Supporting information

Jacalin capped silver nanoparticles minimizes the dosage use of the anticancer drug, shikonin derivatives against human chronic myeloid leukemia

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Materials

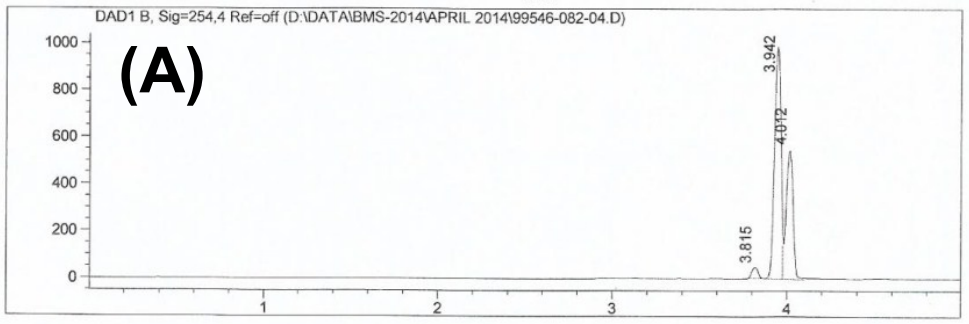
The *Artocarpus integrifolia* (Jack fruit) seeds were obtained from local seed vendors. Guar gum was obtained from Loba, India. Sodium phosphate dibasic and monobasic, sodium chloride, epichlorohydrin, sodium citrate, citric acid, galactose, glucose, lactose and maltose were purchased from Merck. Acrylamide, bis-acrylamide, sodium dodecyl sulfate, acridine orange (AO), ethidium bromide (Et-Br), 2,7-dichlorofluorescein diacetate ($DCFH_2$-DA), DAPI (4',6-diamidino-2-phenylindole), DMSO, SP600125, necrostatin-1, pentoxifylline, rhodamine B were purchased from Sigma, India. RPMI-1640, MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide), *N*-acetyl-L-cysteine (NAC), necrostatin I were purchased from Himedia. FBS, cell culture grade antibiotics were from Gibco, Annexin V-FITC apoptosis kit were from BD Bioscience and TNF-α and IL-10 cytokines kits were from e-Bioscience. All other reagents were of analytical grade.

Lectin activity assay

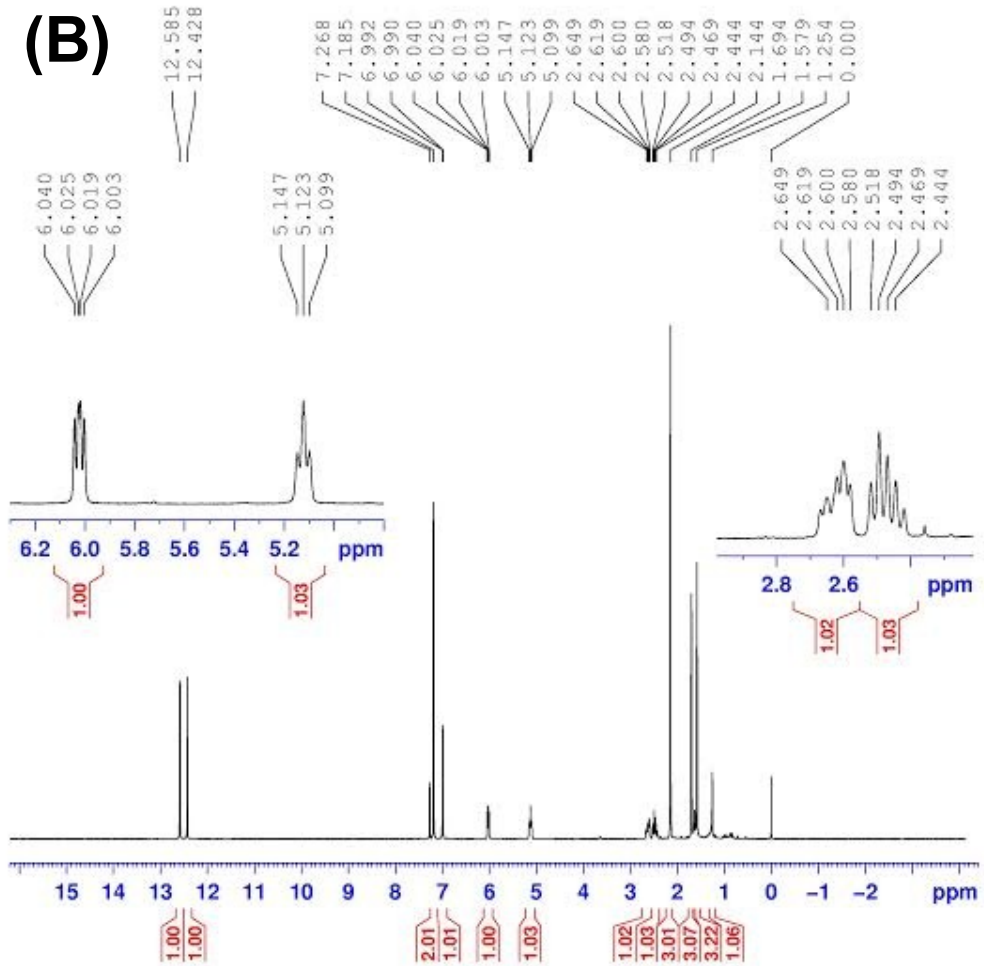
The activity of the protein was checked by hemagglutination and hemagglutination inhibition assays. To determine whether AgNPs binding, altered the sugar-binding activity of the lectin, the hemagglutination experiments were conducted by pre-incubating jacalin with high concentration of AS/BDS as used in the fluorescence studies. Blood sample used in this work were collected by the trained doctor from the volunteers in compliance with institutional guidelines. The experimental protocols received prior approval from the Institutional Biosafety Committee (SASTRA University, IBSC approval Number: SASTRA/IBSC/22/2014).

Cell lines culture and maintenance

Human myeloid leukemia cell lines, K562 (CML) was obtained from NCCS, Pune (India). These cell lines were cultured and maintained in RPMI-1640 complete medium supplemented with 10% FBS, 2 mM L-glutamine, and antibiotics (penicillin, streptomycin and gentamycin) under 5% CO_2 and 95% humidified atmosphere at 37 °C in CO_2 incubator with regular passage.



Peak No	RT min	Area	Area %	
1	3.815	1.172e+002	2.821	**Deoxyshikonin**
2	3.942	2.584e+003	62.196	**β,β dimethylacryl shikonin**
3	4.012	1.453e+003	34.983	**Acetyl Shikonin**



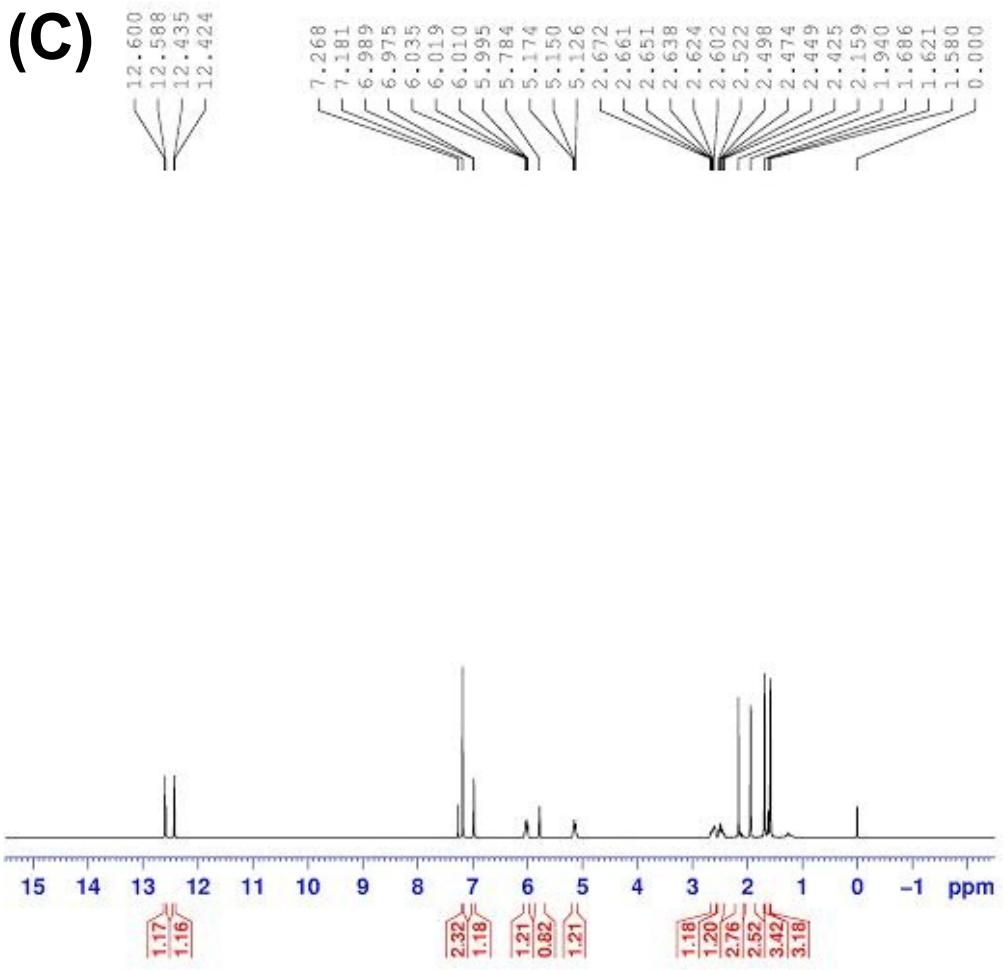


Fig. S1: Purification of acetylshikonin and beta,beta-dimethylacrylshikonin. (A) HPLC profile of *A. nobilis* extract, (B) ^{1}H NMR of acetylshikonin and (C) ^{1}H NMR of beta,beta dimethylacrylshikonin.

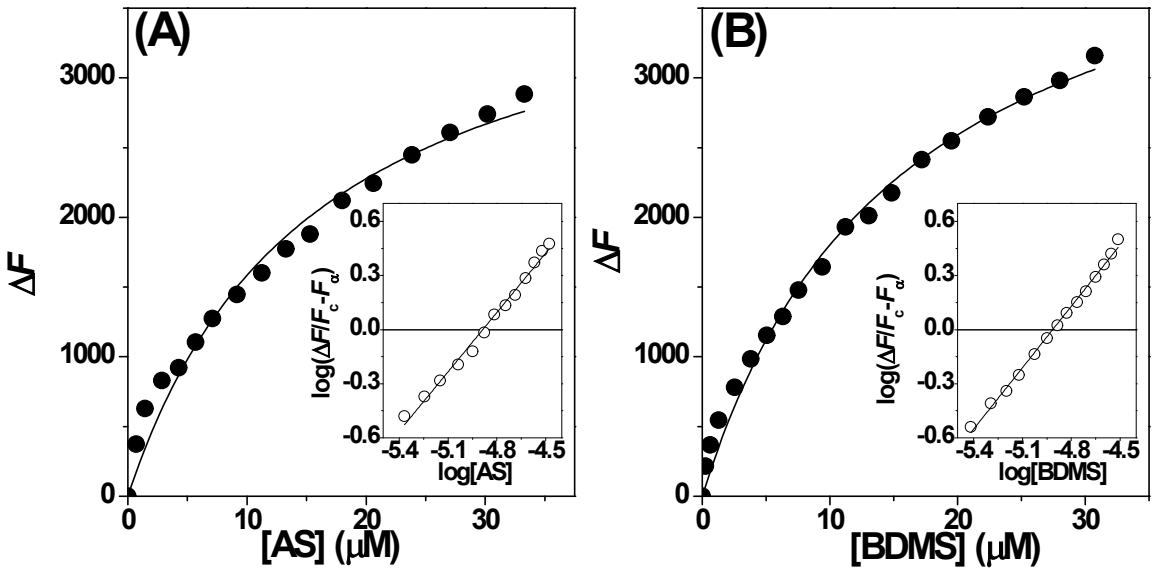


Fig. S2: Binding curve for the interaction of (A) AS and (BDS) with jacalin. The change in fluorescence at 330 nm resulting from the addition of drug to the lectin is plotted as function of the total drug concentration. Inset: Plot of log $[(F_0\text{-}F_c/F_c\text{-}F_\infty)]$ against log [drug]. Binding constant was determined by the equation: $\log (F_0 - F_c/F_c - F_\infty) = \log K_a + n\log[Q]$, where F_∞ is the change in fluorescence intensity at infinite drug concentration, n is the number of binding sites and K_a is the association constant. F_∞ was obtained from the ordinate intercept of the plot of $1/\Delta F$ vs $1/[Q]$.

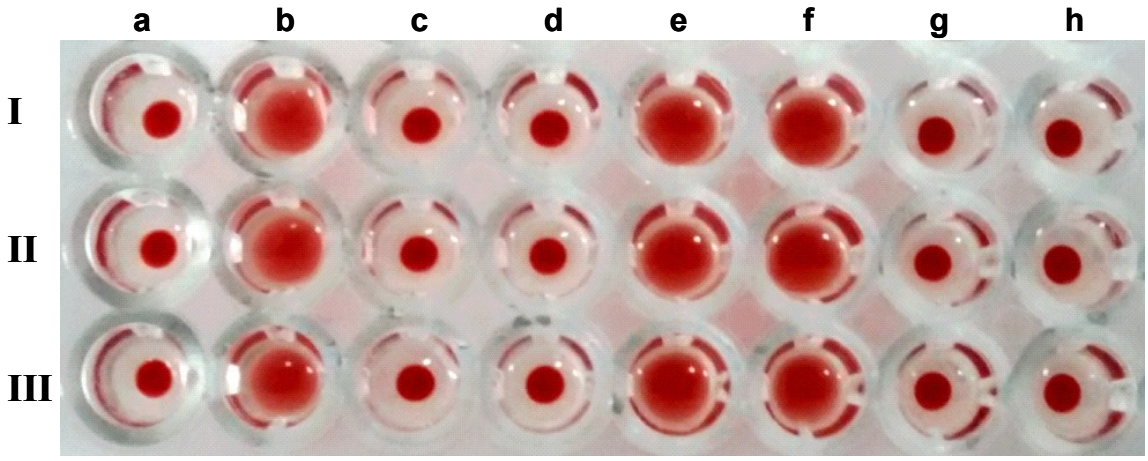


Fig. S3: (A) Hemagglutination assays with human erythrocytes. Lane (a) DMSO, (b) jacalin, (c) DMSO-galactose, (d) jacalin-galactose complex, (e) JAS, (f) JBDS, (g) JAS-galactose complex and (h) JDBS-galactose complex. About 100 µL of 4% human erythrocytes are used in this experiment. Jacalin agglutinates erythrocytes whereas the presence of 100 mM galactose inhibits the agglutination irrespective of the drug presence. As inferred from this data, agglutination was only inhibited by the specific sugar galactose suggesting that AS/BDS and galactose bind at different site. Experiments were performed in triplicate.

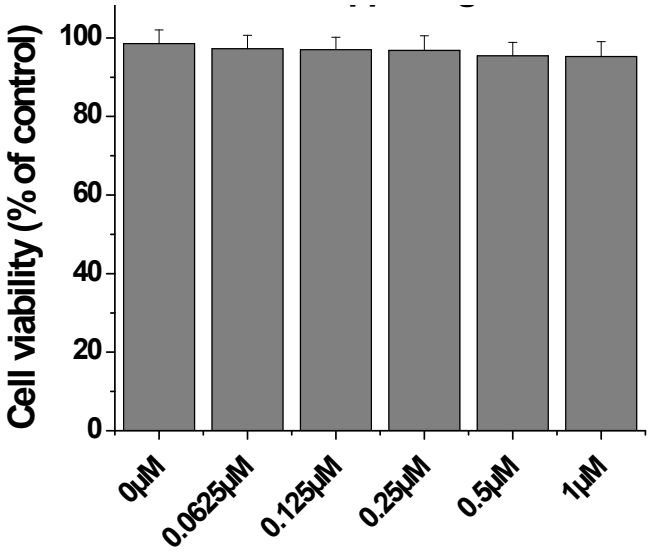


Fig. S4: Jacalin capped AgNPs is unable to induce cytotoxicity in K562 cells. JAgNPs were treated with K562 cells (2×10^4) for 24 hrs and MTT assay were performed to determine the cell viability as described in materials and methods. The experiments were repeated three times, yielding similar results, and data are expressed as means±SEM.

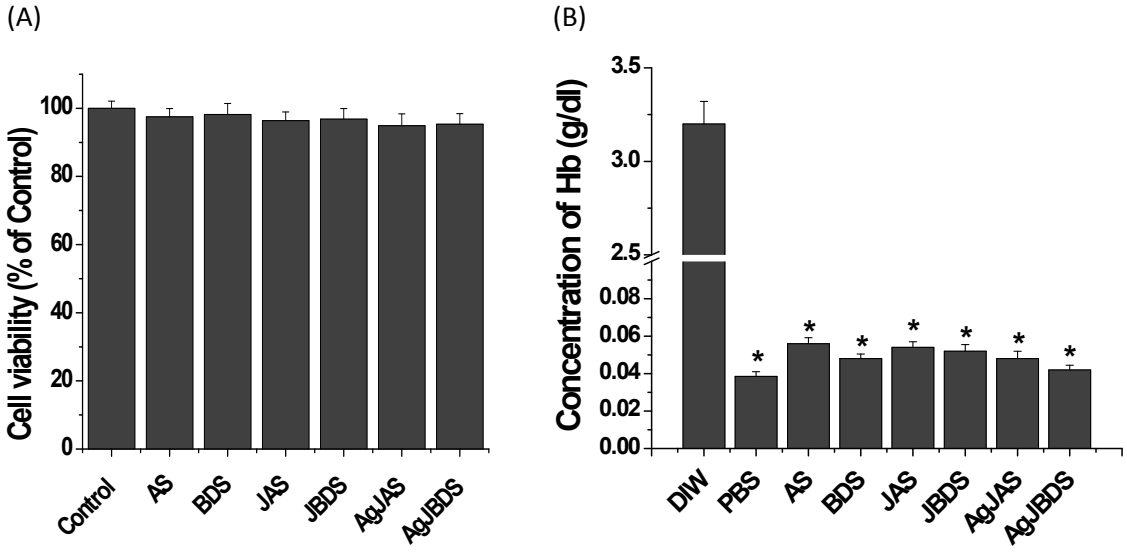


Fig. S5: (A) *In vitro* cell viability of peripheral blood lymphocytes and (B). Hemolytic activity of shokonin derivatives (100 nM). (A) Heparinized human peripheral blood samples were collected from healthy donor. PBL were isolated by standard protocol described elsewhere using Histopaque 1077 (Sigma). Isolated PBL (2×10^6 cells/ml) were remains untreated (Control) or treated with 100 nM shikonin derivatives for 24 h followed by cell viability were measured by MTT assay as described in materials and methods. (B) EDTA-stabilized human blood samples were freshly collected from healthy donor and RBCs were isolated from serum by standard protocol as described earlier. The purified RBCs were diluted to 50 ml with PBS. RBCs were incubated with deionized water (DIW) (positive control), PBS (negative control) and 100nM of different shikonin derivatives at 37°C for 12 h. Finally, cell free supernatants from different groups were collected by centrifugation and concentrations of Hb were measured at 655 nm using microplate reader to determine the hemolytic activity of shikonin derivatives. The experiments were repeated three times, yielding similar results, and data are expressed as means±SEM. *, $p<0.05$ significant difference compared with DIW treated positive control group.

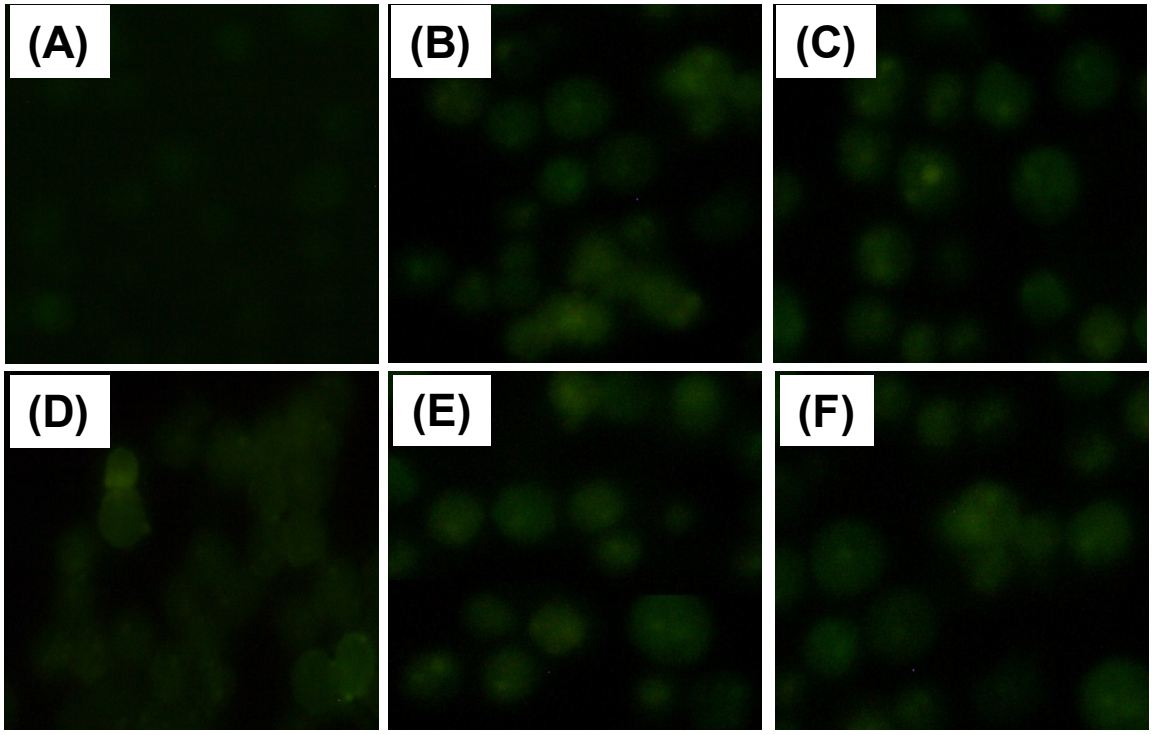


Fig. S6: Qualitative estimation of ROS generation by $DCFH_2$–DA staining using fluorescence microscopy. K562 cells were treated with 100 nM of indicated shikonin derivatives followed by stained with $DCFH_2$–DA as described in material and method. After that, cells were washed with PBS and they were visualized by fluorescence microscopy at an excitation wavelength of 485 nm and an emission wavelength of 520 nm. (A) Untreated control, (B) AS treated, (C) BDS treated, (D) JAgNPs treated (E) JAS treated and (F) JBDS treated K562 cells. From this experiment, we confirm that AS, BDS, JAS and JBDS induced very less amount of ROS generation in K562 cells.

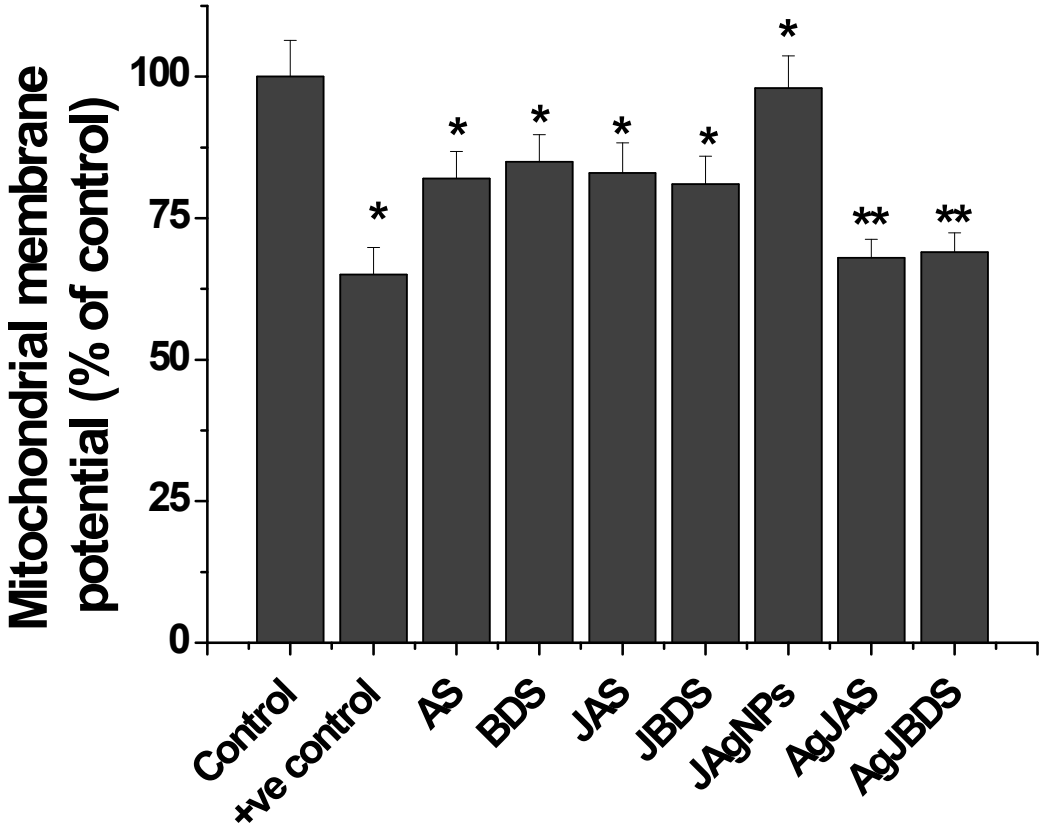


Fig. S7: Estimation of mitochondrial membrane potential (MMP) of shikonin derivatives induced K562 cells. MMP was determined by measuring Rhodamine-123 fluorescence intensity using spectroflurometer as described in materials and methods. The experiments were repeated three times, yielding similar results, and data are expressed as means±SEM. *, $p<0.05$; **, $p<0.01$ significant difference compared with respective untreated control. +ve control is doxorubicin.

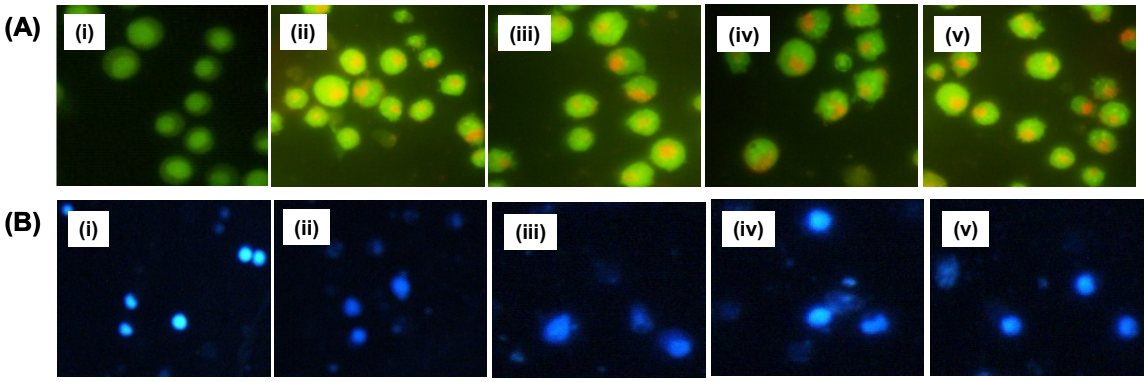


Fig. S8: Qualitative characterization nuclear morphology by (A) EtBr/AO and (B) DAPI staining using fluorescence microscopy. K562 cells were treated with (i) untreated control, (ii) AS, (iii) BDS, (iv) JAS and (v) JBS. After the treatment schedule, K562 cells of different experimental groups were stained with EtBr/AO and DAPI. After that, cells were washed with PBS and they were visualized under fluorescence microscopy at excitation/emission wavelength 490/620 nm and 345/455 nm, respectively.

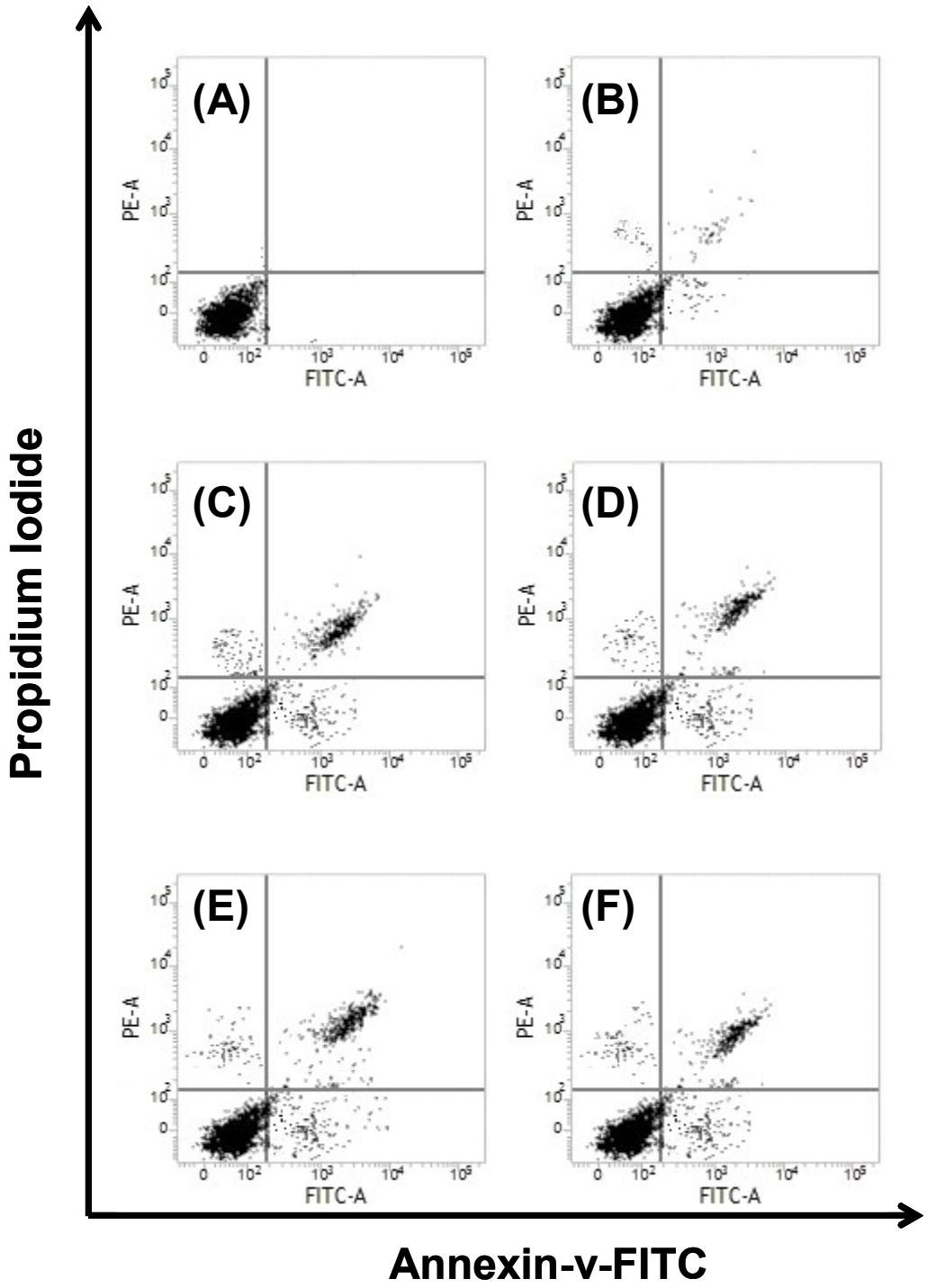


Fig. S9: Shikonin derivatives induced apoptotosis in K562 cells by Annexine-V-FITC/PI dual staining analyzed by FACS. (A) isotype control, (B) untreated control, (C) AS, (D) BDS, (E) JAS and (F) JBDS.

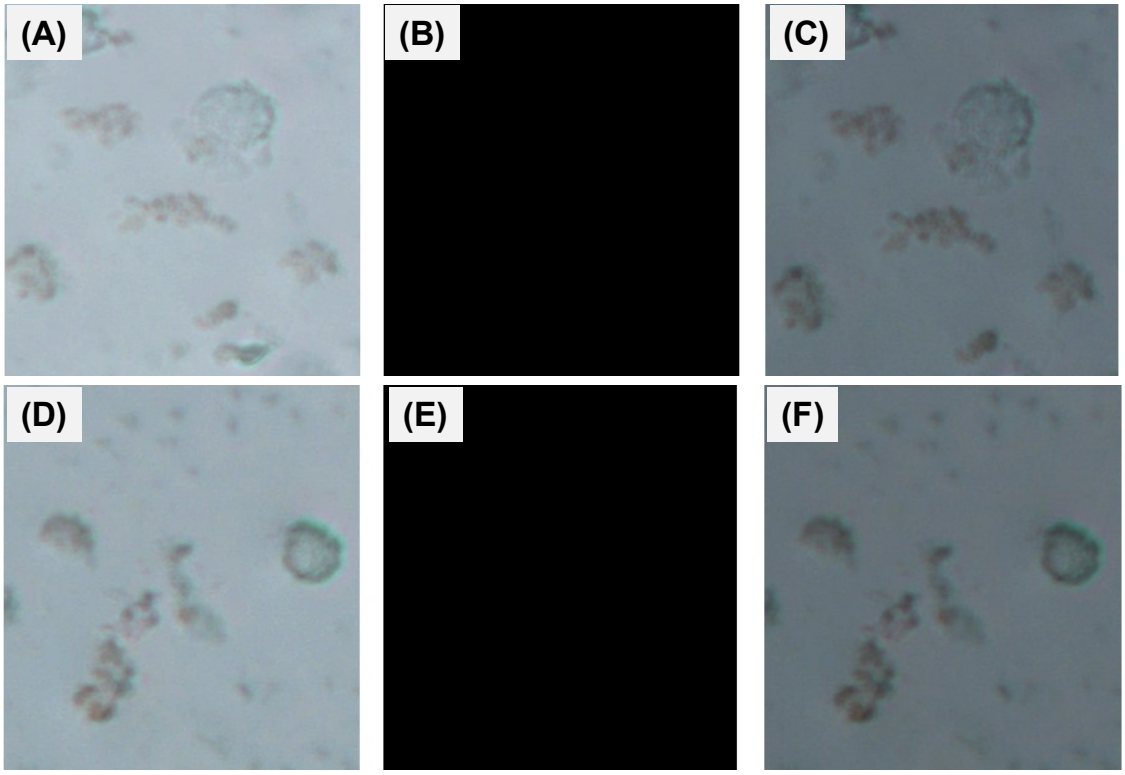


Fig. S10: Intracellular uptake of AgJAS (A-C) and AgJBDS (D-F) in non-tumorigenic HepG2cells by fluorescence imaging. A required amount of cells was treated with Rhodamine B labeled AgJAS/AgJBDS (100nM) for 4 h. Intracellular uptake examined with fluorescence microscope showed no internalization.