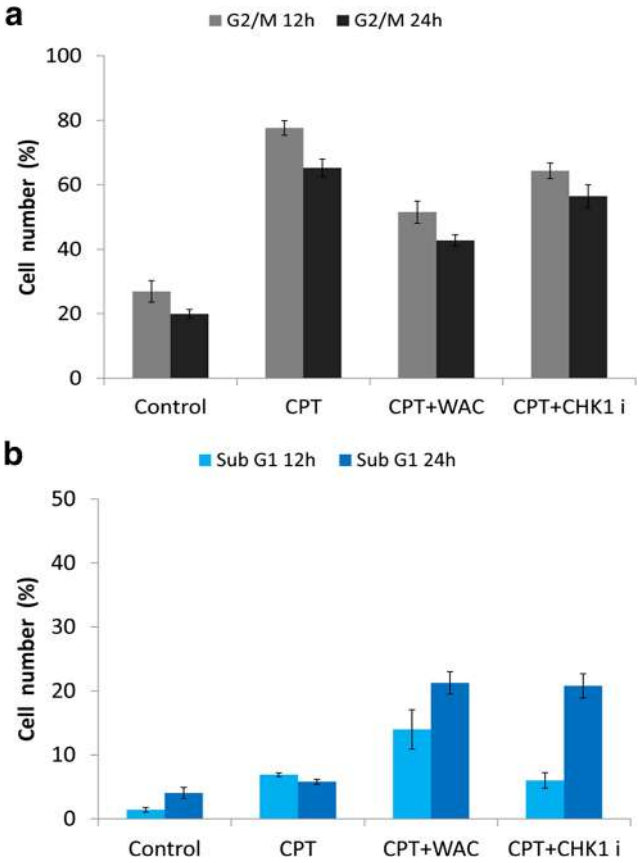

Fig. 8 Time-dependent effect of WAC on camptothecin-induced G2-arrested K562 cells. Quantification of cells in **a** G2/M phase and **b** sub G1 phase after 12 and 24 h treatment with WAC/CHK1i based on data shown in Fig. 7

Equivalent amounts of proteins (50 μg) were electrophoresed on a 12.5% SDS-PAGE gel and then transferred onto a positively charged nylon membrane. Membranes were blocked for 1 h at room temperature in 3.0% BSA in Tris-buffered saline (TBS), and separately immunostained overnight with mouse/rabbit derived primary antibodies (dilution 1:1000) at 4 °C against caspase-3, −9, PARP, ϒ H2AX, cyclin B1, CHK1, and PSTAIR. Antibody against β-actin was used as a loading control to detect the housekeeping function. The membranes were then washed in TBS and incubated for 1 h at room temperature with ALP-conjugated secondary antibody, which was either anti-mouse or anti-rabbit (1:2000). Following another TBS wash, the blots were exposed to BCIP/NBT solution to visualize the immunostained polypeptides.

Statistical analysis

The data are expressed as the mean ± SD from three independent experiments. Results were analyzed for significance by one-way ANOVA using SPSS software version 16.0. Differences with $P < 0.05$ were considered significant.

Results

Effect of *W. arborea* leaf extracts on human cancer cell lines

The cellular toxicity of *W. arborea* leaf extracts—WAA, WAC, WAM, and WAE—was evaluated by performing an MTT assay on three human cancer cell lines—K562, MCF 7, and Hep G2 cells, and the IC 50 concentrations were determined (Table 1). WAC showed the highest cytotoxicity against K562 cells in a concentration and time-dependent manner, with the IC50 decreasing from 40 to 5 μg/ml at 24 and 72 h of treatment, respectively. The values with respect to MCF-7 and Hep G2 cells were found to be enormously higher, around100μg/ml, in comparison. The other three extracts—WAA, WAM, and WAE—however, failed to show any cytotoxicity towards all of the tested cell lines (data not shown).

Cell viability checked by the trypan blue dye exclusion method also corroborated the results of the MTT assay in that, at concentrations ranging from 80 to 100 μg/ml, WAC was found to be highly cytotoxic to K562 cells with about 80–100% cell death observed following 24 h treatment with the extract (Fig. 1). Hence, all subsequent investigations were carried out to better understand the apoptotic potential of WAC extract and its effect on the cell cycle dynamics of K562 cells.

Effect of WAC on hPBLs

The MTT assay performed with the normal control cells—hPBLs—treated with WAC at different concentrations (20, 40, and 60 μg/ml) revealed no significant difference in proliferation rate with less than 2% cells being affected by the extract at the IC50 concentration (Fig. 2). Interestingly, this result provides additional proof of WAC's potential to preferentially kill cancerous, lymphocytic K562 cells compared to normal human lymphocytes.

Effect of WAC on cell morphology

Light microscopy of WAC-treated cells revealed distortions and disruptions in cell structure in addition to cell shrinkage and fragmentation. These changes in cell morphology were observed to increase in a dose dependent manner. The untreated and vehicle (DMSO)-treated control cells showed normal cell morphology with intact cell boundaries (Fig. 3).

Fluorescence microscopy of the WAC-treated cells revealed occurrence of apoptosis induction. The untreated and vehicle (DMSO)-treated control cells were uniformly stained by the nuclear stain—Hoechst 33258—while the extract-treated cells showed morphological changes like nuclear fragmentation and condensation indicative of apoptosis as visualized by intense uptake of stain in such regions (Fig. 4). In

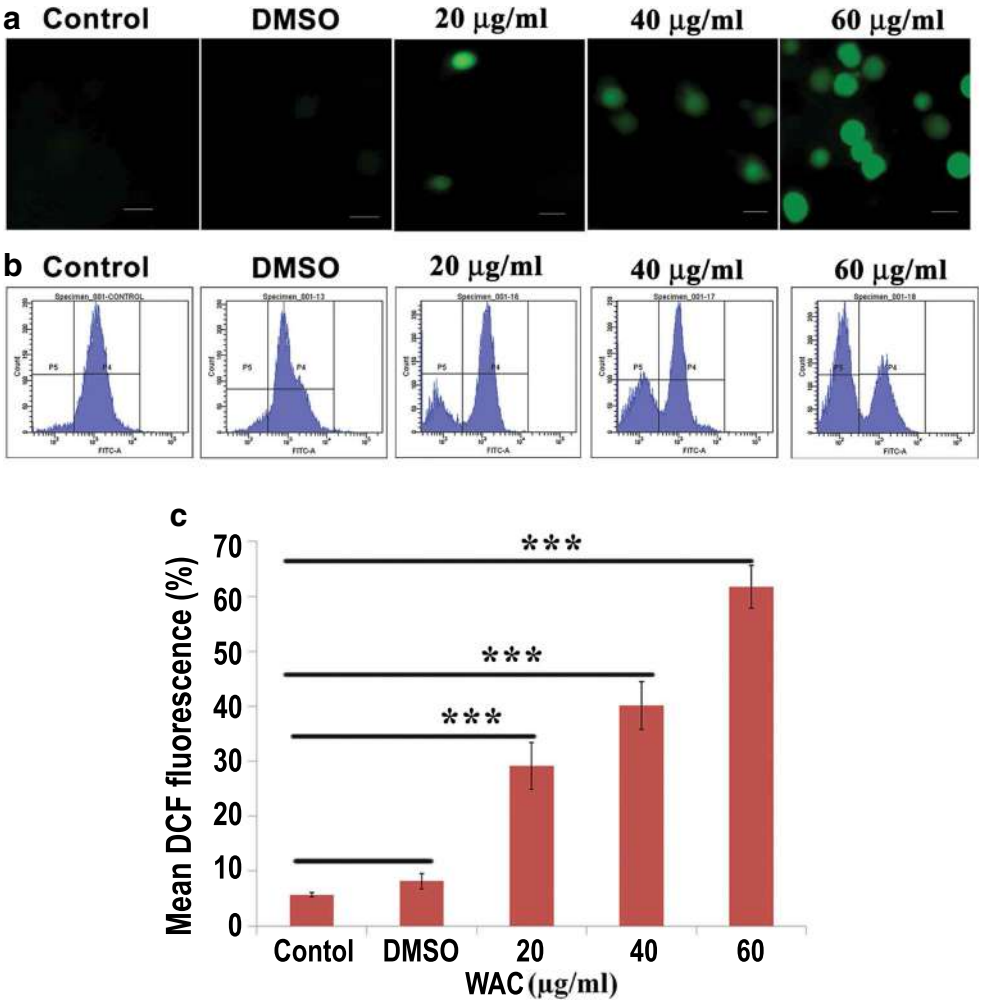

Fig. 9 Measurement of cellular ROS using DCFH-DA following 24 h of WAC treatment. **a** Microscopic images. **b** Flow cytometric analysis. **c** Mean fluorescence intensity of cells stained with ROS indicator. ***$p < 0.001$

acridine orange/ethidium bromide double staining, acridine orange stains only live cells and appear to be green whereas yellow and orange color of dead cells is imparted by ethidium bromide. In WAC-treated cells, yellow stained early-apoptotic and orange stained late-apoptotic cells were observed which were found to increase in a concentration dependent manner. Contrastingly, the control cells appeared green with intact nuclei and normal cell morphology bereft of such yellow-orange stained cells (Fig. 5).

Effect of WAC on cell cycle

FACS analysis of WAC-treated cells revealed discernible changes in the cell cycle phase-specific distribution with respect to the controls. At the IC50 concentration (40 µg/ml), extract treatment resulted in a drastic reduction of about 65% cells in G1 phase as against a marginal increase of only about 1.4% in G2/M phase cell population in comparison to the controls. Interestingly, about 20% cells found in S phase virtually remained unaffected by the treatment. A concomitant appearance of about 30% of apoptotic cells in the sub-G1 population conspicuously reflected the substantial decrease in the G1 population. At a higher dose of WAC (60 µg/ml), a dose-dependent increase of about 70% was observed in the sub-G1 population, which was about sevenfold higher with respect to the untreated cells (Fig. 6).

Effect of WAC on camptothecin-induced G2 arrest

Activation of two checkpoints governing extension of G1-S and G2-M transitions in the cell cycle is dependent on the occurrence of DNA damage leading to G1 or G2 arrest. G1arrest can only be executed in the presence of a functional p53 which is critical for efficient DNA repair. Cancer cells with dysfunctional p53 and G1 checkpoint thus become solely dependent on G2 checkpoint for their survival. On these lines, we have used a strategy similar to that of Jones et al. (2000) to probe the effect of WAC on over-riding of G2 checkpoint—also known as "G2 abrogation." Sublethal concentration of camptothecin (100 nM) was used to activate G2 checkpoint, which resulted in a threefold increase in G2/M population (77.65%) in comparison to that in untreated control

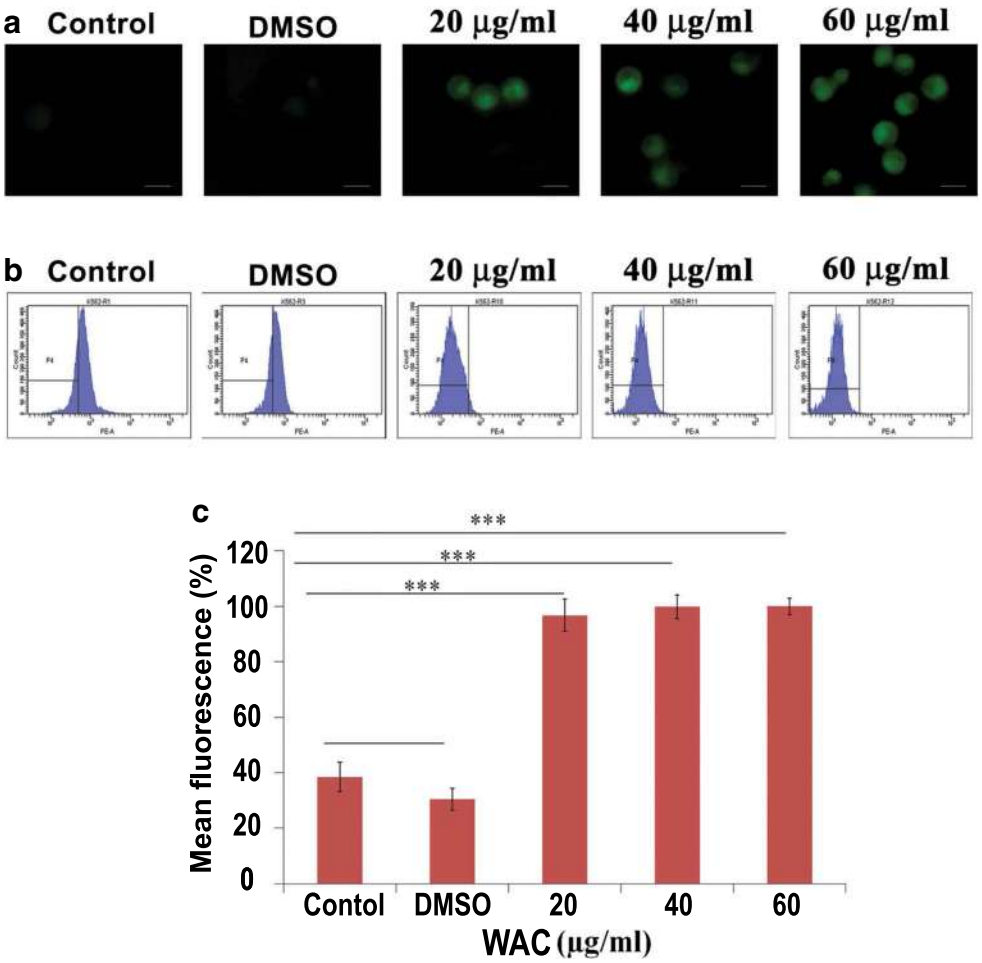

Fig. 10 Measurement of mitochondrial membrane potential using Rhodamine 123 following 24 h of WAC treatment. **a** Microscopic images. **b** Flow cytometric analysis. **c** Mean fluorescence intensity of K562 cells. ***$p < 0.001$

cells (26.94%) (Fig. 7). Following G2 arrest, exposure to checkpoint kinase 1 inhibitior (CHK1i) –PD 407824 for 12 and 24 h resulted in G2 abrogation in 17 and 14% of cells, respectively (Fig. 8). Contrastingly, following WAC treatment for 12 and 24 h, the percentage of G2 abrogated cells was found to be 28 and 36, respectively, which was not only much higher than that of PD 407824, but also showed a time dependence. Strikingly, at the IC50 concentrations employed, WAC clearly surpassed PD407824 as a G2 abrogator (Fig. 8a). With respect to the sub G1 population, a 12 h WAC treatment vis-à-vis PD407824 resulted in two- to fourfold (6–21% approx.) increase in the apoptotic cell population in the former with no apparent change in the latter. However, after a 24 h treatment, a threefold increase in cell death was apparent in both WAC and PD407824-treated cell populations (Fig. 8b).

In the event of G2 abrogation, cells harboring damaged DNA have been reported to enter a kind of aberrant catastrophic mitosis (Kimura et al. 2003; Alves et al. 2016). Though not an ultimate manifestation of cell death, it eventually leads to apoptosis (Skwarska et al. 2007). WAC-induced G2 abrogated cells subjected to Hoechst 33258 staining revealed the presence of intensely stained, irregular multinucleated giant cells similar to those reported by Alves et al. (2016).

Effect of WAC on intracellular ROS generation, Ca^{2+} concentration, phosphatidylserine externalization, and mitochondrial membrane potential

DCFH-DA, after crossing the cell membrane, is hydrolysed by intracellular esterases to non fluorescent DCFH. In the presence of ROS, DCFH is oxidized to highly fluorescent dihydrofluorescein. The emitted fluorescence is directly proportional to ROS generation (Gomes et al. 2005). In WAC-treated cells, a dose dependent increase in fluorescence was observed compared to controls, indicating ROS dependent cell death in K562 (Fig. 9).

To assess the involvement of mitochondria in WAC-induced cell death, the mitochondrial membrane potential was studied using mitochondria—specific and voltage-dependent fluorescent probe rhodamine-123. The fluorescence of WAC-treated cells increased in comparison to the controls which was indicative of loss in mitochondrial membrane potential (Fig. 10).

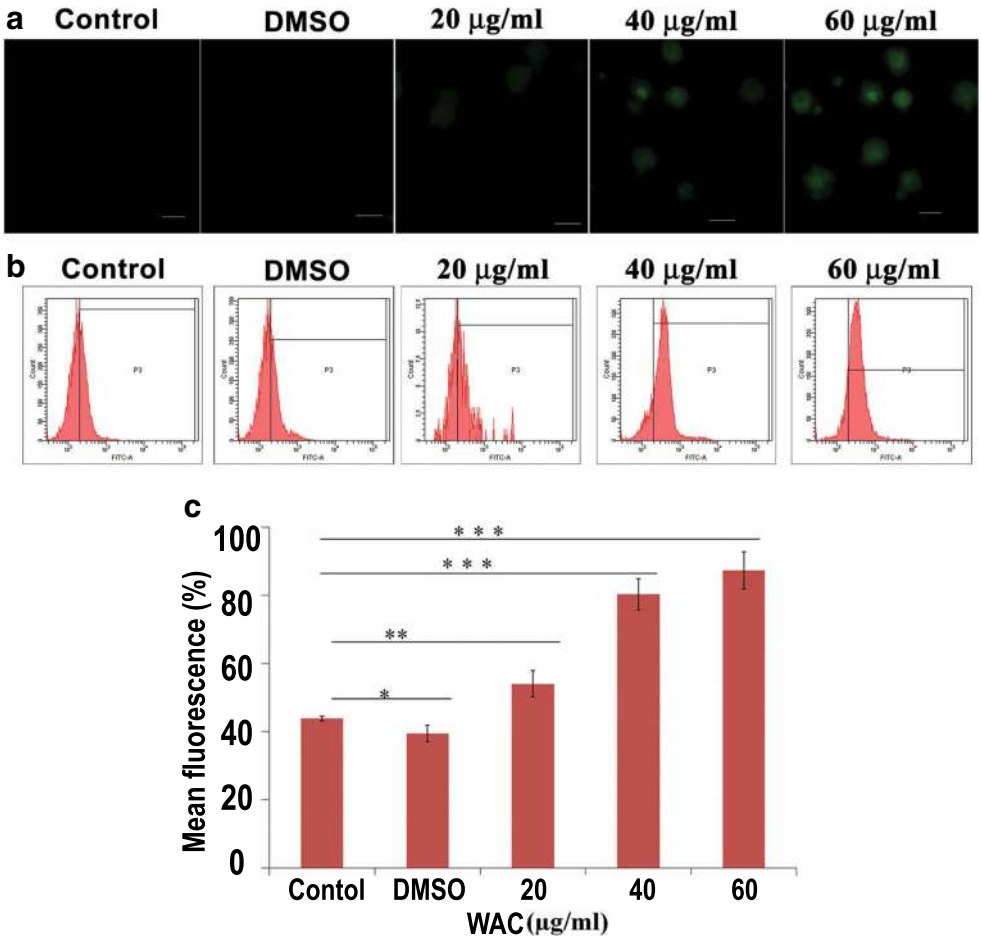

Fig. 11 Measurement of intracellular calcium concentration of K562 cells using intracellular Ca^{2+} indicator Fluo 3 AM, following 24 h of WAC treatment. **a** Microscopic images. **b** Flow cytometric analysis. **c** Mean fluorescence intensity of cells. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$

To determine whether WAC treatment caused intracellular Ca^{2+} release in K562 cells, we stained the cells with Flou-3 AM and fluorescence was measured. Fluo-3 AM dye specifically binds to free Ca^{2+} and shows an increase in emission fluorescence. WAC-treated K562 cells displayed a dose dependent increase in Ca^{2+} production while the untreated and the DMSO-treated controls showed no such fluorescence (Fig. 11).

Translocation of phosphatidyl serine from the inner to the outer phase of plasma membrane during the early stage of apoptosis was determined by staining cells with annexin-FITC (Vermes et al. 1995); inclusion of the otherwise impermeant PI stain assisted further in detecting necrotic cell death as well which is characterized by loss of membrane integrity. Significant increase in early and late apoptotic cells were detected in WAC-treated cells compared to the untreated and vehicle controls (Fig. 12).

Effect of WAC on cellular genotoxicity

Cellular genotoxicity of WAC as evidenced by comet assay revealed DNA strand breaks in the form of diffuse comet tails. The undamaged control and vehicle-treated cells displayed compact DNA within the nucleus. A concentration-dependent increase in the number of comets was observed after 24 h of WAC treatment (Fig. 13).

DNA fragmentation

Further, analysis of DNA isolated from the WAC-treated cells by agarose gel electrophoresis showed DNA laddering, a hallmark of apoptosis, resulting from internucleosomal DNA cleavage. DNA from control cells were bereft of such fragmentation (Fig. 14).

Western blot analysis

To confirm induction of apoptosis, the levels of major apoptotic proteins like PARP, Caspase 3, −9, and ϒH2AX were determined using western blot analysis (Fig. 15). During apoptosis, the 116 kDa PARP undergoes a partial cleavage into characteristic 89 kDa fragment. Our results showed a dose dependent effect of WAC on PARP cleavage at the IC50 concentration (40 µg/ml) and beyond (60 µg/ml) but not at the lower concentration tested (20 µg/ml). Activation of procaspase 9 and −3 in WAC-treated cells was also observed.

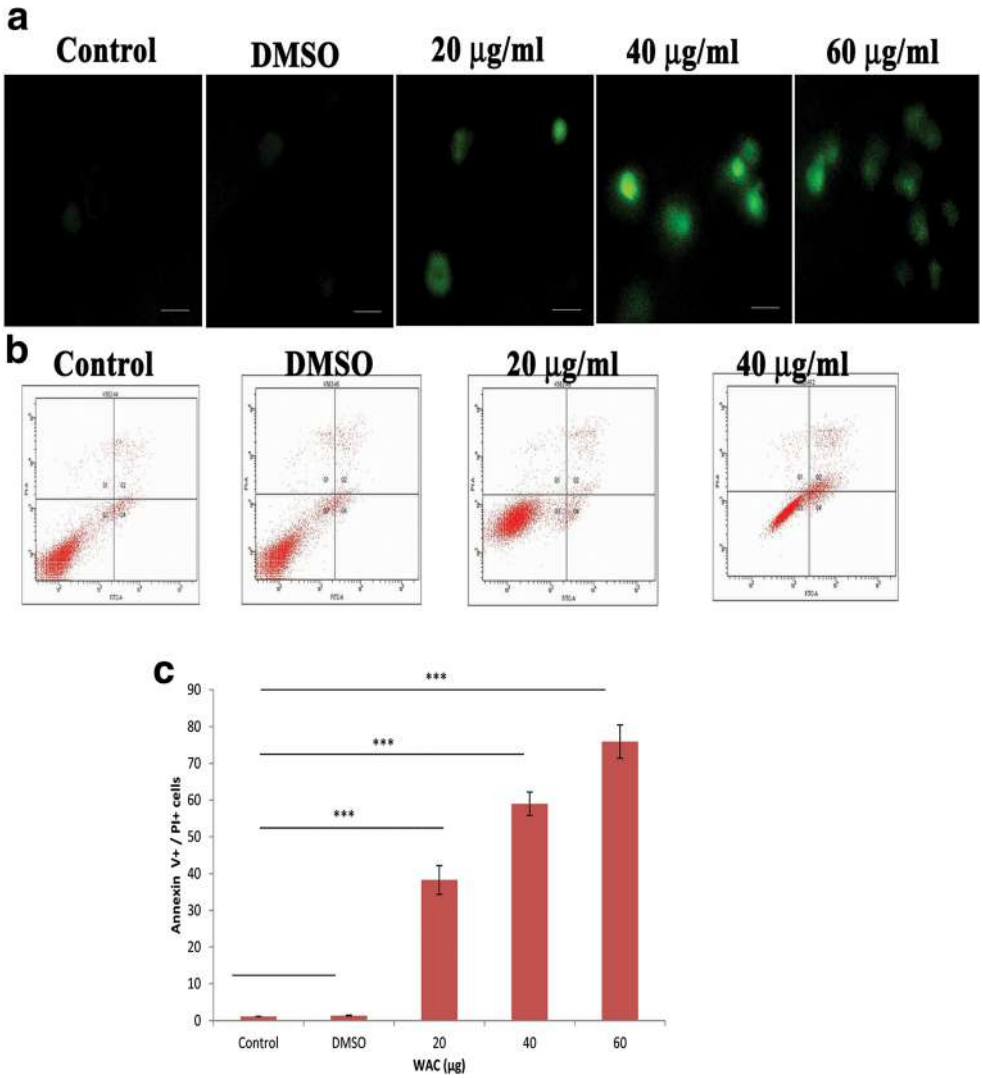

Fig. 12 Apoptogenic effect of WAC on K562 cells after 24 h treatment. **a** Microscopic images **b** Flow cytometric analysis of annexin V-FITC and PI stained cells. **c** Quantitative analysis of apoptotic cells. ***$p < 0.001$

The level of phosphorylated H2AX (ϒ H2AX), indicative of DNA damage, was found increased in WAC-treated cells in a dose dependent manner. This could very likely be due to the occurrence of WAC-induced double stranded breaks in DNA. Together, these results revealed that WAC-induced apoptosis in K562 cells occurred by way of caspase 3- mediated intrinsic pathway through caspase 9 activation.

To further determine the effect of WAC on cell cycle regulation, expression of a few key signaling proteins involved in regulation of G2/M phase of the cell cycle such as Cdk1 bearing PSTAIR sequence, cyclin B1 and CHK1 were also monitored. The activation of CDK1-cyclin B1 complex is known to be the rate-limiting factor of cells for mitotic entry, whereas its inactivation leads to G2/M arrest (Jackman et al. 2003). WAC-treated cells showed an apparent, though marginal, dose dependent reduction in the expression of cyclin B1 and CHK1 in comparison to control samples. PSTAIR expression, however, did not show much variation (Fig. 15).

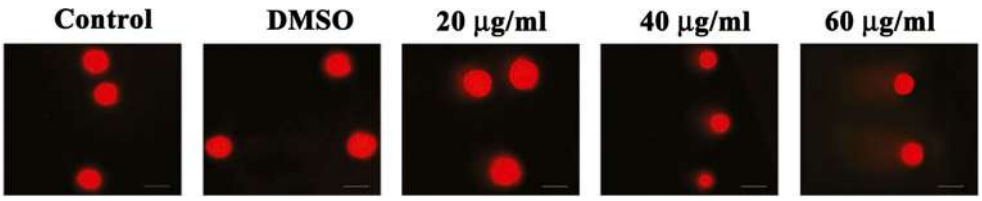

Fig. 13 Detection of DNA damage by alkaline comet assay in K562 cells treated with WAC for 24 h

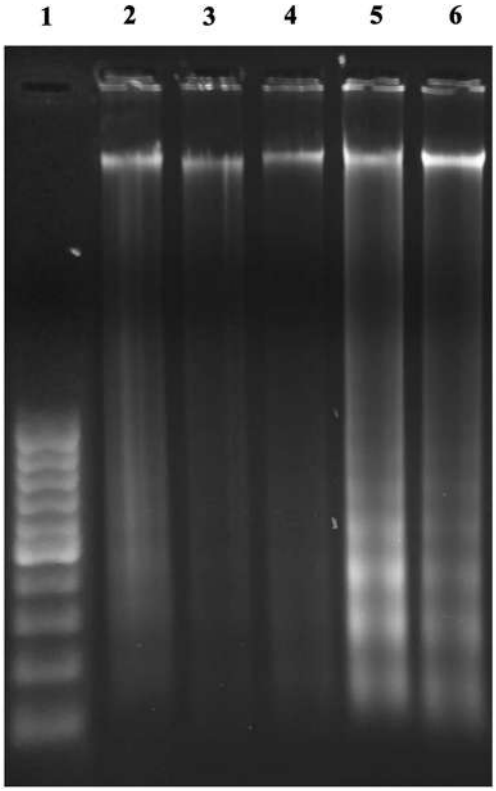

Fig. 14 DNA fragmentation assay for detection of apoptosis in K562 cells. Lane *1* 100 bp DNA ladder (M), Lane *2* DNA from untreated control cells, Lanes *3*, *4*, and *5* DNA from cells treated with 20, 40, and 60 μg/ml of WAC, respectively, and Lane *6* DNA from curcumin treated cells (25 μg/ml) taken as positive control

Discussion

We have evaluated the antiproliferative and apoptosis inducing potential of leaf extracts of *Wrightia arborea*, a traditionally important Indian medicinal plant used to treat a multitude of human ailments. While the acetone, ethyl acetate, and methanol extracts did not show any cytotoxic effect against K562, MCF 7, and Hep G2, the chloroform extract, WAC, showed significant and dose-dependent cytotoxicity against K562 cells. Interestingly, WAC exhibited no toxicity toward normal hPBLs. WAC-treated K562 cells clearly showed morphological features associated with apoptosis such as cell shrinkage, cellular/nuclear fragmentation, and chromatin condensation. WAC-induced genotoxicity was evident by the induction of double stranded DNA breaks (Archana et al. 2013). Upregulation of phosphorylated H2AX, was also indicative of DNA damage (Sharma et al. 2012; Rajan et al. 2015). Phosphatidyl serine externalization, loss of mitochondrial membrane potential, increased intracellular Ca^{2+} and ROS, PARP cleavage, caspase 9, −3 activation, and DNA fragmentation all confirmed apoptosis induction by WAC. These results indicated that WAC induced mitochondria-mediated apoptosis occurred via increase in free intracellular Ca^{2+} and ROS (Lopez and Tait 2015; Hassan et al. 2014). Our results also implicated involvement of cell cycle related proteins such as cyclin B1, PSTAIR, and CHK 1 in WAC-induced cell death.

WAC treatment at 40 μg/ml (IC 50 value) induced a drastic reduction in G1 phase subpopulation (up to 65%) with a concomitant appearance of sub-G1 apoptotic cells comprising

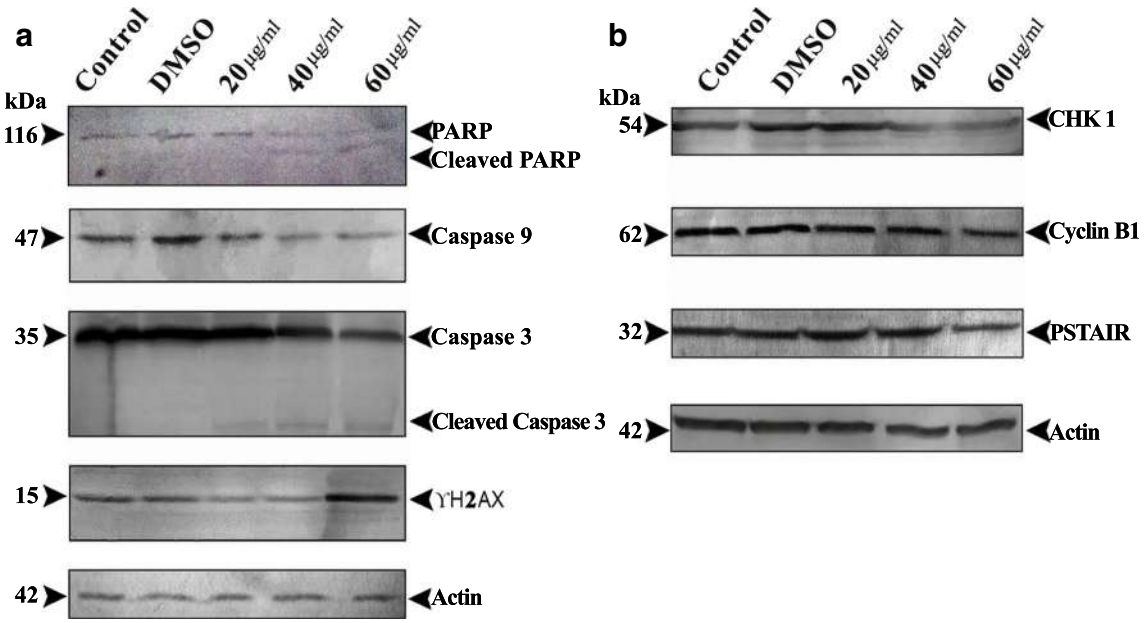

Fig. 15 Western blot analysis of apoptotic (**a**) and cell cycle related proteins (**b**) in K562 cells treated with 20, 40, and 60 μg/ml of WAC. **a** Expression of PARP, Caspase 9, Caspase 3, and ϒ H2 AX. **b** Chk1, Cyclin B1, and PSTAIR. All proteins were normalized against beta actin taken as the loading control

30% of the total population which increased to 70% at 60 μg/ml. FACS analysis, however, did not reveal the presence of a G2 arrested subpopulation of cells, despite the occurrence WAC-induced DNA damage, suggesting thereby the failure of checkpoint activation resulting in non-execution of DNA repair. A similar case of curcumin-induced apoptosis in the absence of G2/M arrest has been reported in BXPC3 cells (Sahu et al. 2009). Notably, both K562 and BXPC3 happen to be p-53 deficient and G1compromised. G2 checkpoint integrity thus becomes critical in development of resistance by tumor cells to radiotherapy and DNA damaging chemotherapeutic agents (Sturgeon et al. 2008). G2 abrogators are currently under extensive evaluation for cancer therapy, both as single agents or in combination with sublethal doses of other drugs or modes of treatment (Kawabe 2008; Sui et al. 2012; Khalil et al. 2012; Daud et al. 2015). Given the fact that K562 cells are p53 null and G1 compromised (Berglind et al. 2008), involvement of G2 checkpoint in WAC-induced apoptosis was further probed. Our results showed that WAC's ability to abrogate G2 arrest was comparatively greater than that of PD407824. Notably, the extent of apoptotic sub-G1 population obtained with a combination of camptothecin and WAC (36%) and with WAC alone (30%) were somewhat comparable. Signs of abortive mitosis were also observed in Hoechst 33258 stained cells from G2 abrogated samples. As proof of principle, G2 abrogation by WAC was also checked on another p-53 deficient leukemic cell line, HL-60. WAC not only elicited G2 abrogation in HL-60 cells to a greater extent (67%) than that observed with K562 cells but also evoked induction of catastrophic mitosis. Additionally, HL-60 cells were found to be much more sensitive to all of the three agents used, namely camptothecin, PD407824, and WAC (Supplementary Fig. 1).

To conclude, of the four extracts, only WAC exhibited selective cytotoxicity against K562 cells among the three cell lines tested leaving the normal control cells—human peripheral blood lymphocytes—unaffected. The dual capacity of WAC both as a DNA damaging agent and as G2 abrogator makes it an attractive drug candidate with promising therapeutic and anti-leukemic potential. It is important to understand whether the bioactive components present in the extract act individually or in combination. Future studies are necessary to characterize the active principles and their mode of action on cellular targets.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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