

Oleanolic acid from black raisins, *Vitis vinifera* with antioxidant and antiproliferative potentials on HCT 116 colon cancer cell line

Kandasamy Sasikumar¹, Vinay Dubey¹, Asit Ranjan Ghosh^{1*}

¹Department of Integrative Biology, School of Bio Sciences and Technology, VIT University, Vellore, Tamilnadu, India

Vitis vinifera (black raisin) is commonly used in traditional medicine for the treatment of various ailments. In the present study, anti-oxidative and anti-cancer efficacy of oleanolic acid from ethyl acetate fraction of black raisins was evaluated and oleanolic acid was isolated without using of any chromatographic techniques and subjected to spectral assessment using UV-Vis spectrophotometer, ¹H NMR, ¹³C NMR, MS and FT-IR for structural confirmation. Antiproliferative efficacy of oleanolic acid against human colon adenocarcinoma HCT-116 cells was assessed using cell viability assay. The minimum inhibitory concentration (IC₅₀) was determined and found to be 40 µg/mL at 48h incubation. Furthermore, antioxidant property of oleanolic acid was analyzed using DPPH method (IC₅₀ is 61.5 µg/mL) by compared to standard antioxidants ascorbic acid, gallic acid, pyrogallol and butylated hydroxytoluene. Hence, the present study aims to establish the use of oleanolic acid as a potential therapeutic agent against human colon cancer.

Keywords: *Vitis vinifera*. Oleanolic acid. Anti-oxidant. Anti-proliferative.

INTRODUCTION

Black raisin (*Vitis vinifera*) belongs to the Vitaceae family. Many berries make up a cluster or bunch of grapes. Polyphenol is an important phytochemical in the grape which promotes many biological activities and has immense health-promoting benefits (Xia *et al.*, 2010). Grapes are available in the form of fresh fruit, raisins, juice and wine. In India, *V. vinifera* has been used as a traditional medicine for human health over 2000 years ago in 'Darakchasava,' a well-known Indian herbal preparation. This 'Ayurvedic' medicine is prescribed as a cardiogenic (Prasad, Tyagi, 2015) and is also used in the prescriptions for cough, problems in the respiratory tract, catarrh, subacute cases of enlarged liver and spleen, as well as being used in alcohol-based tonics (Aasavs) (Wang, 2014).

The triterpenoids are natural compounds that are widely distributed in the skin and seeds of different edible fruits, such as olives and grapes (Allouche *et al.*, 2009). Oleanolic acid (OA) is a major triterpenoid acid found in the fruits and is also present in both virgin olive oils and red wine (Pensec *et al.*, 2016). The role of triterpenoids in the diet helps in the prevention of certain cancers, including

breast cancer (Patlolla, Rao, 2012). OA also has the potential to be used as an antifungal (Tang *et al.*, 2000), anti-HIV (Khasiwada *et al.*, 2000), diuretic (Alvarez, Maria, Saad, 2002), and anti-inflammatory effects (Singh *et al.*, 1992). However there is little literature currently available with respect to their effects on colon carcinoma cells.

The present study was designed to isolate and purify OA in a limited resource laboratory and investigate its inhibitory effects on human colon carcinoma cell line HCT116 and also as an antioxidant potential.

MATERIAL AND METHODS

General experiments

UV-Vis Spectrophotometric analysis was run on a Shimadzu UV-2401 PC Spectrometer. FT-IR spectrum analysis was performed in KBr pellets on a Perkin Elmer FT-IR spectrum BX Spectrophotometer. A ¹H and ¹³C NMR spectra were recorded on BrukerAvance FT-NMR spectrometer operating at 300 MHz and 100 MHz respectively.

Plant materials

V. vinifera was sourced locally from the Vellore

*Correspondence: A. R. Ghosh. Department of Integrative Biology, School of Bio Science and Technology (SBST), VIT University, Vellore -632014, Tamilnadu, India. E-mail: cidcvit@gmail.com

market. It was deseeded manually using a knife and the peel was dried in an oven at 50 °C for around 48 h and stored at 4 °C.

Extraction and isolation of oleanolic acid

20 g of black raisin (*V. vinifera*) was taken and dried at 50 °C for 48 h, pulverized and extracted with MeOH under reflux (6 h). After removal of the solvent using a rotary evaporator, the thick paste of MeOH extract was obtained. The oleanolic acid was isolated from methanolic extract using modified solvent-solvent fractionation without using any sophisticated chromatographic techniques (Sasikumar, Ghosh, 2017; Shoba, Sasikumar, Sathiavelu, 2018). The methanol extract was then resuspended with Milli Q water and the aqueous insoluble extract was further washed with EtOAc. The washed soluble fraction of EtOAc was collected, dried and stored for further isolation process. The diethyl ether soluble fraction was separated from the EtOAc dried extract. The dried diethyl ether fraction was washed with hexane and the hexane insoluble extract was dissolved with the acetone followed by chloroform. The insoluble content of both the solvents were removed and the soluble fraction of chloroform was completely dried and the same was dissolved in dichloromethane. The solvent was removed under reduced pressure which afforded compound **1** (175mg).

DPPH Radical Scavenging Assay

The radical scavenging activity of OA was measured using the DPPH method (Ghafoor, 2014). In brief, 1 mL solution of antioxidants (0.5 mg/mL DMSO) was mixed with 2 mL of 10 mg/L methanolic solution of DPPH. This solution was then mixed thoroughly and kept at an ambient temperature for 5 min followed by absorbance measurement at 517 nm. The control solution for this test was made by mixing the reagent solution with methanol which did not contain sample or standard. The radical scavenging activity (RSA) was obtained using the following formula:

$$\text{RSA (\%)} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100$$

Cell proliferation assay

HCT-116 cells were procured from the National Centre for Cell Science, Pune, India. The cells were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% heat-inactivated Foetal bovine

serum (FBS), penicillin (10 U/mL), streptomycin (10 µg/mL) and 0.2 mM sodium pyruvate. The cultures were incubated in the presence of 5% CO₂ at 37 °C in humidified atmosphere. HCT-116 cells were seeded in 96-well micro plates at a density of 1×10³ cells/well and incubated overnight. The medium was replaced with serum free DMEM supplemented with oleanolic acid at different concentrations (5-1000 µg/mL). After 48 h of incubation, the cell viability was determined using the colorimetric MTT assay as described earlier (Encalada *et al.*, 2011). 15 µL of MTT solution (stock 5mg/mL) was added to each well and subsequently incubated for 4 h. The formazan crystals were dissolved using DMSO and absorbance was recorded at 570nm. The untreated cells (vehicle alone) were chosen as the negative control. Three independent assays were performed and the % cell viability was calculated.

All experiments were carried out in triplicates and expressed as Mean ± SD. The statistical analysis for each experiment was done by using the one-way ANOVA at significance level <0.05.

RESULTS AND DISCUSSION

The methanolic extract of the black raisins was used for the isolation of OA. The methanolic extract was partitioned with water and ethyl acetate to obtain two distinct fractions. The ethyl acetate fraction was subjected to various solvent-solvent fractionation (Figure 1). Without using any chromatographic technique, compound **1** was isolated and the yield (8.7 mg/g) was obtained. The yield was significantly high when compared to other optimized methods (Ghafoor, 2014).

Compound (1): White powder with slight green pigmentation; mp 302-305 °C; UV (MeOH) λ_{max} (log ε) 235, 282 nm; IR (KBr) ν_{max} 3446 (OH), 2926, 2852, 1687, 1641, 1452, 1388, 1271, 1178, 1083, 1035, 997 cm⁻¹; HREIMS *m/z* 456.7032 (calculated for C₃₀H₄₉O₃, *m/z* 342.348); ¹H NMR(CDCl₃) δ 5.27 (1H, t, H-12), δ 3.18 (1H, dd, *J* = 11.0, 5.0 Hz, H-3), δ 2.85 (1H, dd, *J* = 4.0, 14.0 Hz, H-18), δ 0.93 (3H, s), δ 0.839 (3H, s), δ 0.963 (3H, s), δ 0.80 (3H, s), δ 0.996 (3H, s), δ 1.08 (3H, s), δ 0.964 (3H, s); ¹³C NMR δ 39.16 (C-1), δ 27.44 (C-2), δ 78.31 (C-3), δ 38.43 (C-4), δ 55.36 (C-5), δ 18.1 (C-6), δ 33.5 (C-7), δ 46.23 (C-8), δ 48.11 (C-9), δ 36.77 (C-10), δ 23.12 (C-11), δ 122.25 (C-12), δ 143.79 (C-13), δ 41.49 (C-14), δ 26.47 (C-15), δ 22.66 (C-16), δ 45.85 (C-17), δ 32.16 (C-18), δ 41.33 (C-19), δ 30.20 (C-20), δ 32.41 (C-21), δ 22.57 (C-22), δ 28.79 (C-23), δ 14.9 (C-24), δ 14.48 (C-25), δ 16.32 (C-26), δ 25.0 (C-27), δ 180.4 (C-28), δ 32.62 (C-29) and δ 27.34 (C-30). The FT-IR data

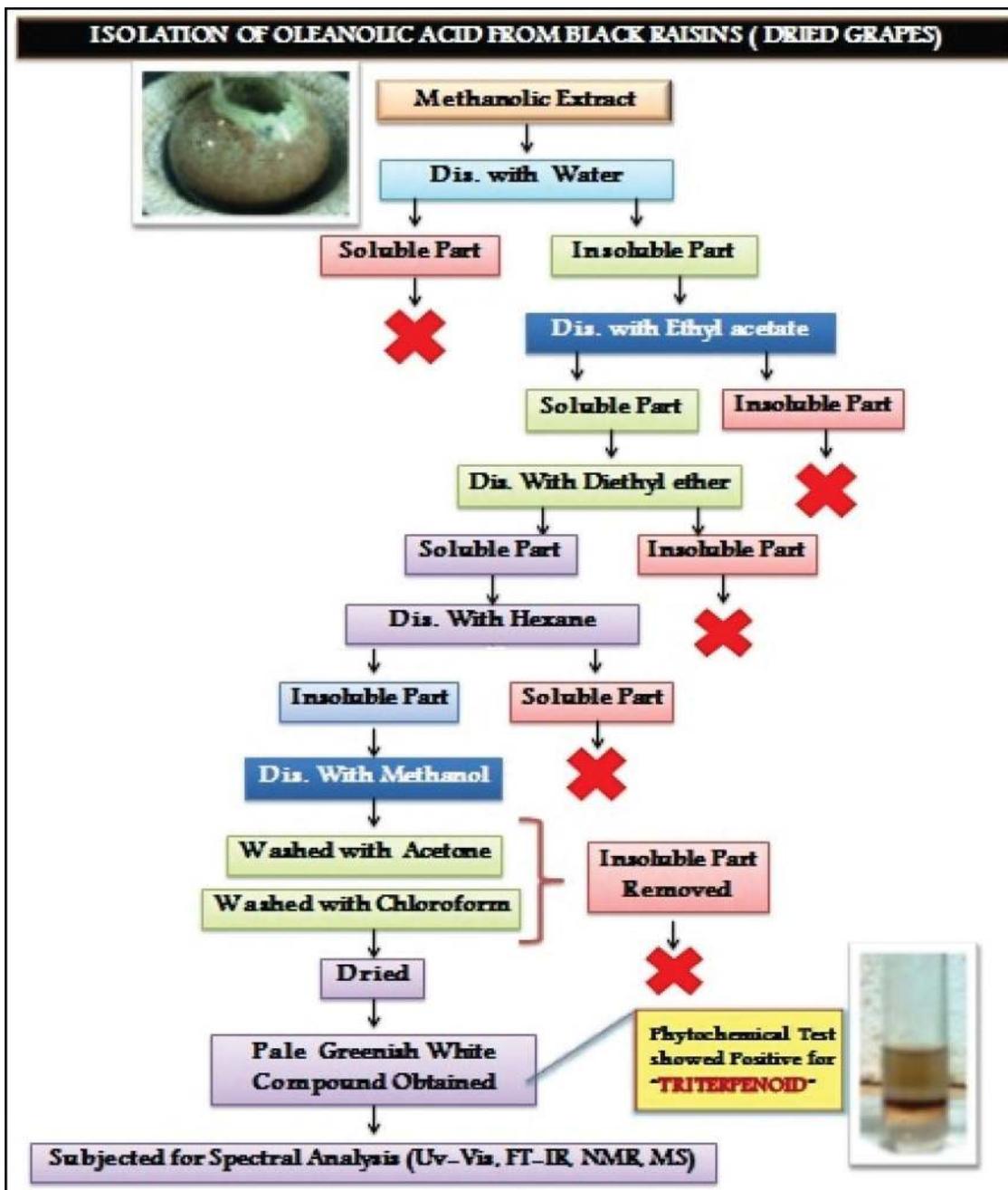


FIGURE 1 - Flow chart of isolation of oleanolic acid from the black raisins.

(Onoja, Ndukwe, 2013) and NMR data of **1** were identical to the published data of 3- β -Hydroxyolean-12-en-18 α -28-oic acid (18 α – Oleanolic acid) (Seebachar *et al.*, 2003; Zhang *et al.*, 2004; Gohari *et al.*, 2009; Maillard, Adewunmi, Hostettmann, 1992).

Structural elucidation of compound (1)

The Infrared (IR) analysis of compound **1** showed a peak at 3446 cm^{-1} , which suggests the presence of a hydroxyl group, while the peak at 1687 cm^{-1} indicates

the presence of a carbonyl group. These two peaks may be due to the presence of carboxylic acid functional group (COOH). The peak at 1388 cm^{-1} may be due to the presence of tri-substituted olefinic group, while the peak at 1462 cm^{-1} may represent the presence of $-\text{CH}_3$ and $-\text{CH}_2-$ signals. The peak at 2926 cm^{-1} showed the presence of C-H stretch for alkanes. This signal suggests that the molecule may be highly saturated. The molecular ion peak was established by HRMS spectrum which showed a molecular ion peak at m/z 456.7032, corresponding to the molecular formula $\text{C}_{30}\text{H}_{48}\text{O}_3$ (calcd. 456.3603).

In the HRMS, the pronounced peak at m/z 418.9207 represents the loss of 38 m. u. corresponding to the COOH group. The prominent baseline peak at m/z 248.6462 represents a typical RDA fragmentation characteristic of Δ^{12} -oleanane type triterpenes with COOH group, at C-17 (Ayatollahi *et al.*, 2011). Another baseline peak at m/z 203.6032 can be attributed to the loss of COOH from the fragment at m/z 248. The $^1\text{H-NMR}$ spectrum of compound **1** shows seven singlets methyl groups at δ_{H} 1.08, 0.83, 0.99, 0.80, 0.96, 0.96 and 0.93 (H-23, 24, 25, 26, 27, 29 and 30). The carbonyl proton at C-3 position resonated at δ 3.18 ($J=11.0, 5.0$ Hz), suggesting its α - and axial configuration and the olefinic proton at C-12 appeared at δ 5.27 as a triplet ($J=3.5$ Hz). A one proton double doublet at δ 2.85 was assigned to H-18 on the basis of its chemical shift as well as the multiplicity pattern reported for H-18 with β -stereochemistry. The $^{13}\text{C-NMR}$ spectrum of compound **1** exhibited thirty carbon peaks. The peak at δ 180.4 may be due to the presence of the carbonyl group assigned to C-28. The two peaks at δ 122.25 and δ -143.79 may be due to the presence of a pair of sp^2 hybridized carbon-carbon atoms assigned to C-12 and C-13. While the seven peaks at δ 28.79, δ 14.9, δ 14.48, δ 16.32, δ 25.0, δ 180.4, δ 32.62 and δ 27.34 can be attributed to the seven methyl groups which are assigned to C-23, C-24, C-25, C-26, C-27, C-29 and C-30 respectively. Therefore compound **1** could be assigned as 3- β -Hydroxyolean-12-en-18 α -28-oic acid (18 α – Oleanolic acid) (Figure 2).

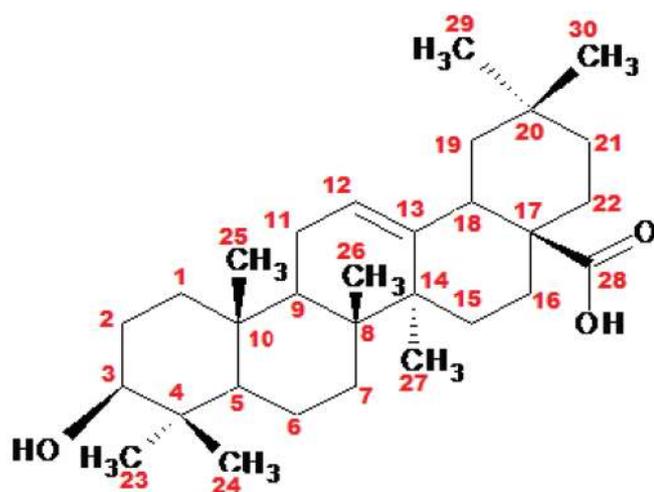


FIGURE 2 - Structure of compound **1** is oleanolic acid (OA).

Free radical scavenging assay

The free radical scavenging activity of oleanolic acid was investigated by DPPH assays and the result exhibited a good free radical inhibited activity when compared to standard antioxidants ascorbic acid (AA), gallic acid

(GA), pyrogallol (PG) and butylated hydroxytoluene (BHT). DPPH involves hydrogen atoms transfer and electrons transfer (Kaviarasan *et al.*, 2007). The result of the present investigation illustrated that oleanolic acid may reduce/inhibit the production of free radicals by donating hydrogen molecules and result in the decolorization in the DPPH assays. Figure 3 demonstrates the abilities of the different antioxidants to scavenge free DPPH radicals. It is evident that oleanolic acid has $88.30 \pm 1.84\%$ DPPH-scavenging activity, which is comparable with that of AA ($93.11 \pm 2.14\%$), GA ($90.85 \pm 1.32\%$), PG ($93.03 \pm 1.12\%$) and BHT ($93.20 \pm 2.33\%$). The minimum inhibitory concentration (IC_{50}) of oleanolic acid was found to be $61.5 \mu\text{g/mL}$.

Anti-proliferative assay

The anti-cancer efficacy of OA was determined using cell-based *in vitro* techniques. The proliferation of HCT-116 was found to be inhibited which was evident from reduced cell viability of OA treated HCT-116 cells when compared with the untreated control cells. This inhibitory effect was concentration dependent and was found to lower the cell viability at the concentration $50 \mu\text{g/mL}$ of OA. The required concentration for the 50% inhibition (IC_{50}) of cells was $40 \mu\text{g/mL}$ (Figure 4). The similar class of pentacyclic triterpenoids like Asiatic acid (AA), betulinic acid (Bet A), boswellic acid (BA), glycyrrhizin, lupeol, ursolic acid (UA) and their derivatives were also reported previously to have a potent anticancer effect (Paduch, Kandeferszerszen, 2014). Asiatic acid (AA) caused about a 50% reduction in the viability of ovarian cancer cells SKOV3 and OVCAR-3 at the concentration of $40 \mu\text{g/mL}$ (Ren *et al.*, 2016). Betulinic acid was active against breast cancer cell lines with an IC_{50} value on MDA-MB-231 ($21.9 \mu\text{M}$) and MDA-MB-468 ($46.0 \mu\text{M}$) cell lines (Weber *et al.*, 2014). It also showed concentration-dependent anti-proliferative activity on paclitaxel-resistant lung H460 cells and IC_{50} was found to be $50 \mu\text{M}$ (Zhan *et al.*, 2018). Glycyrrhizin exhibited a significant inhibition at $100 \mu\text{M}$ on the human lung carcinoma cell line, HCC827 cells after 48 h (Ma *et al.*, 2016) but the cell viability was not affected on the primary mouse mammary epithelial cells at 50, 100 and $200 \mu\text{g/mL}$ (Fu *et al.*, 2014). Lupeol also inhibited the growth of the MCF-7 breast cancer cells with a dose-dependent increase providing an IC_{50} value of the compound as $80 \mu\text{M}$ (Pitchai, Roy, Ignatius, 2014). Oleanolic acid from *Coleus tuberosus*, has an antiproliferative activity through induced apoptosis in the MCF-7 cells and was in a dose-dependent manner. IC_{50} of OA was found at $48.61 \mu\text{g/mL}$ after 48h (Nugraheni *et al.*, 2011). Colon carcinoma HCT15 cells

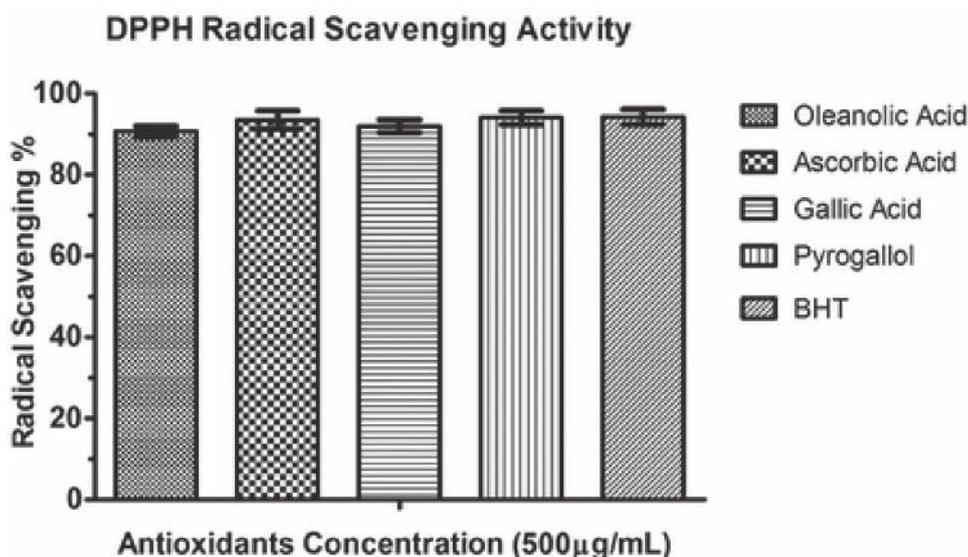


FIGURE 3 - Antioxidant activity (DPPH method) of oleanolic acid.

were treated with various concentrations of ursolic acid (UA) and OA for 48 h. The cell viability was significantly decreased in a dose-dependent manner and IC_{50} of UA and OA was found to be 30 μ M/L and 60 μ M/L respectively (Li, Gai, Yang, 2002). OA administered in a dose and time-dependent manner exhibits a significant decrease in the cell viability of GBC-SD and NOZ (human gallbladder cancer) cell lines. The IC_{50} of the GBC-SD and NOZ cells was found to be 50 μ M/L at 48 hr (Li *et al.*, 2015). The previous report found that OA has similar IC_{50} of 47 μ g/mL against the same cell line HCT 116 (Abdelwahab, Hussein, Kadry, 2015). According to morphological changes in

HCT116 cells, OA has a direct cytotoxic effect which is concurrent with a previous study on it and it has been reported that OA has a cytotoxic activity in HCT15 (Li, Gai, Yang, 2002). Some studies have also supported that oleanane-type triterpenoids had inhibitory effects on DNA polymerase beta and DNA topoisomerases (Wada, Iida, Tanaka, 2001).

CONCLUSION

OA obtained from *V. vinifera* can be established as a suitable drug candidate for anti-colon cancer from

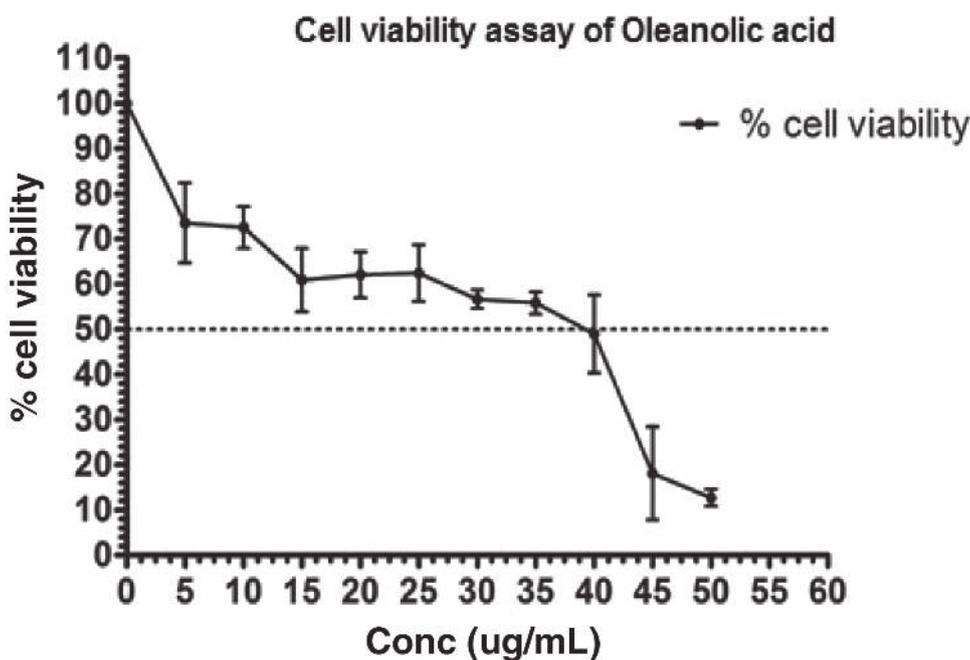


FIGURE 4 - Anti-proliferative efficacy of oleanolic acid against HCT116 colon cancer cells.

based on the inferences drawn from the antioxidant and cytotoxic assays. These findings of the present study show black raisin as a hopeful source to inhibit colorectal cancer. Though OA metabolites are present in low quantity in raisins they possess good anticancer activity due to the synergetic effect of the various metabolites that are present in the black raisins. Further refinements may attribute to elucidate the molecular mechanism of interaction for anti-colon cancer activity of OA. Considering the clinical efficacy and toxicity of numerous anticancer agents which are unknown and uncertain, understanding the fundamental role of herbal extracts has been found to play an essential role in the development of herbal drugs and its use in the treatment of cancer.

Supplementary data: UV- Vis spectra, FT-IR spectra, NMR spectra (^1H and ^{13}C NMR) and HR-ESI-MS spectra for the compound **1** (oleanolic acid).

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