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Research Paper

SECLUSION OF VASICINE- AN QUINAZOLINE ALKALOID FROM BIOACTIVE FRACTION OF *Justicia adhatoda* AND ITS ANTIOXIDANT, ANTIMUTAGENIC AND ANTICANCEROUS ACTIVITIES

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Abstract

In the present study, an alkaloid-vasicine has been isolated from the most bioactive *n*-butanol fraction of *Justicia adhatoda* and analysed for antioxidant, antimutagenic (Ames assay), and anticarcinogenic potential. In all the antioxidant assays, vasicine exhibited strong activity with very low IC_{50} values. In Ames assay that was used to explore antimutagenic activity, it was seen that vasicine exhibited dose dependent antimutagenic activity against 2-Aminofluorine (S9 dependent mutagen) induced mutation in TA98 and TA100 *Salmolnella typhimurium* stains of maximum % inhibition of 98.09±0.088% and 87.3±0.661% respectively. Furthermore, it was also showed the cytotoxic effect in PC-3 cancer cells as well as loss of mitochondrial membrane potential and the features typical of apoptosis. Overall results depict that the vasicine has antioxidant, antimutagenic as well as anticancer potential.

Key words: *Justicia adhatoda, Vasicine, Antioxidant, Antimutagenicity, Anticarcinogenic, Reactive oxygen species (ROS)*.

INTRODUCTION

Justicia adhatoda (Family; Acanthaceae), an Indian shrub, has been used extensively for respiratory disorders including cough, cold, asthma, bronchitis, tuberculosis etc [1]. Its leaves and roots have also been used extensively to treat diabetes in North East of Pakistan [2]. Some investigators have also studied its antimicrobial phytochemicals in essential oil extracted from leaves of *Justicia adhatoda* [3]. It is well established that most active and abundant alkaloid present in the in *J. adhatoda* is a bitter quinazoline alkaloid, vasicine, characterized by the presence of heterocyclic skeleton. Besides this, it contains several other alkaloids like vasicinone, vasicinol, deoxyvasicine, adhatonine, adhavasinone etc. Vasicine is famous for its bronchodilatory and anti-inflammatory activities [4, 5]. Inspite of all that, vasicine has great importance as a uterus stimulant and abortifacient [6, 7]. Kudle et al., 2014 [8] reported that aqueous leaf extract of the *J. adhatoda* acts as reducing agents in biosynthesis of silver nanaoparticles using silver nitrate and moreover these silver nanoparticles exhibited significant antibacterial activity.

Keeping in view the importance of vasicine, coupled with the fact that *J. adhatoda* as an important medicinal plant, the present study was planned to isolate it from the most bioactive n-butanol fraction and explore its antioxidant, antimutagenic and cytotoxic activities.

MATERIALS AND METHODS

Leaves of *Justicia adhatoda* were collected from Mata Kaulan Botanical Garden, Guru Nanak Dev University, Amritsar. The plant was identified and submitted in Herbarium of Department of Botanical and Environmental Sciences, GNDU, Amritsar, where a voucher of specimen (Accession no.7034) was deposited on 27 April 2014.

Chemicals and instruments

Plasmid pBR322 DNA was purchased from Genei Pvt. Ltd., Bangalore. The *Salmonella typhimurium* strains TA98 and TA100 were kindly provided by Prof. B.N. Ames, University of California, Berkeley, USA. Dextrose anhydrous (purified) was purchased from E. Merck (India) limited, Mumbai, Nicotinamide adenine dinucleotide phosphate sodium salt, Calcium chloride (fused) and Ammonium chloride were purchased from Loba chemie Pvt. Ltd., Potassium dihydrogen orthophosphate, monobasic, Agar-agar, Sodium chloride, Luria broth, Disodium hydrogen phosphate dehydrate, Magnesium sulphate heptahydrate extrapure were purchased from Himedia Laboratories Pvt. Ltd. 2-Aminofluorene (2-AF), sodium azide (NaN_3) and 4-nitro-o-phenylediamine (NPD) were purchased from Sigma-Aldrich and dissolved in dimethyl-sulfoxide (DMSO). All the other reagents used to prepare buffers and media were of analytical grade. ^1H, ^{13}C NMR spectra was recorded at 600 MHz Bruker Avance spectrometer. Silica gel 340-400 mesh size was used for column chromatography, HIMEDIA NMR tubes, MeOD for preparation of sample for NMR spectroscopy.

Extraction procedure

The fresh leaves of *Justicia adhatoda* were washed with tap water and then dried at room temperature. Dried leaves were crushed and extracted as per the procedure given in Figure 1.

Isolation and characterization of chemical constituent

Among all the fractions, *n*-butanol fraction, which was highest in yield (9.0 g) and soluble in methanol, exhibited the most significant antioxidant activity in DPPH, reducing power, superoxide scavenging and cupric assays. Therefore, this fraction was subjected to column chromatography and run with solvent system (ethyl acetate: methanol (2:1)) selected after performing Thin layer chromatography (TLC). TLC plates were developed on a silica gel (Merck silica gel 60/UV_{254}). The spots were identified with Dragendorff's reagent which gave orange colour.

Column Chromatography of n-butanol fraction of leaves

The *n*-butanol fraction of *J. adhatoda* was subjected to column chromatography in which silica gel (230-400 mesh) was used as adsorbent. The slurry was prepared by mixing silica gel and ethyl acetate. The 2/3rd of column was packed with the slurry by pouring it slowly from the top of the column. The extract slurry was prepared by mixing minimum amount of methanol and dry silica gel (230-400 mesh) in *n*-butanol fraction and dried over water bath. The extract slurry was poured over silica gel already packed in column. The column was run with best selected mobile phase. The fractions were collected using gradient elution that was monitored by thin layer chromatography. The isolated compound was subjected to analysis by various spectroscopic methods viz. NMR (^1H, ^{13}C), DEPT135, HMQC, HMBC and COSY, along with the comparison of data in literature for the characterization and elucidation of their chemical structure.

NMR (Nuclear Magnetic Resonance) spectroscopy was carried out to determine the structure of isolated compound. The ^1H, ^{13}C NMR was recorded on Bruker Avance 300 machine probes. The sample was prepared by dissolving 30 mg or above of isolated compounds in methanol-*d4* (MeOD), depending upon its solubility. The sample prepared was filtered through 0.2 µM filters and then transferred to NMR tubes (HIMEDIA), 178 mm length and then capped. The ^1H and ^{13}C spectra of compounds were recorded at 600 MHz. Two dimensional spectra (DEPT135, HMQC, HMBC and COSY) were also recorded in order to determine the respective bonding between different atoms and to obtain the accurate structure of compound.

UPLC analysis

Isolated compound i.e. vasicine was subjected to UPLC (Ultra performance liquid chromatography). Standard solution was prepared by dissolving accurately the weighed portion of vasicine. Dried methanolic and *n*-butanol fractions were also dissolved in methanol. Samples were filtered (0.22 µm) and injected into the column and eluted at a flow rate of 0.8ml/min using linear gradient solvent system. Retention time of isolated compound was compared with standard compound for identification.

Antioxidant studies

The antioxidant activity of isolated compound was determined by employing deoxyribose degradation assay, superoxide anion scavenging assay, ABTS radical cation decolourization assay and plasmid DNA protection assay.

Deoxyribose degradation Assay

This assay was used to measure the hydroxyl radical scavenging activity of plant extracts [9]. This method involves non-site and site specific scavenging of ·OH radicals depending upon the use of EDTA. In non-site specific deoxyribose degradation assay, 100µl of EDTA, 20µl of $FeCl_3$, 100 µl of H_2O_2, 360 µL of deoxyribose, 1000 µl extract concentrations (0-1000 µg/ml), 320 µl of phosphate buffer (50Mm, pH 7.4) and 100 µl of ascorbic acid were added. In site-specific hydroxyl radical scavenging assay, EDTA was replaced with same amount of buffer. The reaction mixture was incubated for 1 h at 37ºC. 1 ml of above incubated reaction mixture was mixed with 1 ml of 10% trichloroacetic acid and 1 ml of thiobarbituric acid (0.5% in 25 mM NaOH) and heated for 90 minutes on water bath at 80ºC. The intensity of pink chromogen (TBA-MDA complex) formed was measured spectrophotometrically at 532 nm. The % inhibition was calculated as:

$$\% \text{ inhibition} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where $A_{control}$ was t osorbance in the presence of sample.

Superoxide Anion Scavenging Assay

Measurement of superoxide anoin scavenging activity of isolated compound was based on the method described by Liu [10] with few modifications. This assay involves the generation of superoxide anion radicals in PMS-NADH system and these radicals reduce the nitroblue tetrazolium i.e. (NBT) to produce a blue coloured product (Diformazan). 1ml of NBT solution (144µM in 100mM phosphate buffer , pH 7.4), 1ml of reduced NADH (677µM in100mM phosphate buffer, pH 7.4) and 1 ml of sample extract was mixed and the reaction was started by adding 1 ml of PMS solution (60µM PMS in100mM phosphate buffer, pH 7.4) . The reaction mixture was incubated at 25°C for 5 min and the absorbance of coloured complex was measured at 560nm. Gallic acid was used as a positive control agent for comparative analysis. The inhibition percentage was calculated using the formula,

$$\% \text{ scavenging activity} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where $A_{control}$ was the absorbance of the control and A_{sample} was the absorbance in the presence of sample.

ABTS radical cation decolourization Assay

The method of Re [11] was adopted for ABTS radical cation assay with slight modifications. The stock solutions included 7 mM ABTS solution and 140 mM potassium persulfate solution. Both solutions were added in such a proportion so as to make final concentration of 2.45 mM $ABTS^+$ solution and allowed them to react for 12-16 h at 30ºC in the dark. The solution was then diluted by mixing $ABTS^+$ solution with methanol to obtain an absorbance of 0.706 ± 0.001 units at 734 nm using the spectrophotometer. Plant extracts (0.1ml) were allowed to react with 1 ml

of the ABTS solution and the absorbance was taken at 734 nm using the spectrophotometer. The $ABTS^{+}$ s

$$\text{ABTS radical scavenging activity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where $A_{control}$ was the absorbance of the control and A_{sample} was the absorbance in the presence of sample.

Plasmid DNA protection assay

Plasmid DNA protection assay was performed according to the protocol of Lee [12] by using supercoiled pBR322 plasmid DNA. Plasmid DNA (5 µg) was added in Fenton's reagent (30 mM H_2O_2, 50 mM ascorbic acid and 80 mM $FeCl_3$) containing different concentrations of isolated compound and finally the volume of the mixture was raised up to 20 µl. The mixture was then incubated for 30 min at 37°C followed by addition of loading dye. Electrophoresis was carried out in 1×TBE buffer (0.45 M Tris borate, 0.01 M EDTA, pH 8.0) and DNA was analyzed by ethidium bromide staining. The rutin was used as the standard reference compound.

Antimutagenic studies

The Ames test and S9 mix protocol [13] were performed on bacterial strains (TA98 and TA100) to determine the effect of isolated compound on 2-Aminofluorene (2-AF), sodium azide (NaN_3) and 4-nitro-o-phenylediamine (NPD) induced mutagenicity. The samples were dissolved in DMSO (Dimethyl sulfoxide) while making the different concentrations *viz*. 100, 250, 500, 1000 and 2500 µg/0.1 ml. In brief, 0.5 ml of S9 mixture or phosphate buffer was distributed in sterilized capped tubes in an ice bath, 0.1 ml of mutagen; 0.1 ml of plant extract and 0.1 ml of bacterial culture were added. After, mixing it gently 2 ml of top agar (0.6% agar, 0.5% NaCl, 0.5 mM L-histidine and 0.5 mM D-biotin) was added to each tube and poured immediately on the minimal agar plates. The procedure for the pre-incubation was similar with co-incubation, except that Bacterial strain+ extract+mutagen+S9 mix were incubated for 30 minutes prior to the addition of top agar. The plates were incubated at 37°C for 48 h and the revertant colonies were counted on Protocol Colony Counter. The inhibition rate of mutagenicity (%) was calculated by using equation [14].

$$\text{Inhibiton rate (\%)} = \frac{x - y}{x - z} \times 100$$

where x, is the number of revertants induced by mutagen alone (Positive control), y is the number of revertants induced by mutagen in the presence of extract (Co-incubation or Pre-incubation) and z is the number of revertents in the presence of extract alone (Negative control).

Cytotoxicity studies

The protocol for MTT assay was carried out according to Mossman [15] with slight modifications. This assay is dependent on the ability of viable cells to convert a water-soluble tetrazolium i.e. MTT to a purple formazan product that is insoluble in aqueous solution. This assay measures the cell viability via measuring the cellular metabolic activity of oxidoreductase enzymes. Cells of Human prostate cell line (PC-3) were harvested by centrifugation at 1000 rpm for 5 min at 2-8°C. Cell pellet was resuspended in growth medium to get 1.5 to 2 × 10^5 cells/ml and 100 µl of cell suspension per well was seeded in 96 well culture plate. The plate was incubated for 24 hours in CO_2 incubator (37°C, 5% CO2 and 90% relative humidity). The cells were treated with three different concentrations (100µg/ml, 50µg/ml and 30µg/ml) of test material by diluting with growth medium. The plate was incubated for next 24 hours. After that, 100 µl of MTT was added to each well. MTT was prepared by dissolving 5 mg of MTT in 10 ml of growth medium without FBS. Plate was again incubated for 3-4 hours for the reduction of MTT to formazan. After 3-4 hours, the medium was discarded and 100 ml of DMSO was added to each well in order to dissolve the formazan crystals. Plates were kept at shaker for 10 seconds and the optical density (OD) was taken at 595nm at multipurpose plate reader (Biotek synergy HT). Thus, the cytotoxicity was calculated by using the formula:

$$\text{Cytotoxicity (\%)} = \frac{OD_C - OD_S}{OD_C} \times 100$$

where OD_C = Optical density of control, OD_S = Optical density of fraction.

Reactive oxygen species (ROS)

ROS generation was estimated by flow cytometry using 2', 7'- Dichlorodihydrofluorescin diacetate (DCFDA). The protocol followed was according to Santhini [16] with minor modifications. The PC-3 cells (1×10^6) were incubated in the presence and absence of test compound at different concentrations (IC_{30}, IC_{50} and IC_{70}) for 12 hour at 37 ºC in 5% CO_2 incubator in 24 well plates and then washed with phosphate buffer saline (PBS). After this DCFDA (10µg/ml) was added to each well for 30 minute in serum free media. The cells were analysed in F1 channel (Excitation 485/20 nm; Emission 528/20 nm) on BD Accuri C6 flow cytometer (BD Biosciences Immunocytometry Systems, San jose, CA).

Mitochondrial membrane potential (MMP)

Mitochondrial membrane potential (MMP) was estimated by spectrofluorometry using Rhodamine-123 dye [17, 18]. The PC-3 cells (1×10^6) were incubated in the presence and absence of test compound at different concentrations (IC_{30}, IC_{50} and IC_{70}) for 12 hour at 37 ºC in 5% CO_2 incubator in 24 well plates and then washed with phosphate buffer saline (PBS). Then, Rhodamine-123 (10µg/ml) was added to each well for 1 hour. Samples were measured directly in a multipurpose plate reader (Biotek synergy HT) at the 485/20 nm excitation and 528/20 nm emission wavelength respectively.

Confocal microscopy with DAPI

The confocal laser scanning micrograph showed the difference in distribution of pattern of DNA in the treated cells compared to control. DAPI is a popular cell-permeable nuclear stain that emits blue fluorescence when binds to dsDNA. The PC-3 cells ($1x10^6$) in 1 ml of culture medium were transferred into 6 wells plate and grown to confluence at 37º C with 5% CO_2 and Relative humidity 90% for 8 hours. The cultures were then exposed to different test material at their IC_{50} concentration and incubated for another 12 hours. After, incubation, the cells were washed with phosphate buffer solution (PBS) 2-3 times. Then cells were fixed with 4% para formaldehyde (PFA). Again washed with PBS and stain the slide with DAPI (10µg/ml) for 30 min in dark at room temperature. The confocal fluorescence images of the cells treated with test material were scanned on a Nikon eclipse TiE inverted fluorescence microscope equipped with a Nikon AiR laser scanning confocal microscope system and NIS elememt software viewer.

STATISTICAL ANALYSIS

All the analysis were done in triplicates and averaged. The data was calculated as mean± standard error and compared by one-way analysis of variance (ANOVA) followed by the Tukey test. $P < 0.05$ was regarded as significant.

RESULTS AND DISCUSSION

Butanol fraction was subjected to thin layer and column chromatography. Thin layer chromatograms were developed to select the best mobile phase. Column was then eluted with ethyl acetate: methanol (2:1 ratio), and forty-four fractions were collected. The fraction 26^{th} was recolumned and eluted with chloroform: methanol (7.5:2.5 ratio). Its 18^{th} subfraction was an individual compound which was whitish needle-like crystal. The yield of the compound was 112.3 mg and tested positive for alkaloid in the dragendroff test. The pure fraction was further subjected for characterization by developing ^1H NMR and ^{13}C NMR as well DEPT135, HMQC, HMBC and COSY.

The proton nuclear magnetic resonance (H^1 NMR) spectra of isolated compound (Fig. 2a) in MeOD exhibited four singlet between δ 7.328-7.129 resonances at 7.141 (1H, m, Ar H-8), 7.196 (1H, m, Ar H-7), 7.261 (1H, m, Ar H-6), 7.25 (1H, m, Ar H-9) which were attributed to the four aromatic protons. One proton triplet at 5.106 due to (1H, t, H-1). Two multiplet at δ 2.631 and 2.125 due to methylene (1H, m, H- 3) and (1H, m, H-2) respectively and two multiplet at δ 3.348 and δ 3.740 due to (1H, m, H- 5) and (1H, m, H-4) respectively. One at δ 4.823 due to methylene

(2H, brs, H-10). The δ values of the ^{13}C NMR of vasicine (Fig. 2b) were compared with those of vasicine given in earlier reports [19].The δ values for isolated compound was: δ 30.98 (C-1), 48.91 (C-2), 49.62 (C-3), 52.74 (C-4), 72.74 (C-5), 118.57 (C-6), 118.89 (C-7), 128.41 (C-8), 130.61 (C-9), 133.16 (C-10), and 164.98 (C-11).

Two dimensional spectra (DEPT135, HMQC, HMBC and COSY) were also recorded in order to determine the respective bonding between different atoms and to obtain the accurate structure of compounds. Thus, identity of vasicine was confirmed by various spectral analyses and by comparison of the spectral data with those reported [19, 20].

Methanolic mother extract, *n*-butanol fraction, isolated pure fraction (found to be vasicine) along with vasicine as a standard were analysed by UPLC; the chromatograms are presented in Figure 2(c). The UPLC chromatograms of the various extracts showed the presence of peak common in all extracts with retention time 1.92 min, which confirmed that isolated compound is vasicine.

As shown in figure 3 (a) and 3 (b), concentration dependent inhibition of hydroxyl radical-induced deoxyribose degradation was observed. To identify the mechanisms involved in antioxidant potential of vasicine, and particularly to determine whether it inhibits hydroxyl radical generation by chelating metal ions or by directly scavenging hydroxyl radicals, two different experimental approaches, site-specific and non-site specific assays were used. Site-specific assay was employed to verify whether vasicine was able to protect this deoxyribose through metal chelation using ascorbic acid–iron–H_2O_2–EDTA system. On the other hand, non-site-specific assay was employed to verify the ability of vasicine to scavenge hydroxyl radical directly without EDTA. It was observed that relatively greater antioxidant activity was found in site specific assay (75.08±0.860) as compared to non-site specific assay (69.155±0.941) at 1000µg/ml concentration. This showed that vasicine is not only good hydroxyl radical scavengers but also had strong chelating activity.

As illustrated in Figure 3(c), percentage inhibition increased with increase in concentration. In this method, superoxide anion radicals derived from dissolved oxygen by PMS-NADH coupling reaction reduces NBT in the PMS-NADH-NBT system. Antioxidants are able to scavenge the radicals and inhibit the blue colored NBT formation [21]. The decreases of absorbance at 560 nm with antioxidants indicate the consumption of O_2 in the reaction mixture. In the present experiment, vasicine was found to be a strong scavenger of O_2^{*-} radical. The maximum percentage inhibition of superoxide radical generation at 1000 µg/ml concentration of vasicine was found to be 79.68±0.368% with IC_{50} 539.64 µg/ml. Superoxide, O_2^{*-}, is a free radical and paramagnetic. Compared with other oxygen radicals, superoxide has longer life, can move at longer distance and more dangerous. These Superoxide radicals further initiate the peroxidation of lipids [22, 23].

$ABTS^+$ radical scavenging ability of vasicine is shown in Figure 3(d). This assay measures the relative capacity of antioxidant to scavenge the $ABTS^+$ radicals. The results obtained from this assay revealed the high level of proton radical scavenging ability of the given test material. It was found that vasicine exhibited inhibitory activity of 85.34% at 1000 µg/ml concentration with IC_{50} value of 515.46 µg/ml. Dudonne [24] reported the comparative study of antioxidant properties of 30 plant extracts in $ABTS^+$ radical scavenging assay. The ABTS assay is particularly interesting in plant extracts because the wavelength absorption at 734 nm eliminates color interference [25]. Hence, radical scavenging activities are very important due to the deleterious role of free radicals in food and in biological systems [26]. In the present study, there is significant decrease in the concentration of $ABTS^+$ due to the scavenging capacity of vasicine.

The antioxidant activity of vasicine was further confirmed on pBR322 plasmid DNA. pBR322 is damaged by hydroxyl radicals produced by Fenton's reagent. The radicals cause either the single stranded scission (resulting in nicked circular form II) or the double stranded breaks (resulting in linear form III). The extracts provided protection to DNA. The protective effect of different concentrations of vasicine has been shown in Figure 4. Standard antioxidant compound rutin was used to compare antioxidant potential of vasicine. The addition of different concentrations of vasicine along with Fenton's reagent provided protection to plasmid DNA, resulting in retention of native form. It was found that out of all the concentrations, 500 µg/ml

concentration was effective in preserving the supercoiled form of DNA (69.7%) followed by 1000 µg/ml (58.4%)>800µg/ml (58.2%)>200 µg/ml (52.8%)>100 µg/ml(30.2%).

Mutagenic assays, such as Ames test, have been widely used to assess the antimutagenic activities of various compounds [27, 28, 29]. The antimutagenic activity of *J. adhatoda* leaf extracts against sodium azide (NaN_3) and 4-nitro-O-phenylenediamine (NPD) was evaluated by using two strains of *Salmonella typhimurium*, i.e. TA100 and TA98 respectively. It was observed that, the isolated compound (vasicine) did not exert significant inhibitory activity against direct acting mutagens *viz* sodium azide (NaN_3) and 4-nitro-o-phenylediamine (NPD) in both the tester strains of *Salmonella typhimurium*. The percent inhibition against the direct acting mutagen was found to be in the range of 17-43% in both the strains (Figs. 5a and 5c). The results of antimutagenicity potential of vasicine isolated from leaves of *Justicia adhatoda* against S9 dependent mutagen, 2-Aminofluorene (2-AF) are shown in figure 5(b, d). It was noticed that there was decrease in the number of revertants colonies against 2-Aminofluorine (2-AF) induced mutagenicity in both the strains. In case of TA98 at concentration of 2500 µg/0.1 ml, 46.2±25.69% of inhibition in the revertant colony formation was observed. The inhibition in the revertant colonies was more than two folds as the reaction mixture incubated for 30 min i.e. 98.09±0.088%. Whereas in TA100 strain, percent inhibition at 2500 µg/0.1 ml was found to be 87.3±0.661% and 96.88±0.424% in co-incubation and pre-incubation treatments respectively.

In MTT assay, the potency of the vasicine to inhibit the growth of human prostate cancer (PC-3) cells was measured at different concentrations (30, 50 and 100 µg/ml) and compared with known cell growth inhibitor *viz* camptothecin. It was observed that vasicine inhibited the proliferation of PC-3 cells in a dose dependent manner, by inhibiting the 50% cell growth at their IC_{50} concentration i.e. 81.11 µg/ml (Figure 5e). A mitochondrial enzyme in the living cells, i.e. succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells [15]. Many investigators reported the cytotoxic effect on different cell lines *viz* MCF-7, A549, RAW264.7 [16, 18, 30]. Wang [31] reported significant cytotoxicities of two alkaloids, Pegaharmalines A & B, on HL-60 cells with IC_{50} values of 9.4 and 13.6 µM respectively.

Effect of isolated fraction on mitochondrial membrane potential was evaluated by membrane-permeable Rhodamine-123 stain. This dye is used to measure membrane polarization in live cells assay. It penetrates into the cells and decrease the fluorescence because of mitochondrial membrane potential loss which was analyzed in a multipurpose plate reader (Biotek synergy HT) at the 485/20 nm excitation and 528/20 nm emission wavelength. Upon treatment with given fraction, depolarization of the mitochondria (loss of membrane potential) was observed in PC-3 cells. The spectrofluorometer analysis showed that the given fraction exhibited disruption in mitochondrial membrane potential of PC-3 cells after 12 h treatment. As shown in Figure 5(f), the untreated PC-3 cells (control) were 100% active with maximum fluorescence while camptothecin (positive control) and vasicine shifts the mitochondria membrane potential to 43.42% and 38.43% respectively at IC_{70} concentration.

As shown in Figure 6, the DCFH-DA fluorescence of cells increases significantly with increase in vasicine concentrations. In case of vasicine, a dose dependent ROS generation was observed with maximum value of 18.7% at IC_{50} concentration. Treatment with camptothecin at different concentrations was found to enhance ROS generation by 8.8%, 21.2% and 31.4% after 24 h exposure at their respective IC_{30}, IC_{50} and IC_{70} concentration and less ROS generation i.e. 4.5% was observed in untreated cells. It was discussed by many investigators that overwhelming anticancer activity is initiated by ROS generation, leading to the induction of both the intrinsic and extrinsic apoptotic pathways [32, 33, 34]. The results showed that cell-death via apoptosis increased significantly on treatment with vasicine for 24 hours. The results suggested that vasicine could induce ROS accumulation in PC-3 cells.

Apoptosis is characterized by a series of morphological changes including cytosol shrinkage, chromatin condensation, nuclei fragmentation and the formation of apoptotic bodies [35]. As seen in figure 7, upon treatment with vasicine, PC-3 cells were compared with control (without treatment) and observed that PC-3 cells seems to be less adherent, DNA fragmentation, lost

polygonal shape, significant shrinkages, minor surface blebs and some more characteristics of apoptosis.

CONCLUSION

The leaves of the plant *Justicia adhatoda* were extracted serially with the solvent of increasing polarity. Vasicine was isolated from most active *n*-butanol fraction by column chromatography and subjected to different antioxidant assay, antimutagenic assay and cytotoxic assay. Results from the study showed that vasicine exhibited excellent free radical scavenging activity, antimutagenic activity and antiproliferative activity with minimum IC_{50} value. With this in view, vasicine may be used as potential medicine to treat a dreaded disease i.e. cancer.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests regarding the publication of this paper.

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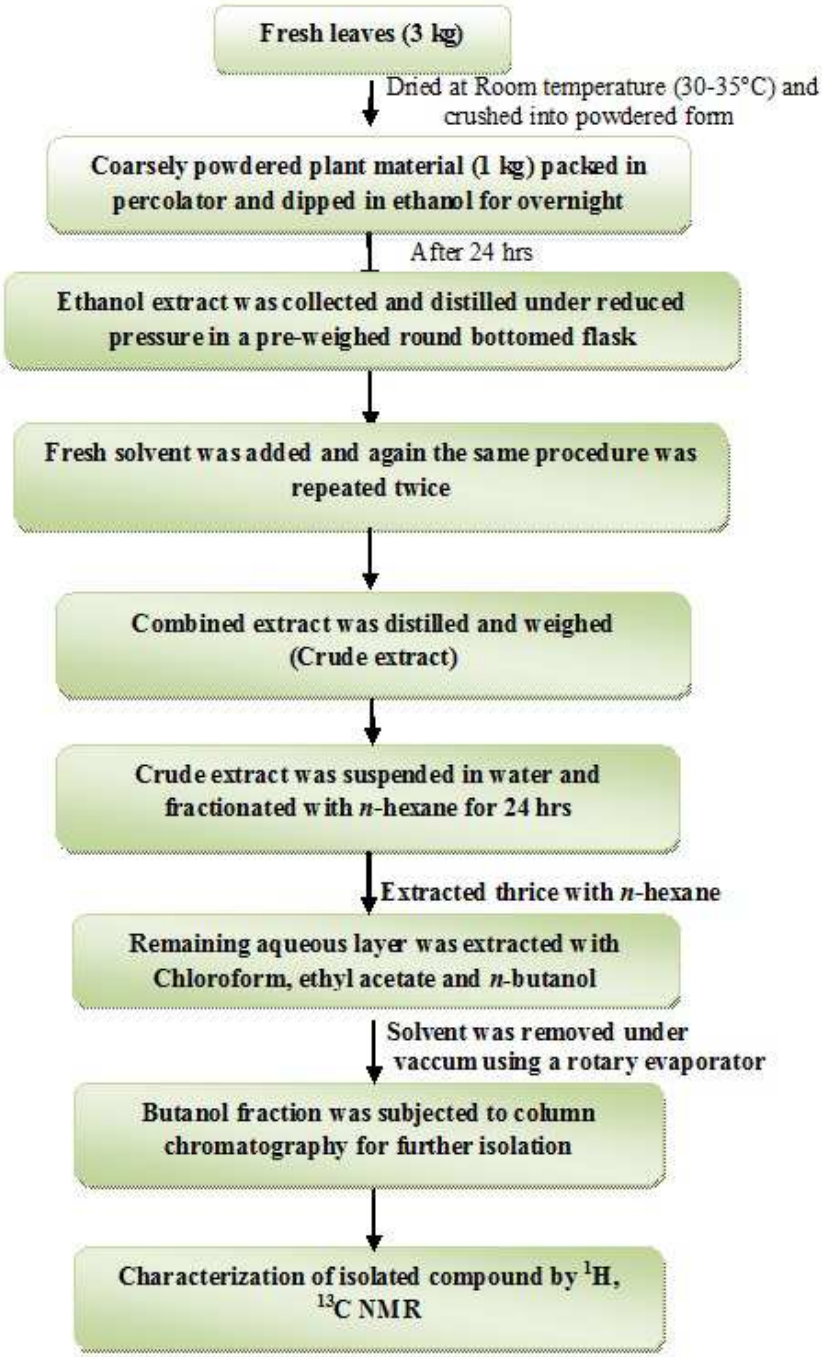

Fig. 1. Preparation of crude extracts of *Justicia adhatoda* by alcoholic extraction method

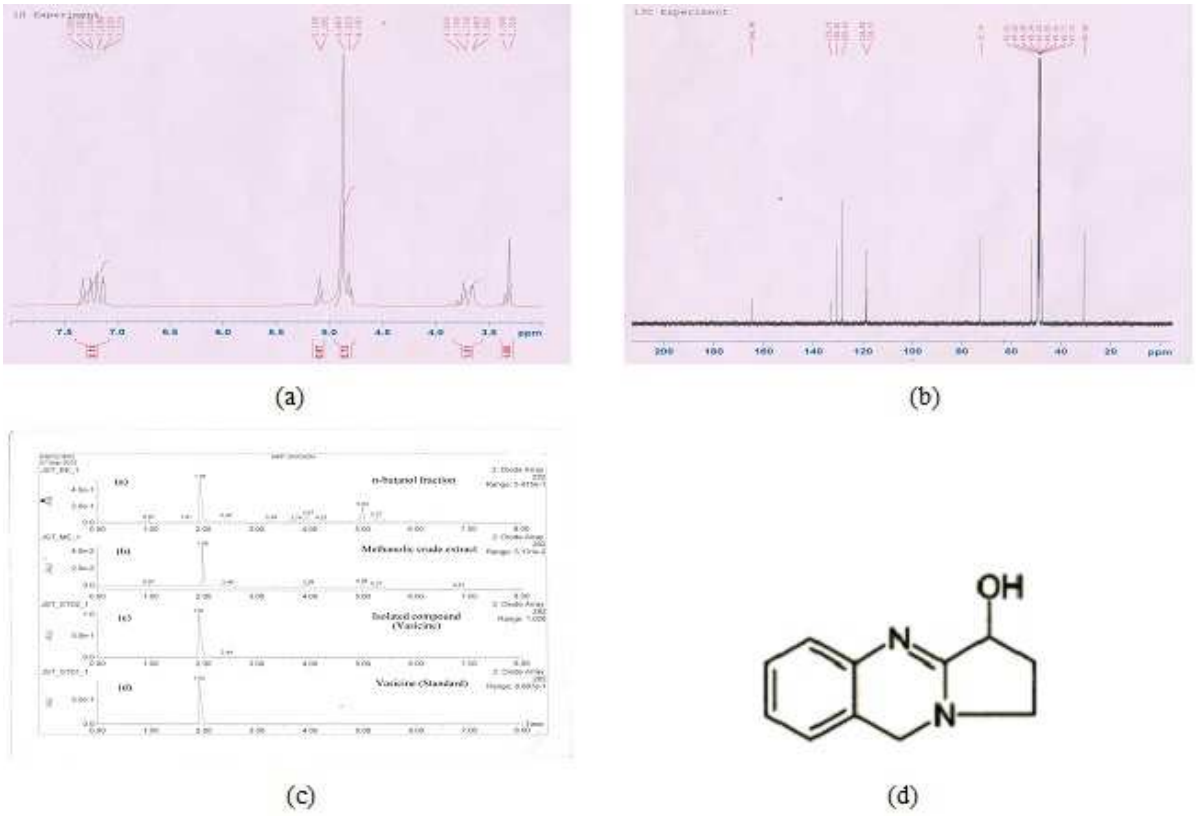

Fig. 2. (a) ^{1}H NMR spectrum of 18th subfraction of *n*-butanol extract of *Justicia adhatoda*; (b) ^{13}C NMR spectrum 18th subfraction of *n*-butanol extract of *Justicia adhatoda*; (c) UPLC chromatogram of *n*-butanol fraction, Methanolic crude extract, isolated compound (Vasicine) and pure vasicine as a standard; (d) Structure of vasicine

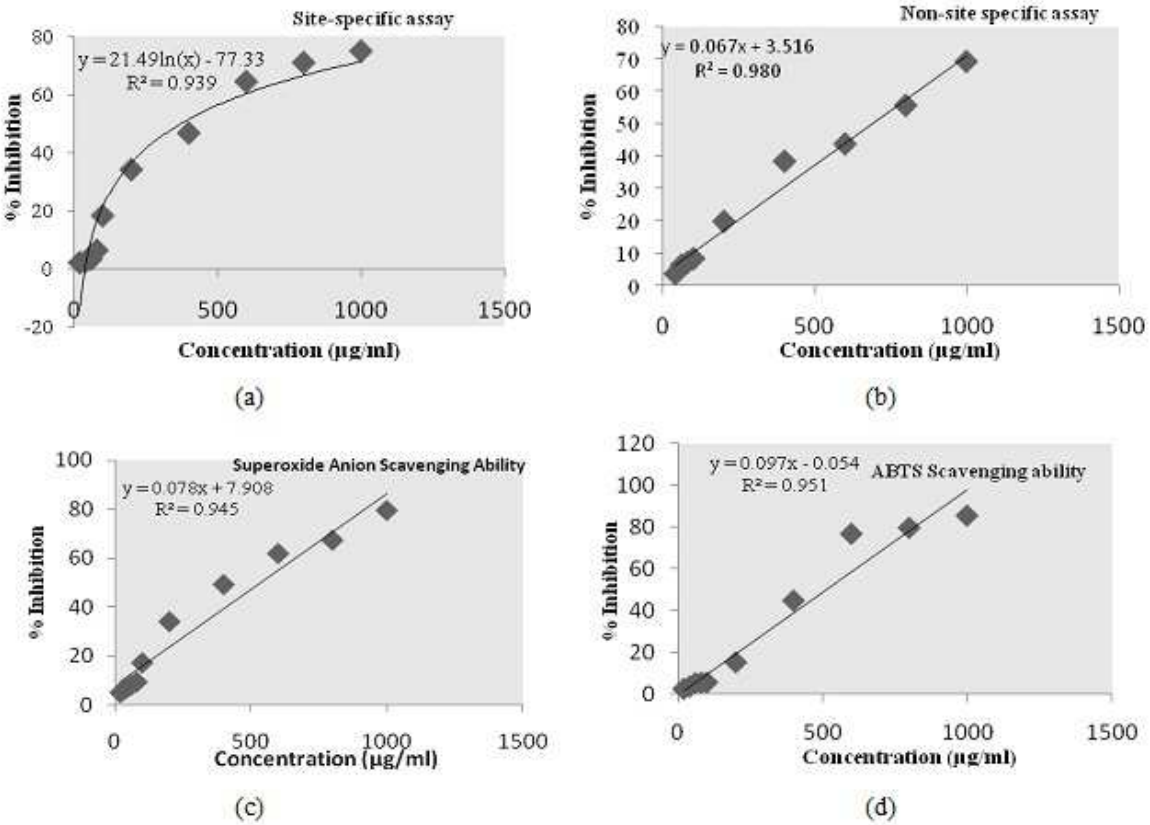

Fig. 3. Inhibitory effect of isolated vasicine compound; (a) Site-sjpecific hydroxyl radical-mediated deoxyribose degradation; (b) Non-site specific hydroxyl radical-mediated deoxyribose degradation. (c) Superoxide anion radical; (d) ABTS scavenging radical

Fig. 4. DNA nicking assay with different concentrations of Vasicine

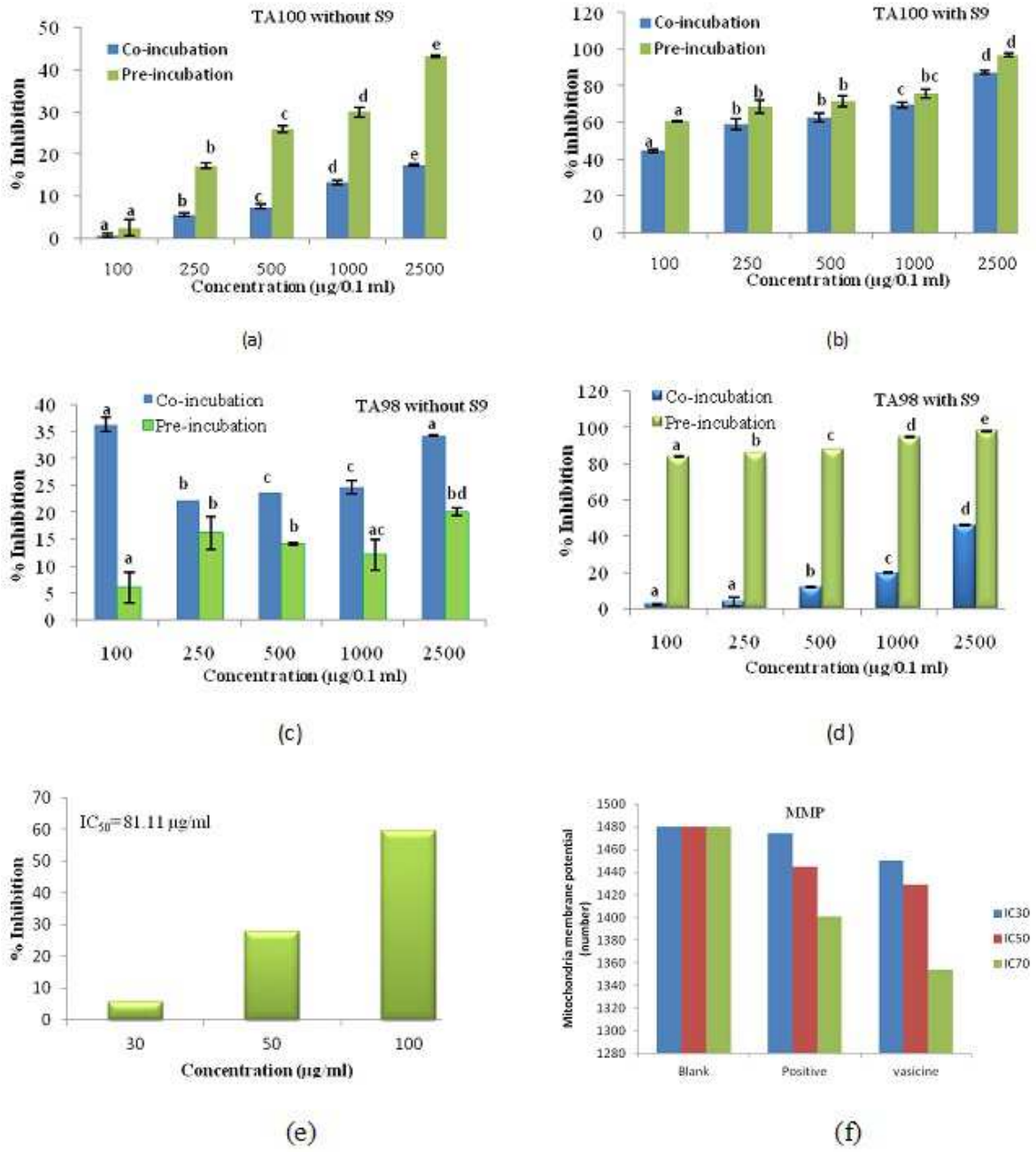

Fig. 5. (a) Antimutagenic potential of vasicine on *Salmonella typhimurium* strain TA100 without S9 against 4-nitro-O-phenylenediamine (NPD); (b) Antimutagenic potential of vasicine on bacterial strain TA100 with S9 against 2-Aminoflurine (2-AF); (c) Antimutagenic potential of vasicine on bacterial strain TA98 without S9 against Sodium azide; (d) Antimutagenic potential of vasicine on bacterial strain TA98 with S9 against 2-Aminoflurine (2-AF); (e) Antiproliferation of PC-3 cell line at low IC_{50} value of vasicine by MTT assay; (f) Mitochondrial membrane potential in PC-3 cells at its IC_{30}, IC_{50} and IC_{70} concentrations

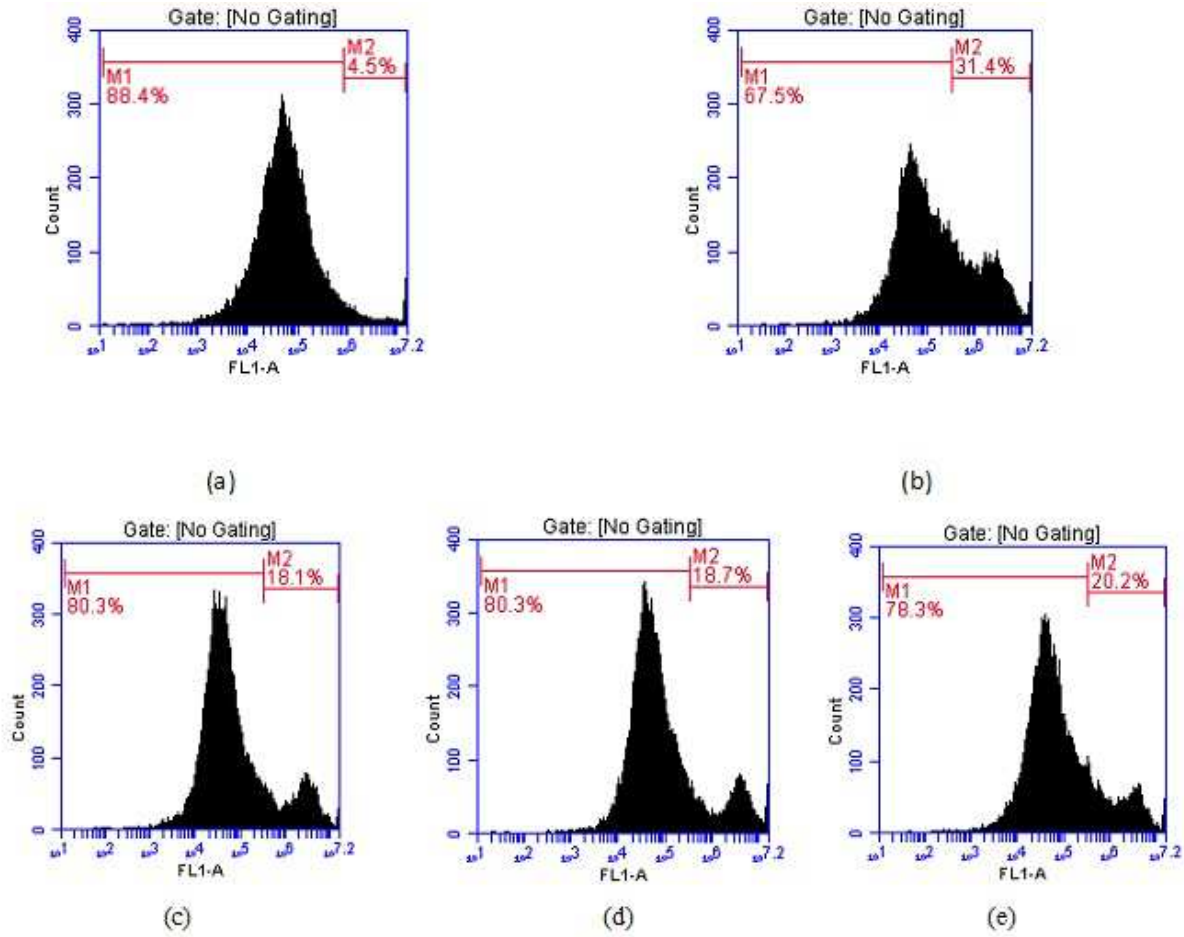

Fig. 6. Effect of vasicine on generation of ROS in PC-3 cells at three respective concentrations (c)-(e) (IC_{30}, IC_{50} and IC_{70}), compared with untreated control cells (a) and positive control camptothecin treated cells (b).Cells were stained with DCFH-DA, 10,000 events acquired and gated population using flow cytometer.

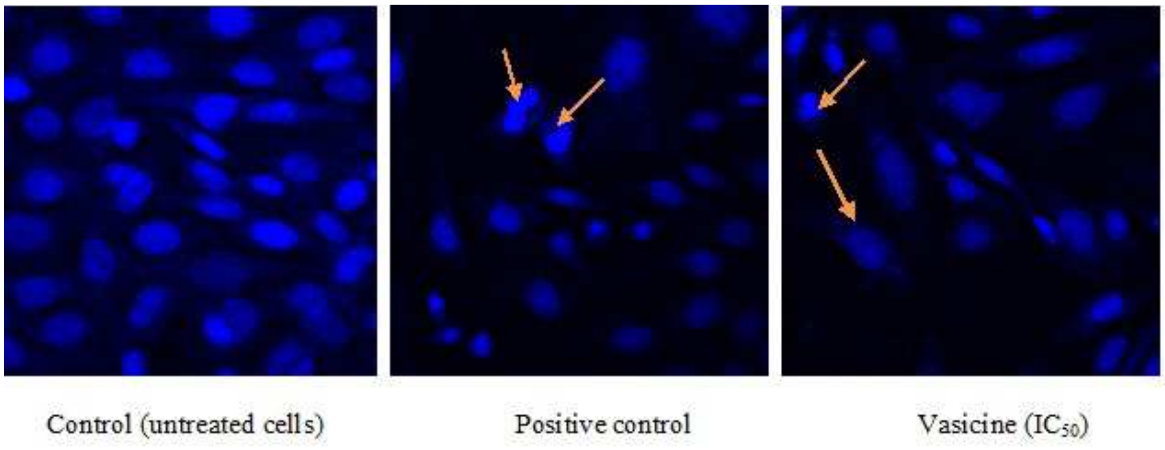

Fig. 7. DAPI fluorescence staining of PC-3 cells treated with camptothecin (Positive control) and vasicine at their IC_{50} concentration. Red arrows shown in pictures indicates apoptotic features like nucleus fragmentation, loss of surface projections and shrinkage

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