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### Abstract

Osteosarcoma shows resistance to chemotherapy and many side effects. The ethyl acetate fraction of ethanolic extract of *Chrysophyllum cainito* was taken in the present work. The extract was characterized for its phenolic content, antioxidant capacity (ferric reducing antioxidant power and 2,2-diphenyl-1-picrylhydrazyl assays), ability to form reactive oxygen species and annexin V assay. The extract contained phenolic content of  $30.1 \pm 0.2$  mg GAE/g extract. Ferric reducing antioxidant power assay and DPPH assay showed  $213.2 \pm 12.4$  mM Fe<sup>2+</sup> equivalent per gram extract and  $85.6 \pm 4.9$  TE/g respectively. For production of reactive oxygen species in the osteosarcoma cells, extract at 50 µg GAE/mL concentration was statistically equivalent to 100 µM H<sub>2</sub>O<sub>2</sub>. EC<sub>50</sub> of extract was calculated to be 133 µg GAE/mL in cell viability studies.

### Introduction

One of most common primary bone sarcoma is osteosarcoma which is a leading cause of cancer death among adolescents, young adults and elder population. It leads to 20% mortality among primary bone cancer and 2.4% among all malignancies in pediatric patients (Huang et al., 2012). Molecular etiology of osteosarcoma is complex and not established (Jones et al., 2012). Chemoresistance is the main cause of treatment failures and is found very common with unclear molecular mechanisms (Huang et al., 2012).

The herbs and their extracts, which are polyphenol rich, have been shown to have effect against hypertension (Ichimura et al., 2006; Oboh et al., 2012), cancer (Ayoub et al., 2010; Hafeez et al., 2006; Martin et al., 2013), Alzheimer's disease (Wang et al., 2008) and cardiovascular diseases.

One of the plant in the category of polyphenolic enriched class is *Chrysophyllum cainito* L., belonging to family Sapotaceae (Lawal et al., 2010), commonly known as star apple (Parker et al., 2010). Its various parts have

great amount of polyphenolic compounds e.g. 387.1 ± 223.2 mg/100 g in fruit pulp, 73.5 ± 52.0 mg/100 g seed (Parker et al., 2010). *C. cainito* plant as such or its extracts has been studied to cure many diseased conditions (Bautista-Banos et al., 2002; Einbond et al., 2004; Lawal et al., 2010; Luo et al., 2002; Meira et al., 2014; Nguessan et al., 2009).

In the present research work, the polyphenolic extract fraction of *C. cainito* fruit was evaluated for anti-cancer activity against osteosarcoma both *in vitro* and *in vivo*.

### Materials and Methods

#### Materials

Gallic acid, vincristine, propidium iodide, formalin, crystal violet, fetal bovine serum (FBS), chloroquine, penicillin, streptomycin, 2', 7'-dichloro-fluorescein diacetate, Folin-Ciocalteu's phenol reagent and annexin V-FITC apoptosis detection kit were purchased from Sigma-Aldrich. Remaining chemicals and reagents utilized in this research work were of analytical grade and were used as such.



### Preparation of polyphenolic fraction of extract

Fruits of *C. cainito* were collected from Hunan Province, China and were supplied by Wu Shi Pharmacy Ltd. Co., China. The identification of the plant was done by Prof. Ding-Xian Han, College of Life Science and Technology, Huazhong University of Science and Technology, China. Fruit pulp was separated from the seeds and cut into small pieces for drying in shades for 120 hours and the dehydrated by lyophilization (freeze drying). Such dried pieces were ground in an electrical grinder and passed through sieve number 5 (4 mm diameter). The polyphenolic fraction of extract was prepared by the method found in literature which was followed with little modifications (Antonio and Brito, 1998; Dhamija et al., 2013). First the alcoholic extract was prepared using coarse, dried powder of fruit of the plant *C. cainito* (250 g) for the hot extraction process (soxhlet) with ethanol (1000 mL) for 20 hours. The concentrated extract was dried by lyophilization. 20 g of ethanolic extract was suspended in water and was fractionated successively and exhaustively with ethyl acetate using separating funnel resulted in extract-fraction of *C. cainito*. The total phenolic content in the extract was estimated by Folin-Ciocalteu method. Gallic acid was taken as standard (standard, 10-150 µg/mL). Total phenolic content was estimated as mg Gallic acid equivalents (GAE)/g of extract (Saeed et al., 2012).

### Determination of total phenol content

The total phenolic content of the extract was estimated by Folin-Ciocalteu method as given in literature (Saeed et al., 2012; Tedesco et al., 2013). In brief, 0.5 mg/mL of extract was taken and heated at 90°C for 10 min. 50 µL of extract was mixed with 50 µL distilled water and 500 µL of complex forming reagent (50:50:1:1 of 2% Na<sub>2</sub>CO<sub>3</sub>, 2% NaOH, 1.5% CuSO<sub>4</sub>, 2.5% sodium potassium tartrate) and incubated at 37°C for 10 min. After incubation 100 µL Folin-Ciocalteu reagent was added and incubated at 37°C for next 30 min. Absorbance was taken at 750 nm. Gallic acid was taken as standard (10-150 µg mL<sup>-1</sup>). Total phenolic content was estimated as mg gallic acid equivalents (GAE)/g of extract.

### Ferric reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay

Reducing or antioxidant capacity of extract was determined by FRAP assay which is based on reduction of ferric tripyridyl triazine (FeIII TPTZ) complex to ferrous form (deep blue color) due to reduction by antioxidants. In particular 100 µL of extract (5 mg/mL) were added to 1 mL of FRAP reagent (TPTZ 1 mM, Fe<sup>3+</sup> 2 mM in acetate buffer of 30 mM pH 3.6) and incubated for 6 min at room temperature. The change in color and intensity can be monitored by taking absorbance at 593 nm in UV spectrophotometer. The results were expressed in millimolar of ferrous sulfate (standard) (Tedesco et al., 2013).

The scavenging/antioxidant capacity of extract for the stable free radical DPPH was monitored. 100 µM DPPH in methanol was mixed with extract solution (0.5 mg extract/mL) by vortexing for 15 sec and incubated for 30 min in the dark at room temperature. Absorbance was taken at 517 nm. The results were expressed in terms of equivalents of trolox (TE) µmol/g of extract (Alomar, 2015).

### U-2 osteosarcoma cell culture

The U-2 osteosarcoma (ATCC HTB-96™) cell line of human osteosarcoma was obtained from ATCC, USA and maintained in Dulbecco's modified Eagle's medium (DMEM) along with fetal bovine serum (10% v/v), L-glutamine (2 mM), penicillin (100 U mL<sup>-1</sup>), streptomycin (100 µg/mL) at 37°C, in a 5% CO<sub>2</sub> humidified atmosphere and harvested at approximately 90% confluence.

### Determination of reactive oxygen species (ROS)

ROS production in the cells was assayed with 2', 7'-dichlorofluorescein diacetate, a non-fluorescent reagent with free permeation to cells (Abdullah et al., 2015). ROS stimulation by treatment with 0, 10, 50, 100 and 200 µg GAE/g extract and H<sub>2</sub>O<sub>2</sub> 10 µM (positive control) for 24 hours was determined in the U-2 osteosarcoma cells. Treated cells were washed with 2',7'-dichlorofluorescein diacetate (10 µM) in phosphate buffer and incubated for 30 min to induce oxidation of 2',7'-dichlorofluorescein diacetate to dichlorofluorescein (DCF) by ROS. The cell lysis was carried out using lysis buffer (50 mM Tris-HCl, 1% Triton X-100, 100 mM NaCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 300 mM sucrose, pH 7.4). At an excitation wavelength of 485 nm the fluorescence (Trilogy, Turner Designs Inc) of suspensions was taken at 530 nm.

### Cell viability assay

Viability experiment protocol was taken from literature (Hsu et al., 2013; Tedesco et al., 2013). In brief, 0.5 mL of cell suspension of 2 × 10<sup>5</sup>/mL was prepared and incubated for 24 hours. Cells were again incubated for 24 hours with lyophilized extract fraction of *C. cainito* of different GAE concentrations (200, 150, 100, 50, 25, 10 and 5 µg GAE/mL in phosphate buffer pH 7.0, 5 mM), taking appropriate control. The cells were separated from medium and washing was done phosphate buffer pH 7.0, 5 mM. Fixing was done by treating the cells with 10% formalin and incubating them for 15 min at room temperature. Formalin treated cells were then treated with crystal violet (0.1% w/v) and was incubated for 30 min at room temperature. Images of cells were taken with inverted microscope in bright field at 400x magnification (Olympus, Japan). After washing, 10% acetic acid was used to solubilize with and absorbance was taken at 590 nm in UV-Vis spectrophotometer.



### Apoptotic assays

Expression of phosphatidylserine on outer layer of plasma membrane has been a marker for apoptosis and was measured using annexin-V (assay protein, having strong affinity for phosphatidylserine) labeled with fluorescein isothiocyanate (FITC, a reporter molecule) (Szliszka et al., 2011; Tedesco et al., 2013). The experiment was carried out as per the manufacturer's protocol. In brief, U-2 osteosarcoma cells ( $1 \times 10^6$  /mL) were allowed to grow in 24-well plates for 48 hours then incubated with extract at different concentrations (10, 50, 100 and 200  $\mu$ g GAE/mL) and standard for 48 hours. The U-2 osteosarcoma cells were washed in saline phosphate buffer and re-suspended in 200  $\mu$ L of binding buffer (10 mM HEPES, pH 7.4; 140 mM NaCl; 2.5 mM  $\text{CaCl}_2$ ). Cells were allowed to incubate with annexin-V FITC (2  $\mu$ L) and propidium iodide (10  $\mu$ L) for 10 min in the dark at room temperature. Then, 400  $\mu$ L of binding buffer was poured to each reaction mixture and mixed properly. Flow cytometer (BD Biosciences, USA) was used for analysis of annexin V-FITC at 530 nm. Low fluorescence debris and necrotic cells, which were permeable to propidium iodide, were omitted out from analysis.

Colorimetric assay kit (Beyotime) for caspase-3 activity determination was utilized. Measurement of p-nitro-anilide (pNA), produced after the caspase-3 activity on the labeled substrate Ac-Asp-Glu-Val-Asp-pNA (DEVD-pNA), was the basis of this assay. U-2 osteosarcoma cells ( $1 \times 10^6$  /mL) were incubated with extract (50, 100, 200 and 300  $\mu$ g GAE/mL) and standard (vincristine 10  $\mu$ g/mL) for 16 hours. Cells were pelleted and lysed with cell lyses buffer (10 mM HEPES, pH 7.4; 2 mM ethylenediamine tetraacetic acid; 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; 5 mM dithiothreitol; 1 mM phenylmethyl sulfonyl fluoride; 10  $\mu$ g/mL pepstatin-A; 10  $\mu$ g/mL aprotinin; 20  $\mu$ g/mL leupeptin) and incubated on ice for 20 min. After 20 min incubation, centrifugation was carried out (10,000x g for 30 min). The supernatant was taken for caspase activity determination and protein estimation. 300  $\mu$ L reaction mixture was containing 10 mM DTT, 5 mM DEVD-pNA (substrate) in phosphate buffer (pH 7, 5 mM) and cellular lysate of 10  $\mu$ g protein. Cell lysate was added in last, mixed and incubated immediately at 37°C for 30 min. Absorbance was taken with an ELISA micro-plate reader (Emax, Molecular Devices) at 405 nm. The activity of caspase-3 was determined by subtraction of appropriate control (untreated) and compared with positive control (vincristine) (Ramamamy et al., 2013).

### Statistical analysis

The results are expressed as the mean  $\pm$  SD obtained from at least triplicate of experiment. Significance was evaluated statistically using analysis of variance i.e. ANOVA (Dunnett's or Tukey's test wherever required).

The difference with p value less than 0.05 was taken as significant.

## Results

The polyphenolic content in ethyl acetate fraction of alcoholic extract of *Chrysophyllum cainito* was found to be  $30.1 \pm 0.18$  mg GAE/g. The antioxidant capacity in the ethyl acetate fraction of alcoholic extract of *C. cainito*, was found to be  $213.2 \pm 12.4$  mM  $\text{Fe}^{2+}$  equivalent/g extract or  $7.1 \pm 0.4$  mM  $\text{Fe}^{2+}$  equivalent/mg GAE and DPPH assay which showed  $85.6 \pm 4.9$  TE/g.

The effect of extract concentration on production of ROS was found to be increased with increase in concentration of extract (Figure 1). The extract at concentration 10  $\mu$ g GAE  $\text{mL}^{-1}$  was showing increase but insignificant in light intensity i.e. ROS concentration in U-2 osteosarcoma cells. While treatment of extract at concentration 50  $\mu$ g GAE/mL and above were showing ROS concentration significantly higher as compared to control i.e. no treatment. In another viewpoint, extract at 50  $\mu$ g GAE/mL concentration was statistically equivalent to positive control i.e. 100  $\mu$ M  $\text{H}_2\text{O}_2$  while higher concentrations of extract were significantly more effective than that of positive control. When the production of ROS with concentrations of 100 and 200  $\mu$ g/mL were compared, the two were found to be statistically same.

In Figure 2, there was clearly observable difference in the growth of the cells between untreated and treated (100 and 200  $\mu$ g GAE/mL). The percentage of viable cells was also determined by taking absorbance of extracted cell associated dye at 590 nm. Results showed that extract caused no cell death at concentration 5 and 10 GAE  $\mu$ g/mL (represented as 'ns' as non significant) taking control (no extract) as 100% cell viability. While the higher concentrations from 25 to 200 GAE  $\mu$ g/mL showed significant (Figure 3) and linear relations with cell death (Figure 4). From the linear relation  $\text{EC}_{50}$  extract was also determined (133 GAE  $\mu$ g/mL).

The effect of extract concentration on apoptotic cell count has been shown in Figure 5. The lowest concentration used for extract (10  $\mu$ g GAE/mL) was not significant as compared to control in terms of annexin V positive cell counts but all the higher concentrations were showing significantly higher number of annexin positive cell counts. In comparison to standard positive control (vincristine 10  $\mu$ g/mL) only extract of 100 and 200  $\mu$ g GAE/mL was statistically equivalent.

Results showed that highest effect as increased caspase specific activity was observed at extract concentration 300  $\mu$ g GAE/mL ( $43.8 \pm 6.6$   $\mu$ mol/min/mg in comparison to  $5.7 \pm 1$   $\mu$ mol/min/mg untreated control i.e. approximately 8-fold) as shown in Figure 6. Moreover, caspase specific activity at this extract

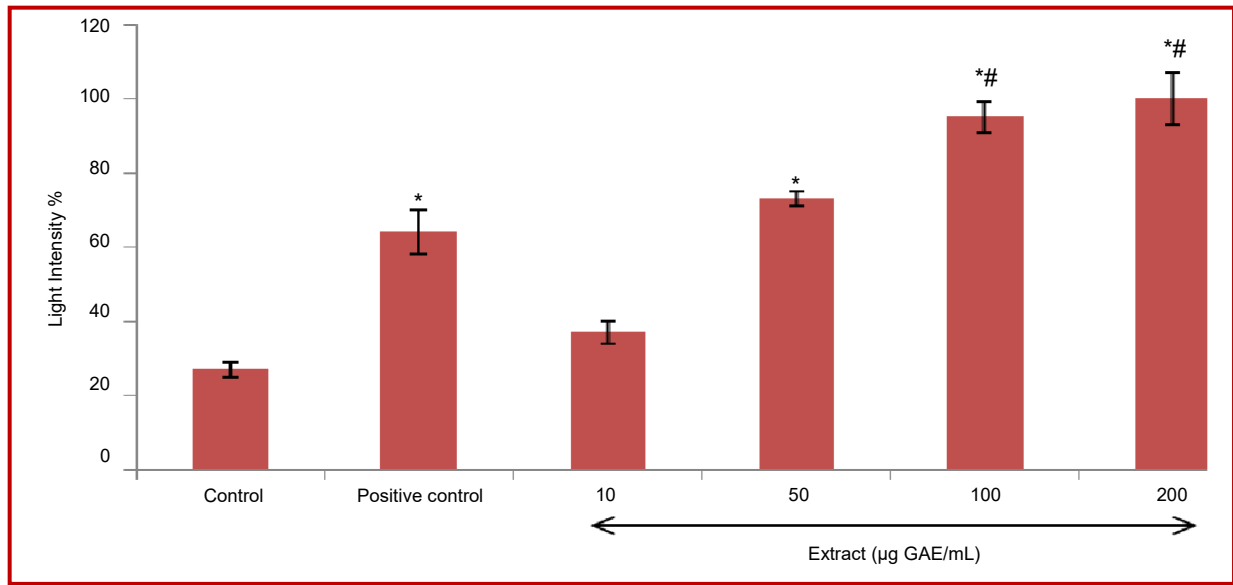


Figure 1: Effect of extract of different GAE concentrations on amount of reactive oxygen species in terms of intensity of fluorescence by DCF taking maximum (extract 200 µg GAE/mL) as 100%. Results represent the mean DCF fluorescence with error bars representing SD from three experiments. Positive control represented the treatment of the cells with 100 µM H<sub>2</sub>O<sub>2</sub>. \*represents significant difference at  $p < 0.05$  from the untreated control and #represents the significance in comparison to standard (H<sub>2</sub>O<sub>2</sub> 100 µM) positive control

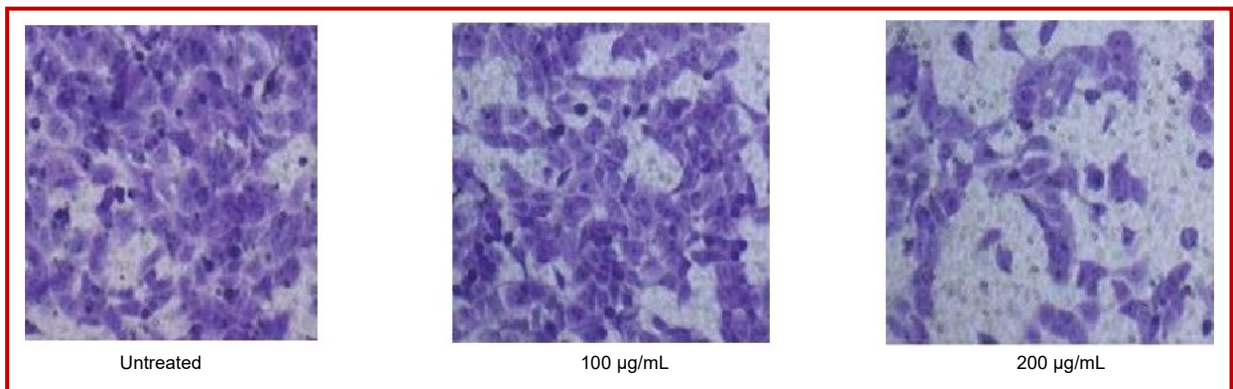


Figure 2: Images of U-2 osteosarcoma cells after 24 hours of untreated cells and treatment with extract of 100 and 200 µg/mL. Images of cells were taken with inverted microscope in bright field at 400x magnification (Olympus, Japan)

concentration i.e. 300 µg GAE/mL was significantly higher ( $43.8 \pm 6.6$  µmol/min/mg) than that of the positive control ( $32.5 \pm 3.5$  µmol/min/mg).

## Discussion

Clinical reports have shown the relation of polyphenolic compounds intake through food with prevention of particular cancers, decreased risk in terms of different cancer types (Arts et al., 2002; Su and Arab, 2002) or a reduced recurrence (Le Marchand et al., 2000). There are many polyphenolic compounds such as gallic acid, isoflavones, stilbenes, tannins and curcuminoids have been shown to have strong chemopreventive properties. Studying the mechanism of action as anticancer of these compound is an

uncovered area of considerable interest (Yar Khan et al., 2012).

The effect of extract concentration on production of ROS which can be correlated with apoptosis. Oxidative stress usually cause the cells to enter apoptosis and ultimately to cell death. In contrast, one research showed the stress caused by H<sub>2</sub>O<sub>2</sub> (an oxidative stress) in presence of mammalian p38α caused up-regulation gene responsible for antioxidant defenses in the cells viz. superoxide dismutase 1 (SOD-1), SOD-2 and catalase which naturally protects the cell from ROS accumulation and leads to cell survival (Gutierrez-Uzquiza et al., 2012). Similarly, antioxidants are thought to protect the cells from oxidative stress (Dhamija et al., 2014; Lowes et al., 2013) but gallic acid, a phenolic compound with antioxidative activity, has been

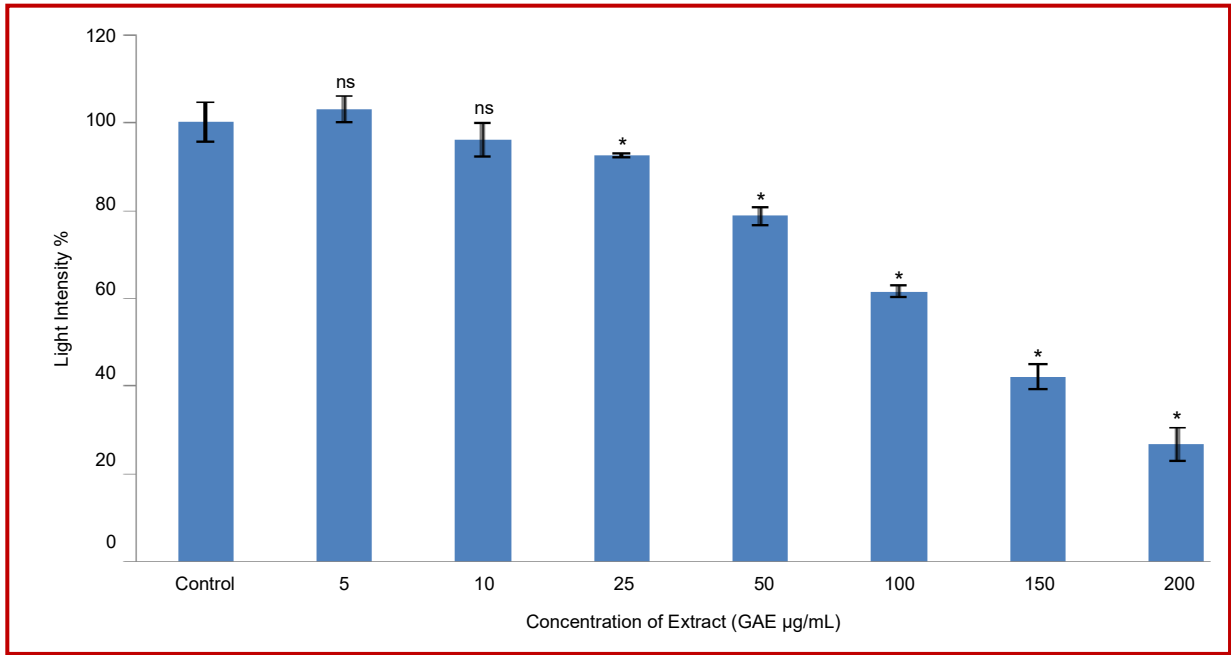


Figure 3: Effect of different concentration of extract in terms of GAE on U-2 osteosarcoma cell viability. The value was average of three samples with standard deviation as error bars. \*represents the significant difference and ns means non-significant compared to control with  $p < 0.05$  using Dunnet multiple comparison test

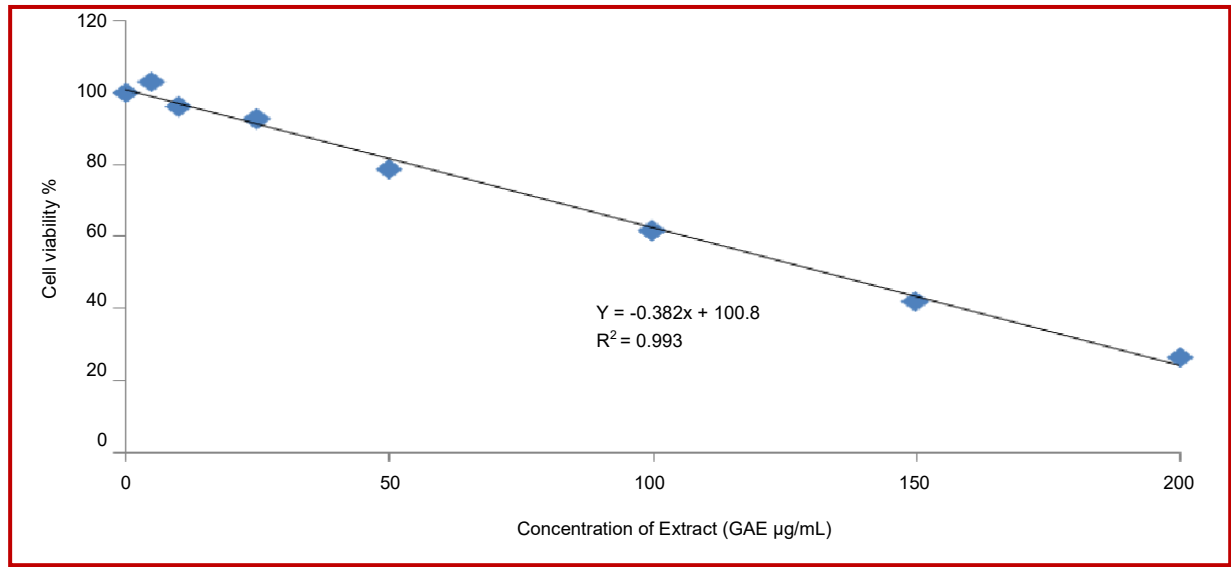


Figure 4: Linear relation between extract concentration in terms of GAE on U-2 osteosarcoma cell viability

reported to cause apoptosis in promyelocytic leukemia cells. This assay of cell death/inhibition was the proof of involvement of reactive oxygen species such as  $H_2O_2$ , superoxide anion in addition to  $Ca^{2+}$  ion and calmodulin-dependent enzymes. Its structure activity relationship studies gave the idea of apoptosis induction in this particular cell line was depending on apoptosis activity was derived from the structure, not from its antioxidative property (Inoue et al., 1994). Similar to cytotoxicity study done in this research, the

cytotoxicity of curcumin, a well-known representative natural polyphenolic compound, was checked at 5, 10, 25, 50, 75, and 100  $\mu M$  concentration and comparison was established between healthy human osteoblast cells and osteosarcoma (MG-63 cell line). As a consequence of curcumin treatment, result showed the 10  $\mu M$  concentration retaining less than 50% viability of osteosarcoma cell compared to the control while healthy osteoblast cells retained at least 80% viability at all concentrations of curcumin (Chang et al., 2014).

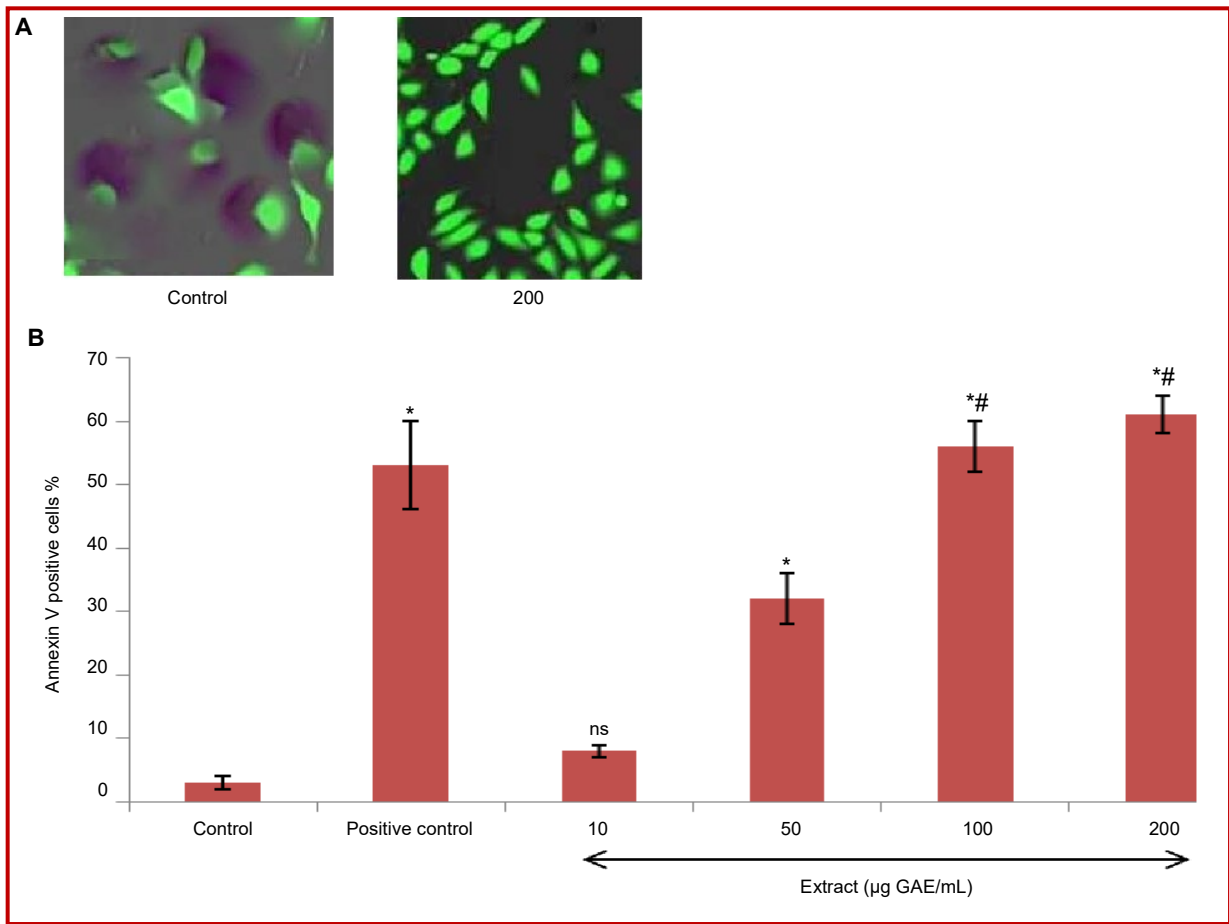


Figure 5: A. Fluorescent image of U-2 osteosarcoma cells. B. Effect of extract of different GAE concentrations on apoptotic cells in terms of annexin V positive cells. Results are represented as the mean of %annexin V positive cells with error bars representing SD from three experiments. Positive control represented the treatment of the cells with 10 µg/mL vincristine. \*represents significant difference and 'ns' represented non significant difference at  $p < 0.05$  from the untreated control and #represents the non significance i.e. equivalence in comparison to standard (vincristine 10 µg/mL) positive control

The phosphatidylserine (PS) translocation to the outer membrane from cytosolic membrane is one of important signature of apoptosis at very early stage. The assay of PS over outer membrane has been used to evaluate the potential drugs or extracts. Its assay exploits the affinity of annexin V which is labeled with FITC and this FITC attached cells cause green fluorescence and separation in flow-cytometer for calculation of cell count which are annexin V positive which means PS positive or apoptotic cells (Tedesco et al., 2013) while the propidium iodide gives red fluorescence and is representative of late apoptotic cells or necrotic cells were omitted from the analysis. It was also noteworthy that the pattern of result of extract on apoptotic cells was similar to that obtained from production of ROS. This relation suggested that apoptosis by ROS is the mechanism used by the compounds in the extract.

Increase in caspase activity has also been one of path line of the cell's pathways to undergo apoptosis. In a study by Tedesco et al. (2013) the caspase activity was

found to be significantly decreased (unexpectedly) at 200 µg lyophilized dealcoholated red wine mL<sup>-1</sup> concentration than lower concentrations. The interpretation behind this unexpected was excessive killing of cells at higher concentrations while optimal time for increase in caspase activity had already been achieved (Tedesco et al., 2013).

## Conclusion

The plant *Chrysophyllum cainito* has a great potential in aurvedic medicines and could be great source of lead novel molecule for allopathic medicines for treatment of osteosarcoma. The significance in each result obtained in this study clearly depicts its candidature for further exploration.

## Conflict of Interest

Authors declare no conflict of interest

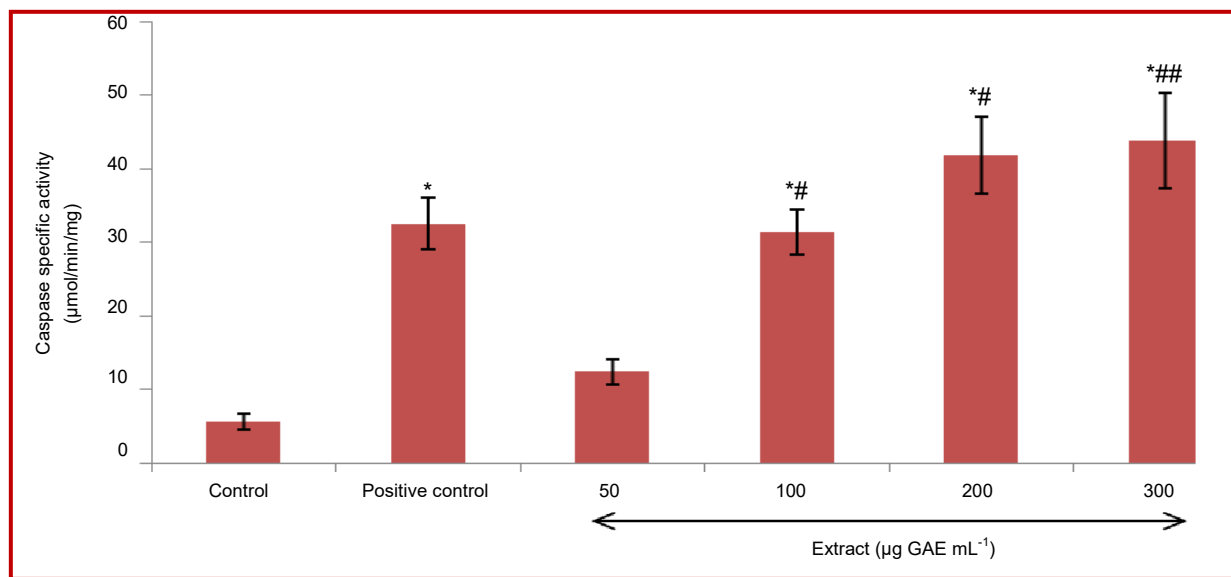


Figure 6: Effect of extract of different GAE concentrations on apoptotic cells in terms of caspase specific activity in cells. Results are represented as the mean of caspase specific activity ( $\mu\text{mol}/\text{min}/\text{mg}$ ) with error bars representing SD from three repeats. Positive control represented the treatment of the cells with  $10 \mu\text{g}/\text{mL}$  vincristine. \*represents significant difference at  $p < 0.05$  from the untreated control and #represents the non significance i.e. equivalence in comparison to standard (vincristine  $10 \mu\text{g}/\text{mL}$ ) positive control while ##represents the significantly higher caspase specific activity than that from standard or positive control

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