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Original Research Article

Reactive Oxygen Species Scavenging and Anti-Proliferative Potential of Veratric Acid: An *in vitro* Approach

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ABSTRACT

Medicinal plants and their bioactive constituents play a vital role in the prevention of oxidative stress mediated diseases such as cancer and diabetes mellitus. The aim of this study is to investigate the reactive oxygen species (ROS) scavenging and anti-proliferative potential of veratric acid *in vitro*. The study analysed the antioxidant potential of veratric acid using *in vitro* free radical scavenging assays. The anti-proliferative potential of veratric acid was assessed by utilizing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, mitochondrial membrane potential (MMP) changes, intracellular ROS generation measurement and by determining the morphological alterations using Acridine orange/Ethidium bromide (Ao/EtBr) staining in the subline of Keratin-forming tumour cell line HeLa (KB). Veratric acid showed a good antioxidant activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), superoxide and hydroxyl radicals and the effect was found to be much comparable to that of the reference drug, ascorbic acid. Veratric acid significantly reduced the cell viability of KB cells and IC_{50} was 80 µg/ml. Veratric acid reduced the cell viability by generating excess ROS through activation of MMP depolarization and by inducing the apoptotic cell death of KB cells. The *in vitro* antioxidant and antiproliferative effect of veratric acid could be used further to validate its anti-carcinogenic potential using ideal experimental animal models.

Keywords: Veratric acid, Reactive oxygen species, Cell viability, Apoptosis

Introduction

Cancer, a life-threatening pathological disease, is characterized by abnormal and uncontrolled proliferation of cells and is responsible for significant morbidity and mortality of human population. Each year around 19.3 million cancer patients are newly diagnosed worldwide and around 10 million death due to cancer are reported to occur each year worldwide. The annual incidence as well as annual mortality rate are rapidly increasing throughout the world.[1] Cancer arises due to genetic changes in the single cell, which further proliferate to form a mass or clone, termed as tumour. While benign tumour is harmless, the malignant tumour is fatal due to the characteristic features of invasion and metastasis. Moreover, the cancer cells do not obey the normal cell growth regulatory mechanisms. While well differentiated carcinoma cells retain the functional characteristic features of normal cells and thus grow slowly, poorly differentiated cancer cells do not retain such characteristic features and thus grow rapidly.[2]

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A chemical species with unpaired electron is known as free radical. It plays a dual role in the body based on its cellular or circulatory concentrations. Human body generates free radicals continuously as cellular metabolic by-products and they play a dual role in the body based on their cellular or circulatory concentrations. At physiological concentrations, they have a vital role in a number of cellular activities; however, excessive ROS generation could harm the body tissues and thus contribute several clinical disorders and diseases.[3,4]

ROS are oxygen derivatives that include both oxygen radicals (free radicals) and chemical species without unpaired electrons (H_2O_2, hypochlorous acid etc.). Over production of ROS has been documented in diverse pathological disorders such as cancer, diabetes mellitus and neurological diseases.[5] ROS mainly targets biomembrane phospholipids, where it initiates a chain reaction known as lipid peroxidation. ROS accumulation causes damage to macromolecules especially to DNA and is also linked to imbalance in cellular homeostasis, loss of membrane fluidity and integrity.[6,7] An imbalance between antioxidants and lipid peroxidation could result in a condition known as oxidative stress, which has now been considered as non-specific biomarker of several pathological conditions including cancer.[8] To counteract the effect of abnormal lipid peroxidation, human body is endowed with enzymatic and non-enzymatic antioxidants defence mechanism.[9,10]

Reduced glutathione serves as an effective scavenger of ROS including hydroxyl radical and single oxygen. Vitamin E, chain-breaking non-enzymatic antioxidant, terminates the chain reaction of the lipid peroxidation process.[11,12] Superoxide dismutase (SOD) is essential for removing superoxide, whereas glutathione peroxidase (GPx) and catalase scavenge hydrogen peroxide. While catalase scavenge H_2O_2 at higher concentrations, GPx scavenges the H_2O_2 effectively at physiological concentrations. Catalase hydrolyses H_2O_2 into water and

oxygen whereas GPx converts H_2O_2 into water by converting reduced glutathione into oxidized glutathione.[13]

A large number of medicinal plants or their active principles are reported to have anticancer properties.[14] Veratric acid is a derivative of hydrophobic benzoic acid. It is a phenolic phytocompound and found in the *Tabebuia impetiginosa* stem and in the *Sparassis crispa*, a medicinal mushroom.[15] Smaller amounts of it can also be found in fruits and vegetables. Veratric acid, a polyphenolic has diverse pharmacological properties, including anti-inflammatory, antioxidant, anti-microbial, cardioprotective, anti-hypertensive and anti-hyperlipidaemic properties. Veratric acid can treat mouse hepatic ischemia, according to Yu et al.[16] They concluded that veratric acid activated the Nrf2 signalling pathway, protecting the liver from oxidative stress. Saravanakumar and Raja[17] reported that veratric acid exhibited significant antioxidant and anti-hyperlipidaemic potential in hypertensive rats. Raja et al.,[18] demonstrated the anti-hyperlipidaemic and antioxidant potential in atherogenic diet fed Wistar rats. Lee et al., [15] suggested that veratric acid has the ability to prevent UV mediated premature aging of the skin. Choi et al.,[19] demonstrated that veratric acid has the ability to suppress iNOS expression in LPS-stimulated macrophage cells and suggested that the inhibitory consequence was through the regulation of histone acetylation and p13K activation. Veratric acid controls the formation of nitric oxide in LPS-stimulated macrophage cells, according to Choi et al.,[20] Veratric acid has been proven to be effective in protecting mice's acute lung injury caused by lipopolysaccharide by Ran et al.,[21] and suggested that the protective effect was due to its inhibitory effect on NFk-B expression to attenuate inflammatory injury. Palko-Labuz et al.,[22] pointed out that conjugation of veratric acid with phospholipid significantly increased its anticancer potential in melanoma cells. The free radical scavenging potential of veratric acid in KB cells has been studied in vitro using specific colorimetric assays in this investigation. The molecular structure of veratric acid is given in Figure 1.

KB cell line was thought to have been developed from a throat cancer called a human oral epidermal carcinoma of the nasopharynx. KB cells produce keratin and exhibit the morphology of epithelial cells. It has been pointed out that KB cell line has the marker chromosome of HeLa cells. Through DNA fingerprinting, it has been identified that KB cells have HeLa cell contamination and is now recognized as an inbred of the keratin-forming tumour cell line HeLa. This cell line is utilized as a model cancer cells to evaluate the anti-cell proliferative effect of several bioactive phytoconstituents.[23] Veratric acid's *in vitro* anti-proliferative activity on KB cells was assessed in the current investigation. Veratric acid's anti-proliferative activity and cytotoxic efficacy on KB cells was also assessed in vitro in this investigation.

The antiproliferative effect of veratric acid has not been evaluated so far in KB cells and thus the current investigation evaluated the effectiveness of veratric acid under *in vitro* conditions. The free radical scavenging potential of veratric acid was evaluated using specific colorimetric assays to reveal its antioxidant efficacy.

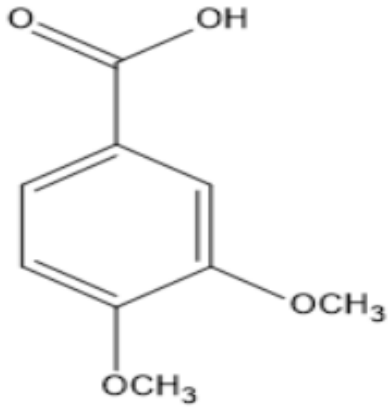

Figure 1: Structure of veratric acid

Materials and Methods

Chemicals

Foetal bovine serum, Dulbecco's Modified Eagle Medium, acridine orange and ethidium bromide stains, 2,7-diacetyldichlorofluorescein, Rhodamine-123, penicillin and streptomycin antibiotics, and other biochemical reagents such as phenazine methosulphate, ascorbic acid, potassium ferricyanide, ferric chloride, glacial acetic acid, hydrogen peroxide, and ethanol were purchased from Himedia laboratories, Mumbai, India. Veratric acid was acquired from Sigma-Aldrich, Pvt. Ltd. India. The analytical grade chemicals were purchased from Fisher Inorganic and Aromatic Limited in Chennai and S.D. Fine Chemical in Mumbai.

DPPH radical scavenging activity

Veratric acid's capacity in scavenging DPPH radicals was determined using the Blois[24] method as a reduction in absorbance at 517 nm. Veratric acid was added to methanolic DPPH solution at different doses (10–50 µg/ml), and the optical density was read at 517 nm after keeping at 30 minutes in the dark. Methanolic DPPH solution without veratric acid served as control and ascorbic acid as a standard reference drug. The following formula was used to examine the DPPH scavenging effect of veratric acid.

$$\frac{\text{Optical density of control} - \text{Optical density of test}}{\text{Optical density of control}} \times 100$$

ABTS radical scavenging assay

Veratric acid's overall antioxidant capacity was assessed using the ABTS radical scavenging method (Miller *et al*.,[25]). The ABTS radicals generated during the reaction between ABTS and potassium persulphate was determined in the presence of veratric acid (10-50 µg/ml) and the obtained results were compared with ascorbic acid measured at 734 nm after five minutes of incubation.

Hydroxyl radical scavenging assay

Veratric acid's capacity to eliminate hydroxyl radicals was determined using the Halliwell *et al*.,[26] method. This method is based on the capacity of the hydroxyl radical to break down deoxyribose into products that forms a pink colour by reacting with TBA was measured at 532 nm.

Superoxide anion scavenging activity

The Nishikimi et al.,[27] method was modified to determine the superoxide anion radical scavenging capacity of veratric acid. The assay relies on phenazine methosulfate-nicotinamide adenine dinucleotide coupling process to produce superoxide anions that forms purple formazan by reducing nitroblue tetrazolium, which was detected spectrophotometrically at 560 nm.

Cell culture

The KB cancer cell line was procured from National Centre for Cell Science, Pune, India. The KB cells were placed in 75 cm^2 tissue culture flasks containing a Dulbecco's Modified Eagle Medium supplemented with foetal bovine serum, glutamine, and penicillin-streptomycin and cultured at 37 °C in a humidified, 5% CO_2 atmosphere.

Determination of effective cytotoxic dose of veratric acid

According to Mosmann[28] procedure, cytotoxic efficacy of veratric acid (20-200 µg/ml) was evaluated on KB cells using MTT assay. The range of veratric acid concentration (20-200 µg/ml) was fixed after standardization of the dose. MTT assay quantifies the amount of mitochondrial succinate dehydrogenase or mitochondrial reductase that reduces yellow colored MTT to dark purple coloured insoluble formazan product which was measured at 590 nm.

Determination of ROS generation

The method of Rastogi et al.,[29] measures the intracellular ROS levels. Briefly, KB cells ($1x10^5$ cells/ml) were treated for 24 hours with veratric acid at the IC_{50} concentration (80 µg/ml). A known volume of 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) was added and incubated for

30 minutes at 37°C. Using a blue filter and excitation (480 nm) and emission (530 nm) wavelengths, the cells were examined under a Nikon fluorescent microscope.

Analysis of mitochondrial transmembrane potential (Δψm)

ΔΨm measurement was done in accordance with Scaduto and Grotyohann's procedure.[30] Veratric acid at the IC_{50} concentration (80 µg/ml) was treated with the KB cells ($1x10^6$ cells/m) in a 6-well plate for 24 hours and subsequently stained using Rh-123 dye. It was then incubated in a CO_2 incubator for 30 minutes. The alterations in MMP of untreated and treated KB cells were then studied under a blue filter on a fluorescence microscope.

Apoptotic morphological changes assessment by AO/EtBr staining

According to Baski et al.,[31] method, apoptotic index was carried out using a staining technique that included AO and EtBr. A 1:1 ratio of AO/EtBr was used to stain the KB cells after they had been cultured in 6 well plates ($1x10^5$ cells) for a day with an IC_{50} concentration of veratric acid (80µg/ml). The cells were then trypsinized, fixed in methanol: glacial acetic acid for 30 minutes at room temperature, and washed in phosphate buffer saline (PBS). Following an immediate PBS wash, stained cells were examined using a fluorescence microscope under a 40x blue filter. The number of cells exhibiting the signs of apoptotic cell death was analysed.

Statistical analysis

The mean ± SD was used to express the values. ANOVA followed by DMRT was used to assess the statistical significance between the groups. A value of $p < 0.05$ was considered to have statistical significance.

Results and Discussion

Any compounds that have a potent antioxidant effect are considered to have a wide therapeutic efficacy against several pathological disorders including carcinogenesis. Worldwide researchers are therefore seeking good antioxidant principles from the natural resources. The antioxidant can be evaluated using a spectrum of *in vitro* free radical scavenging assays (DPPH, ABTS, superoxide and hydroxyl radical scavenging assays). The effect of veratric acid was evaluated using the above-mentioned assays in this study.

Figure 2 shows veratric acid's DPPH radical scavenging activity. IC_{50} value of veratric acid. (38.1 µg/ml) was nearer to ascorbic acid's IC_{50} value (29.3 µg/ml). The effect of veratric acid on DPPH was found to be dose dependant. DPPH assay is widely utilized to examine medicinal plants or their active constituents' antioxidant capacity (Baldisserotto *et al*.,[32]). This method is based on the ability of the test compound to reduce DPPH into diphenyl hydradizine (violet to yellow colour). The present study observed that veratric acid had a significant potential to scavenge DPPH radical and the effect was found to be dose dependent. The present findings also revealed the ability of veratric acid to counteract the effects of DPPH radicals by donating or transferring the hydrogen atom or electrons. The effect of veratric acid was also much nearer to that of ascorbic acid value.

Figure 3 shows veratric acid's capacity to scavenge ABTS radicals. Veratric acid (24.5 µg/ml) significantly scavenged ABTS radical. Veratric acid's IC_{50} value was comparable to that of ascorbic acid, a reference antioxidant (24.3 µg/ml). Veratric acid was revealed to have a dose-dependent impact on ABTS radical scavenging. It is possible to assess the test compound's overall antioxidant effectiveness using the ABTS radical scavenging assay.[33] This approach involved measuring the test substance's capacity in scavenging the ABTS radicals produced during potassium persulfate and ABTS salt interaction. In comparison to ascorbic acid, the present investigation demonstrated a significant ABTS radical scavenging potential, and the effect was assessed to be dose dependent.

Veratric acid's hydroxyl radical scavenging ability is shown in Figure 4. Veratric acid has potent hydroxyl radical scavenging effect with the IC_{50} value (34.6 µg/ml), which was much comparable with ascorbic acid IC_{50} value (33.2 µg/ml). The effect of veratric acid on hydroxyl radical scavenging was found to be dose dependant. Hydroxyl radical has been regarded as one of the potent ROS and can cause severe damage to protein structure. It can also serve as a promoter of lipid peroxidation by interacting with membrane phospholipids.[34-36] Over production of hydroxyl radicals has been implicated in the abnormal or altered membrane permeability and fluidity.[37,38] In the present study, veratric acid effectively scavenged hydroxyl radicals and the scavenging effect was found to be dose dependant as well as comparable to the scavenging effect of ascorbic acid. The current research thus explores veratric acid's ability of elimination of hydroxyl radicals under *in vitro* conditions.

Figure 5 shows superoxide radical scavenging effect of veratric acid. Superoxide radicals were effectively scavenged by veratric acid in a dose dependent manner. IC_{50} value of veratric acid (25.1 µg/ml) showed potent free radical scavenging activity, which was much similar to the standard ascorbic acid IC_{50} value (23.3 µg/ml). Superoxide radicals and hydroxyl radicals, the harmful ROS can produce potent oxidative damage to DNA, proteins and lipids. The test compounds ability to scavenge superoxide radical was examined through formation of NBT using PMS/NADH system.[34]

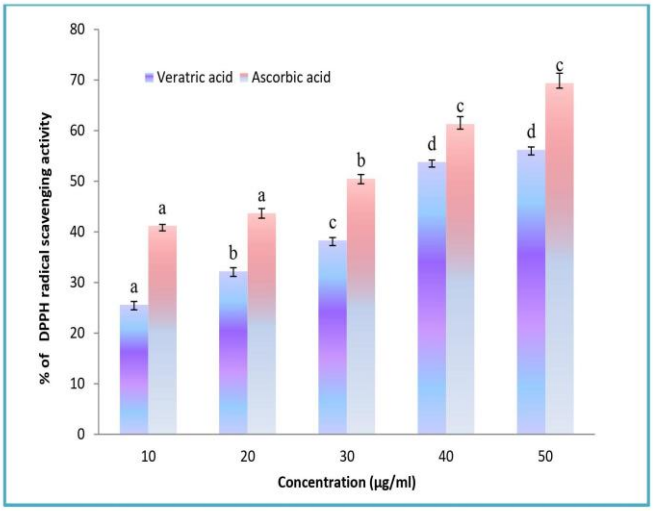

Figure 2: DPPH scavenging potential of veratric acid. Data represents mean ± SD for three samples (n=3). Two different superscript letters in the increasing concentrations of the test samples indicate the statistical significant ($p<0.05$).

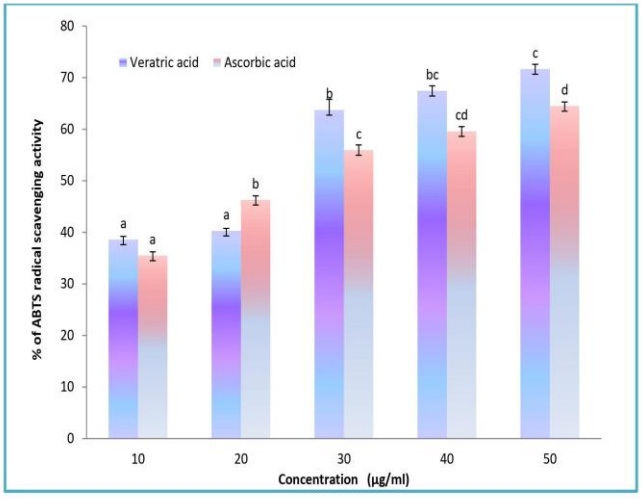

Figure 3: ABTS scavenging potential of veratric acid. Data represent mean ± SD for three samples (n=3). Two different superscript letters in the increasing concentrations of the test samples indicate the statistical significant ($p<0.05$).

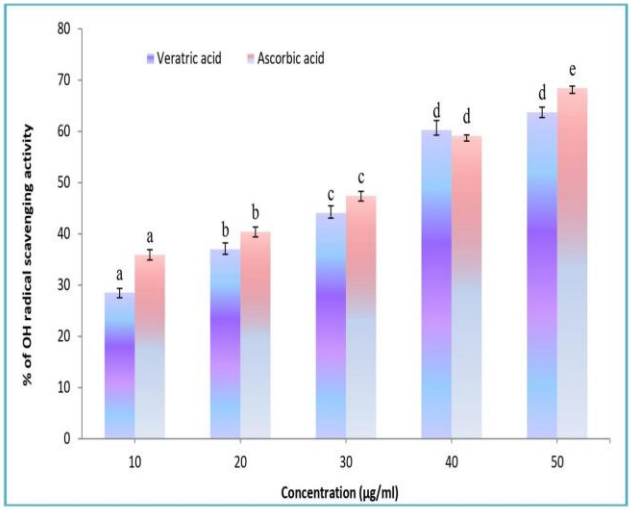

Figure 4: Hydroxyl radical scavenging potential of veratric acid. Data represent mean ± SD for three samples (n=3). Two different superscript letters in the increasing concentrations of the test samples indicate the statistical significant ($p<0.05$).

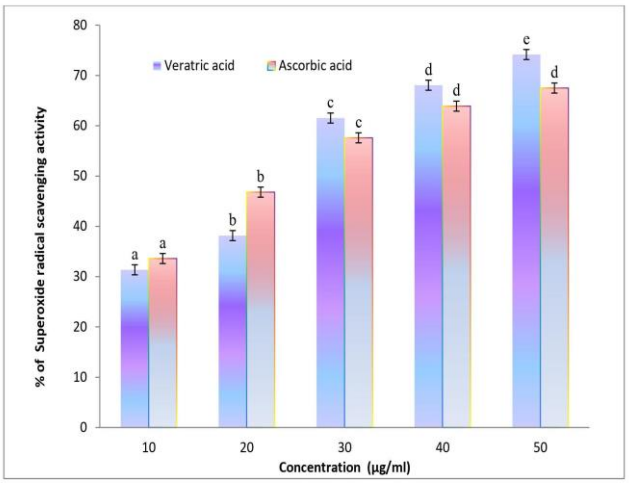

Figure 5: Superoxide radical scavenging potential veratric acid. Data represent mean ± SD for three samples (n=3). Two different superscript letters in the increasing concentrations of the test samples indicate the statistical significant ($p<0.05$).

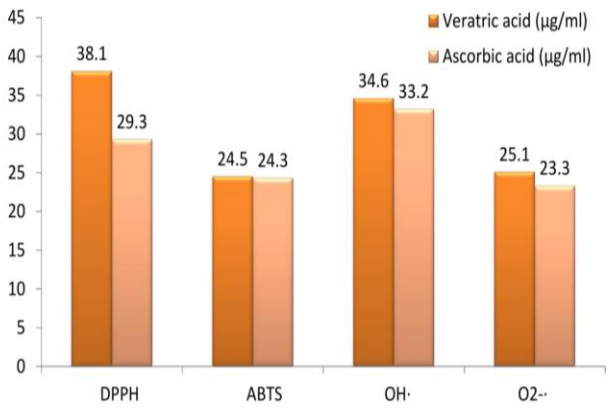

Figure 6: IC_{50} values for the different radicals tested

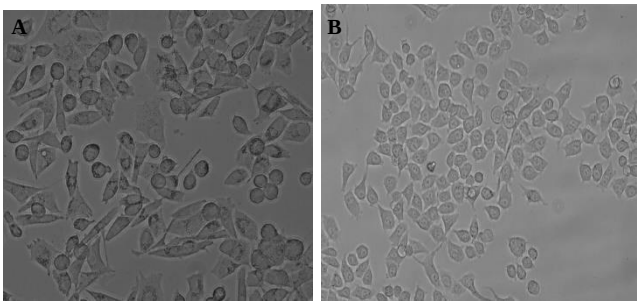

Figure 7: Morphology of untreated KB cells (A) and veratric acid treated KB cells (B). Untreated KB cells showed uniform monolayer. Veratric acid treated KB cells exhibited severe morphological changes and reduced cell survival.

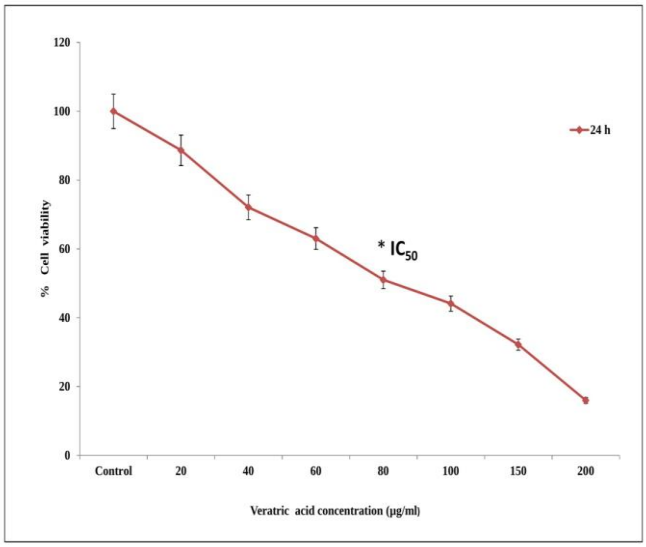

Figure 8: Effect of veratric acid on proliferation of KB cells (MTT Assay). Data represent mean ± SD for three samples (n=3). Two different superscript letters in the increasing concentrations of the test samples indicate the statistical significant ($p<0.05$).

The present study noticed an appreciable superoxide scavenging potential of veratric acid and the effect was found to be dose-dependent. The superoxide scavenging potential of veratric acid was much closer to that of the standard drug ascorbic acid values.

Figure 6 shows IC_{50} values of veratric acid for the various radicals tested in the assay. The obtained results were compared with ascorbic acid. Antioxidant effect of veratric acid at IC_{50} concentrations was nearer to that of ascorbic acid.

Figures 7 and 8 show the morphology of KB cells and the cytotoxic effect of veratric acid on KB cells respectively for 24 hour incubation. Veratric acid (20–200 µg/ml) exhibited antiproliferative effect in dose dependent manner. The IC_{50} of veratric acid was observed at 80 µg/ml for 24 h incubation. The current study assessed the anti-proliferative effects of veratric acid against KB cells *in vitro* in addition to its potential to eliminate free radicals. Using MTT assay, the present study showed the anti-cell proliferative potential of veratric acid against KB cells *in vitro*. Veratric acid markedly decreased the cell viability of KB cells, in a dose dependant manner and IC_{50} was found to be at 80 µg/ml. The production of ROS in both KB cells and veratric acid treated KB cells for 24 hr incubation is shown in Figure 9. The IC_{50} value of veratric acid (80 µg/ml) showed the highest ROS production. The percentage of fluorescence intensity for ROS generation is given in Figure 10. An increase in fluorescence intensity was noticed in the veratric acid treated KB cells. The IC_{50} concentration was further utilized to study the ability of veratric acid on ROS generation, changes in MMP and apoptosis

induced morphological changes in KB cells. Excessive ROS production was noticed in the veratric acid treated KB cells as evidenced by intensity in green fluorescence due to oxidation of H_2DCF (2,7 dichloro dihydro fluorescein to DCF (2,7dichloro fluorescein) by ROS.

Veratric acid treatment caused significant changes in MMP in KB cells (Figure 11). Green fluorescence was found in cancer cells with a high MMP. The percentage of fluorescence intensity for MMP was observed to be decreased at IC_{50} concentration of veratric acid treated KB cells (80 µg/ml) for 24 h as compared to untreated KB cells (figure 12). Veratric acid treated KB cells showed a reduction in MMP as evidenced by decreased green fluorescence. Rhodamine fluorescence was used as an end point to assess the changes in MMP. Accumulation of fluorescence in KB cells and decreased fluorescence in veratric acid treated KB cells indicates the membrane polarization and membrane depolarization respectively, as Rhodamine accumulates in the membrane of live cells.

Figure 13 depicts enhanced apoptotic cell deaths in KB cells following treatment with veratric acid at the IC_{50} concentration (80µg/ml) for 24 hours. The colour of the DNA-binding dyes in each cell, Ao/EtBr stain, was used to identify the modifications. Bright green dots were visible in live cells. Veratric acid was administered to KB cells at the IC_{50} concentration for 24 hours. This resulted in an increase in apoptotic cell deaths (figure 14). The fluorescent DNA binding dyes, acridine orange and ethidium bromide can be used to measure cell membrane integrity and apoptotic index. All cell types can be penetrated by acridine orange, which gives them a green appearance. However, only in injured or cells that have lost the integrity of their membrane can ethidium bromide stain the nucleus red in colour. Thus, it is possible to distinguish between above cells (typical green nucleus), early apoptotic cells with chromatin condensation (brilliant green nucleus) and late apoptotic cells with broken chromatin, also known as necrotic or dead cells (orange nucleus). In the current investigation, KB cells treated with veratric acid displayed brilliant green nuclei and orange chromatin, demonstrating the substance's capacity to cause apoptotic cell death in KB cells

The present study thus explored the antioxidant and anti-proliferative potential of veratric acid in KB cells using specific *in vitro* free radical scavenging assays and *in vitro* cell proliferative assays.

Conclusion

Veratric acid explored a good antioxidant efficacy against DPPH, ABTS, superoxide and hydroxyl radicals and the antioxidant effect was found to be much closer to that of the reference drug, ascorbic acid. Veratric acid showed a substantial anti-proliferative capability in the current investigation, which was demonstrated by reduced cell viability, excessive ROS production, decreased MMP, and enhanced apoptotic cell death in veratric acid treated KB cells.

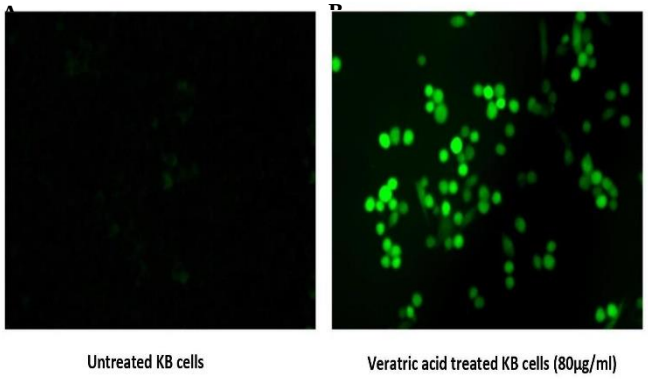

Figure 9: Efficacy of veratric acid on ROS generation in KB cells (A) and veratric acid treated KB cells (B) by DCFH-DA staining. Veratric acid treated KB cells exhibited bright green fluorescence due to excessive generation of ROS.

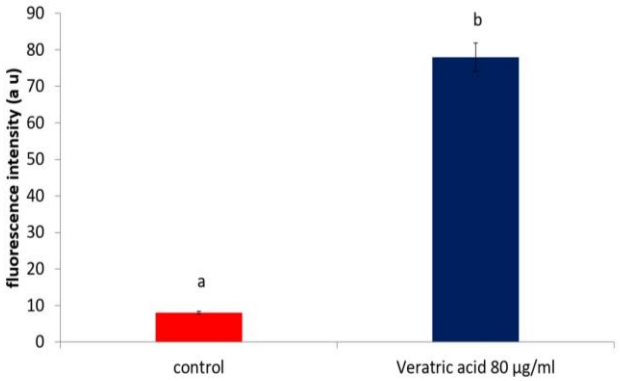

Figure 10: Percentage fluorescence intensity for ROS generation in KB cells and veratric acid treated KB cells. Data represent mean ± SD for three samples (n=3). Two different superscript letters in the increasing concentrations of the test samples indicate the statistical significant ($p<0.05$)

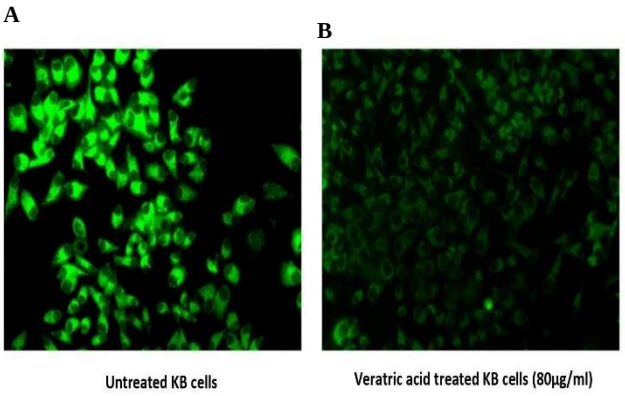

Figure 11: Efficacy of veratric acid on MMP in KB cells (A) and veratric acid treated KB cells (B) by Rhodamine-123 Staining. Veratric acid treated KB cells showed reduction in green fluorescence due to changes in MMP

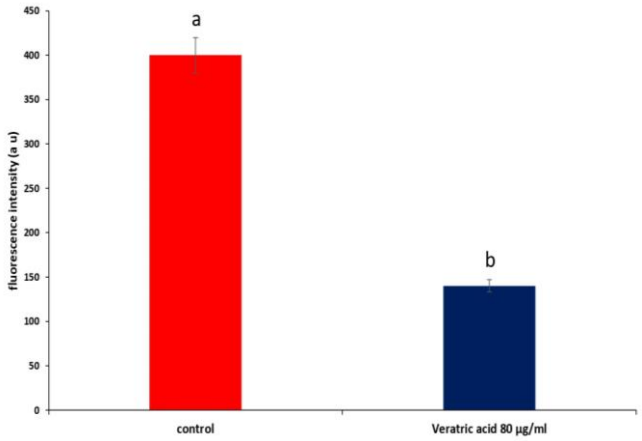

Figure 12: Percentage fluorescence intensity for MMP in KB cells and veratric acid treated KB cells. Data represents mean ± SD for three samples (n=3). Two different superscript letters in the increasing concentrations of the test samples indicate the statistical significant ($p<0.05$).

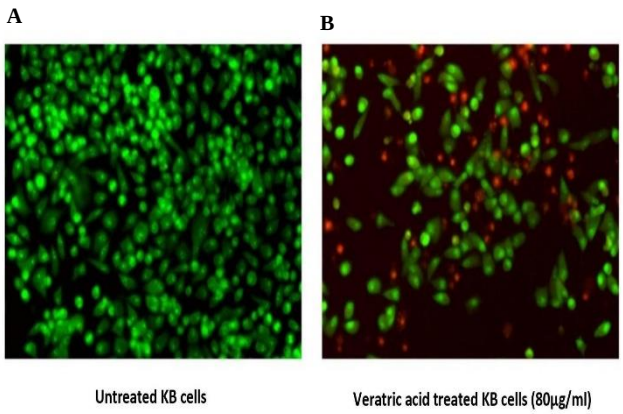

Figure 13: Dual staining showing efficacy of veratric acid on morphological changes in the KB cells. Untreated KB cells (A) and veratric acid treated KB cells (B). Veratric acid treated KB cells revealed more number of apoptotic cells.

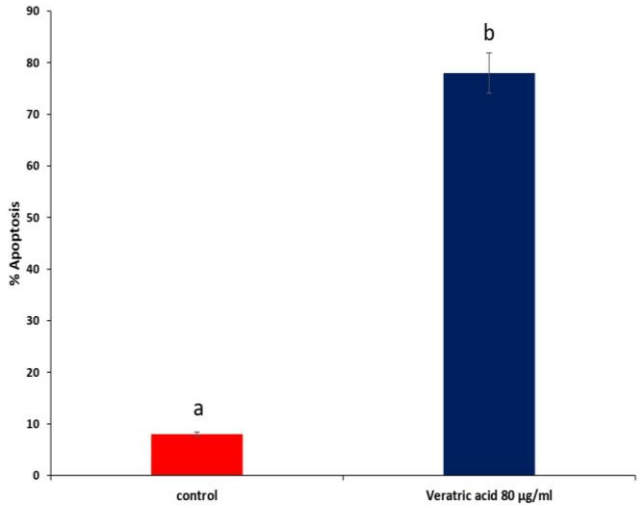

Figure 14: Percentage of apoptotic index in KB cells and veratric acid treated KB cells. Data represent mean ± SD for three samples (n=3). Two different superscript letters in the increasing concentrations of the test samples indicate the statistical significant ($p<0.05$).

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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