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Free Radical Scavenger Solanidine Modulates Cell Proliferation in Murine Solid Lymphoma Model by Activating Bax and Bad to Induce Tumor Regression.

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ABSTRACT

The pharmacological and antioxidant property of the natural steroidal alkaloids from Solanaceae family gain prime importance. In the current study Solanidine were investigated for its antioxidant and anti-proliferative efficacy. Various *in-vitro* antioxidant assay such as DPPH, Total antioxidant, Super radical scavenging assay, lipid peroxidation inhibition assay were carried out with varied concentration of Solanidine. Results signify that Solanidine could significantly affect the scavenging capacity on different free radicals. The anti-neolpastic effect was evaluated using DLA solid tumor model system where Solanidine effectively halts the tumor growth by promoting apoptosis. Solanidine treatment resulted in the altered gene expression of the important candidate pro-apoptotic protein i.e., Bax and Bad and also exhibited typical apoptotic hallmark by inducing DNA fragmentation. Results showed that Solanidine could significantly hinders the cancer progression by exerting the free radical scavenging capacity.

KEYWORDS: Solanidine; Antioxidant; DPPH; Apoptosis.

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INTRODUCTION

Free radicals are extremely reactive chemicals that have the likely to damage cells. Free radicals are shaped naturally in the body and play an vital role in many normal cellular event [1]. At higher concentrations, conversely, free radicals could be dangerous to the body and injure all major apparatus of cells, including DNA, proteins, and cell membranes. The harm to cells caused by free radicals, especially the damage to DNA, may plays a crucial role in the cancer cell progression and other health conditions [2]. Antioxidants are indispensable and significant for plants and animals development. They are core substance that look after to the normal cellular function and protects the cells from the damage caused by unstable molecules known as free radicals [3]. Cancer develops when cells multiply in the presence of oxidation and other damage [4]. Certain "antioxidant" substances, such as vitamin C, are able to exploit the differences between cancer and healthy cells; they kill cancer cells while helping healthy cells [5]. Antioxidants interact with and stabilize free radicals and may prevent some of the damage free radicals [3]. Different types of natural antioxidants are there in many edible fruit and vegetables they have synergistic effect that are important due to their activity and regenerative potential [6]. The plants belonging to Solanaceae, or nightshades, family acts as rich reservoir for many salutary steroidal alkaloids with vast pharmacological and antioxidant potential[7]. The medicinal values of plants belonging to this family has assumed a more important dimensions in the past few decades owing largely to the discovery that extracts from plants contains not only minerals and primary metabolites but also a diverse array of secondary metabolites with antioxidant potential. In the current investigation one of the such steroidal alkaloid called Solanidine was investigated for its free radical scavenging activity with various *in-vitro* antioxidant assays and anti-proliferative effect against solid tumor model was investigated. Results indicated that Solanidine emerged as novel antioxidant molecule, thereby by inhibiting cancer cell progression by inducing apoptosis.

MATERIALS

Thio barbituric acid (TBA), and 2.2-diphenyl-1-picrylhydrazyl (DPPH), were purchased from Sigma Aldrich (Poznan´,Poland). Trichloroacetic acid (TCA), was procured from Himedia (India). Anti Bax and Anti Bad from B.D Bioscience, USA. Immunostaining kit from Leica Biosystems, Germany. All other chemicals used in the current study is of analytical grade. All the experiments were performed at minimum three independent times and analyzed. All bright field and fluorescence image were taken EVOS FL cell imaging, Thermo Scientific, USA and results were assessed by ImageJ software.

METHODS

DPPH radical Scavenging Assay

Solanidine was screened for free radical scavenging activity by DPPH radical scavenging assay [8]. Solanidine at varied concentration (0 -50 µM) were added to each test tube and volume was made up to 2 ml using the distilled water. To this 3 ml of 0.004% DPPH in 95% ethanol was added and the mixtures were incubated at room temperature under dark condition for 30 min. The scavenging activity on the DPPH radical was determined by measuring the absorbance at 517 nm. Radical scavenging activity was calculated using the formula: % of radical scavenging activity = ([(A $_{control}$−A $_{test}$)/A $_{control}$]) × 100, where A $_{control}$ is the absorbance of the control sample and A $_{test}$ is the absorbance of the test sample. The DPPH radical scavenging activity of BHA was also assayed for comparison. Test was performed in triplicate and the results were averaged.

Superoxide radical scavenging

Super oxide anion radical scavenging activity of Solanidine was measured as reported earlier [9]. All the reagents used in this experiment were prepared in phosphate buffer (pH 7.4). 1 ml of NBT (156 µM), 1 ml of NADH (468 µM) and 3 ml of Solanidine at varied concentration (0 -50 µM) were added. The reaction was started by adding 100 µl of PMS (60 µM) and the mixture was incubated at 25°C for 5 min. And the absorbance was measurement of at 560 nm. Decreased absorbance of the reaction mixture indicated increased super oxide anion radical scavenging activity. The percentage inhibition was calculated from the formula, % of radical scavenging activity = ([(A $_{control}$−A $_{test}$)/A $_{control}$]) × 100, where A $_{control}$ is the absorbance of the control sample and A $_{test}$ is the absorbance of the test sample. The Super oxide anion radical scavenging of ascorbic acid was also assayed for comparison. Test was performed in triplicate and the results were averaged.

Lipid peroxidation inhibition assay

In-vitro lipid peroxidation assay was carried out as reported earlier [10]. 10% of liver homogenate was prepared using 0.15 M KCl. 0.5 ml of liver homogenate and 2 ml of Solanidine at varied concentrations (0 -50 µM) were taken in test tubes. 100 µl of ferric chloride (0.2 mM) was added to each test tube and incubated at room temperature for 30 min to induce lipid peroxidation. The reaction was stopped by adding 2 ml of ice-cold HCl (0.25 N) containing 15% TCA, 0.38% TBA, and 0.5% BHA. The content was mixed thoroughly and heated on boiling water bath for 60 min. Reaction mixture was cooled and centrifuged at 3000 rpm for 10 min. Absorbance of the supernatant was measured at 532 nm. Percentage of inhibition was calculated from the formula, % of

inhibition = ([(A $_{control}$−A $_{test}$)/A $_{control}$]) × 100, where A $_{control}$ is the absorbance of the control sample and A $_{test}$ is the absorbance of the test sample. Test was performed in triplicate and the results were averaged.

Total antioxidant capacity

Total antioxidant capacity of Solanidine was performed by phosphomolybdenum method as described earlier [11]. Solanidine at different concentrations (0 -50 μM) was taken. To this, 3 ml of reagent mixture containing 4 mM ammonium molybdate, 0.6 M sulfuric acid and 28 mM of sodium phosphate was added. Test tubes were kept for incubation at 95°C for 90 min. and allowed to cool. Absorbance of the content in each test tube was measured at 695 nm against blank. Antioxidant capacity of each extract is expressed as equivalents of ascorbic acid. Ascorbic acid equivalents are calculated using the standard graph of ascorbic acid. Test was performed in triplicate and the results were averaged.

Animal tumor development and treatment

The BALB/c mice (27-30g) were used throughout the study and maintained as per CPCSEA guidelines with ethical clearance (NCP/IAEC/CL/101/05/2013-14). *In vivo* efficacy of Solanidine was tested with murine solid tumour model(DLA) in Balb/C mice by adopting reported procedure [12]. To validate the ability of Solanidine to regress the tumor development, DLA solid tumor model was used by injecting DLA cells (1×10^6 cells/animal) were grown in Balb/c mice by intramuscularly in left thigh of animals for developing solid tumor. After the visible development of the tumor the animals were randomly allocated for treatment and control (n=6/group). Six doses of Solanidine (50 mg/kg b.w.) were administered i.p. on alternative days after the tumor inoculation and the tumor progression was monitored. On 25th day after tumor cell implantation animals were sacrificed and thigh tissue were dissected for further processing such as histological examination. The number of tumor bearing animals survived and the duration after the treatment regimen were documented in a separate analysis.

DNA fragmentation assay

DNA from cells treated with and without Solanidine were isolated by employing organic phase extraction method as reported earlier, and resolved on 1% agarose gel and documented [13].

Endonuclease Assay

The Solanidine induced DNAase activation was assessed by endonuclease assay**.** In brief, Solanidine treated nuclear fraction from DLA solid tumor tissue were incubated with salmon sperm DNA suspended in agarose gel at 37^{o}C for 18 h and lysis zone was measured and documented [13].

Immunohistochemistry (IHC) analysis

The tissue level expression of apoptotic genes detection from tumor tissues of *in-vivo* system of Solanidine treated were carried out by IHC studies as reported earlier [14] with appropriate antibodies as per the manufacturer's instructions. Images were captured using bright field microscopy; changes in intensity of the antibody staining were evaluated by two independent pathologists with blind folding method.

Statistical analysis

Values were represented as mean ± standard deviation (SD). Statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by 2-tailed 13 Student's t-test. MS excel 8.1 version software was used for data analysis and statistical significant values were expressed as $*p < 0.05$ and $**p < 0.01$

RESULTS

Solanidine exerts free radical scavenging activity

Antioxidant potential of Solanidine was investigated with various *in-vitro* antioxidant assay viz., DPPH radical scavenging assay, superoxide radical scavenging assay, lipid peroxidation inhibition assay, total antioxidant capacity by phosphomolybdenum . Results from the above assays indicated the free radical scavenging ability of Solanidine.

Solanidine was screened for free radical scavenging activity using the DPPH method and it is based on the measurement of the reducing ability of antioxidants on DPPH free radical. Fig 1A represents the percentage of DPPH scavenging activity of Solanidine, where Solanidine exhibited significant radical scavenging activity which increases with increasing concentration of Solanidine.

Solanidine was screened for superoxide radical scavenging activity using ascorbic acid as a standard. Superoxide radical is identified to be very destructive as a originator of more reactive oxidative species that have potential to react with biological macromolecules and thereby inducing

tissue damage. Superoxide radical scavenging activity of extracts is presented in Fig. 1B. Solanidine showed noteworthy dose dependent activity.

Total antioxidant ability of Solanidine was carry out by phosphomolybdenum method. Antioxidant aptitude are expressed as equivalents of ascorbic acid. Solanidine had shown significant antioxidant activity as equivalents to ascorbic acid with increasing concentration. The results of total antioxidant activity were presented in Fig.1C.

Lipid peroxidation, a well-established mechanism of cellular damage animals is used as an sign of oxidative stress in cells and tissues.

The results of the Solanidine to check lipid peroxidation were shown in Fig.1D. The lipid peroxidation inhibitory percentage increased with the increased concentration of Solanidine.

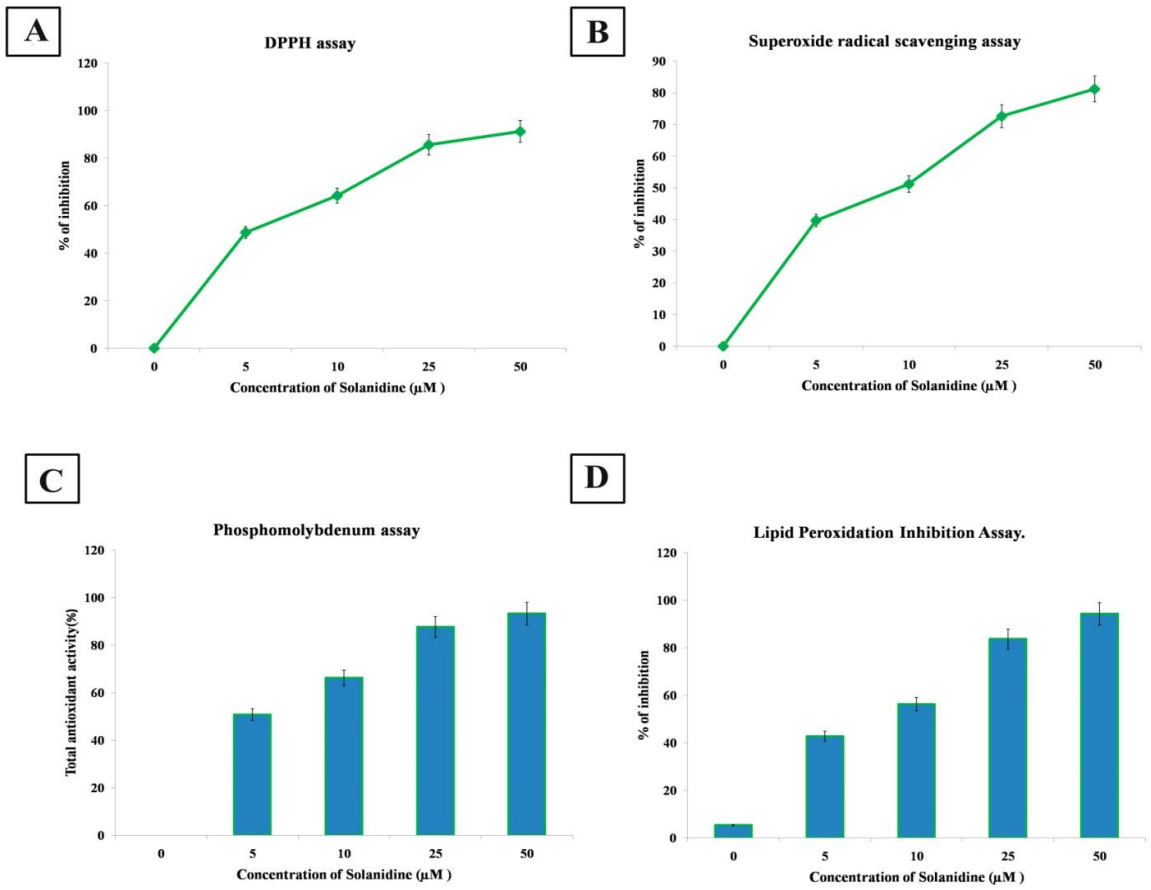

Figure 1: Antioxidant activity of Solanidine (A) DPPH radical scavenging activity, (B) Superoxide radical scavenging activity (C) Total antioxidant capacity (D) Lipid peroxidation inhibition activity.

Solanidine halts the Daltons Lymphoma solid tumor progression

The tumor inhibitory efficacy of Solanidine in in-vivo condition, DLA solid tumor model was implicated in the current investigation. The treatment of Solanidine on DLA solid tumor model attenuated the tumor growth in a dose dependent conduct (Fig 3A). The day to day variation in tumor establishment after each treatment was visibly noticeable (Fig 3A) , Solanidine treatment leads to the decrease in solid tumor mass, (Fig 3B) Additionally Solanidine treatment enhanced survival period of the animals (Fig 3C).

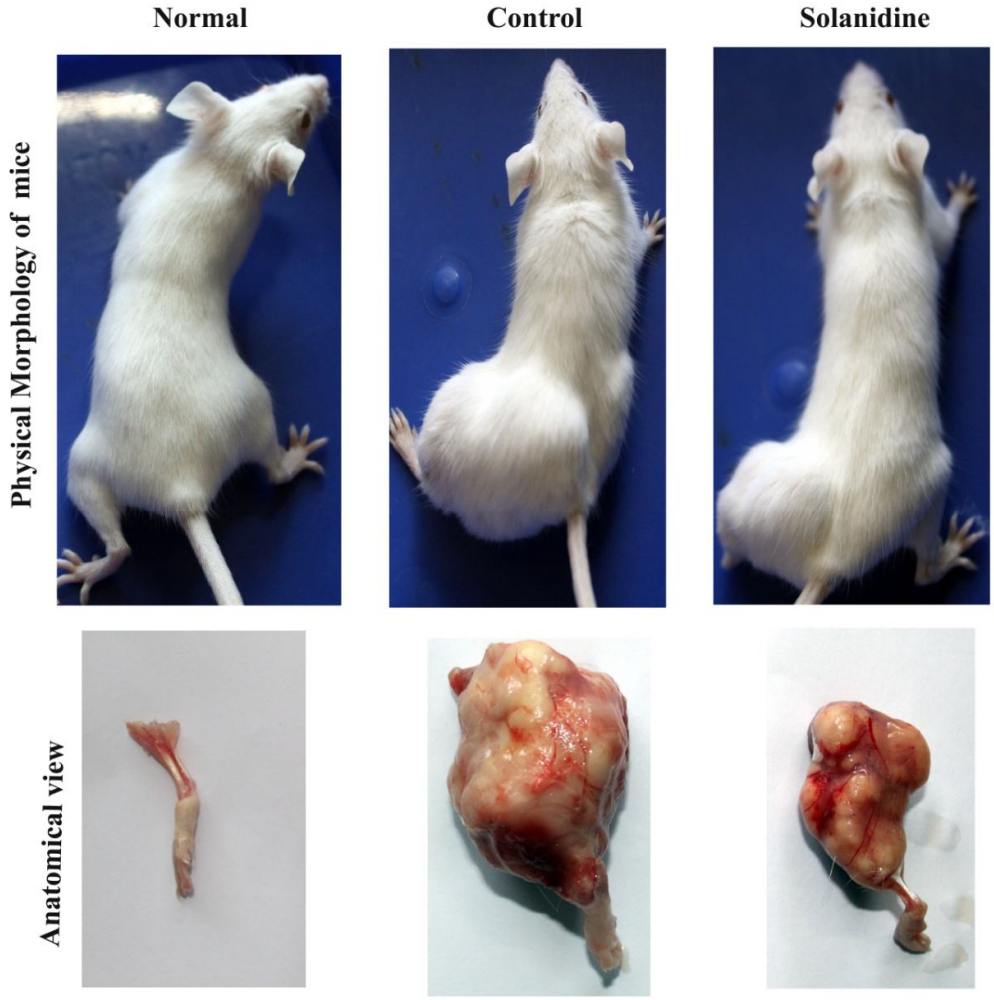

Fig 2: Effect of Solanidine on DLA Solid tumor: Physical morphology of normal, control and Solanidine treated animal and anatomical view of thigh tissue.

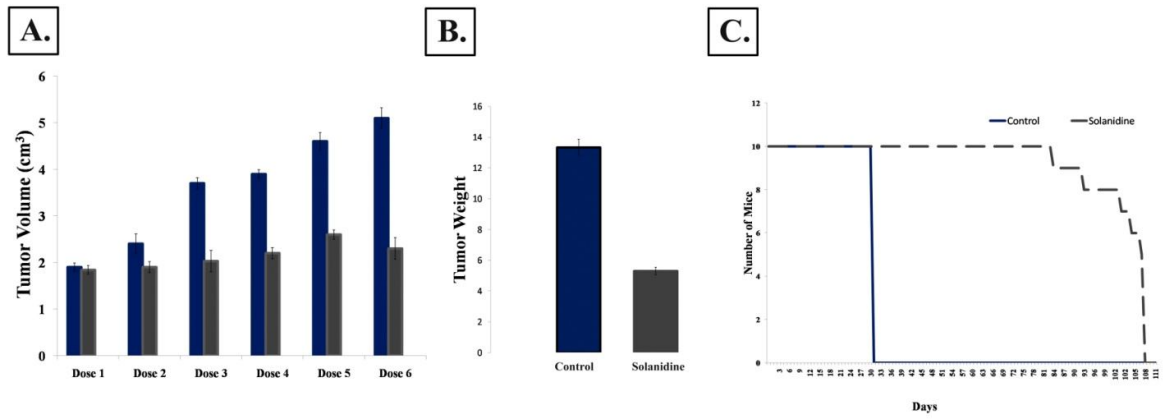

Fig 3: Solanidine treatment alters tumor parameters and increases the survivability of animals : (A) Tumor volume of control and Solanidine treated mice (B) Decreased tumor mass of Solanidine treated animal, (C) Increased survivability of Solanidine treated animals.

Solanidine turn on the apoptotic cell death in the tumor tissues.

The antitumor effect was due to altered cellular signaling in tumor cells inferred through the relevant gene expression studies. Changes in the expression level of pro-apoptotic proteins Bax, Bad (Fig.4A) The eventual nucleosomal disruption observed through fragmented DNA and increased DNA lysis zone of 0.8cm after Solanidine treatment as indicated through endonuclease assay (Fig 4B and 4C).

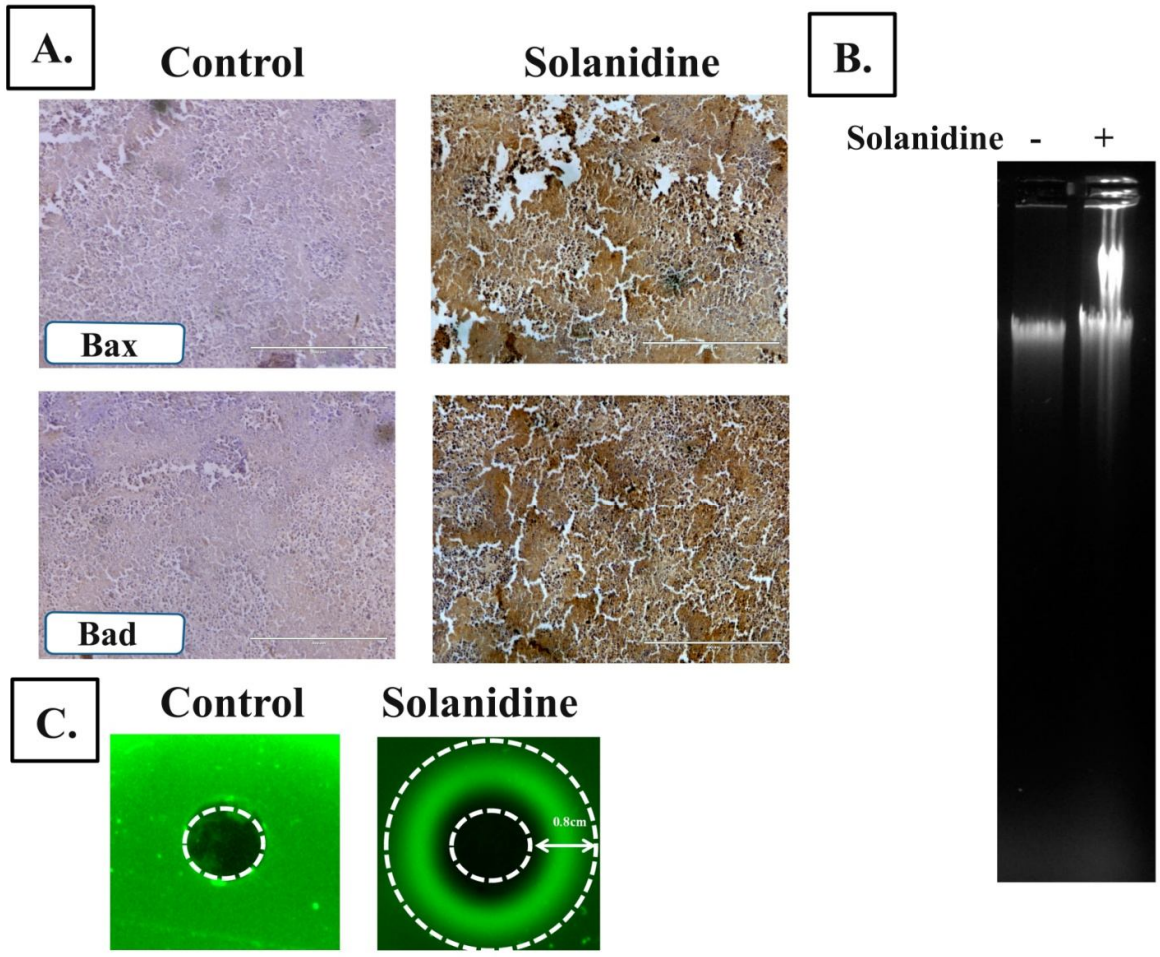

Fig4: Solanidine activates apoptosis in tumor cells. (A) Altered gene expression of pro-apoptotic gene Bax and Bad (B) Nucleosomal degradation (C) Increased DNA lysis zone.

DISCUSSION:

In the normal physiological event of the human body are constantly experience to numerous degradative stresses[15]. These reactive species comprehensively cause the oxidative injure to the biomolecules and make a payment to the pathogenesis of oxidative stress related illness such as cancer, ageing[16]. Antioxidants are well thought-out as possible defensive agents against oxidative injure to the human body. As a result, there is a increasing attention towards agent display antioxidant properties that are supplemented to human. In that concern, natural antioxidants have become one of the major areas of scientific research.

Identifying the molecules from the natures library and exploring the novel characteristics of such compound gained attention in the current day research. Solanidine, is a biophysically well characterized steroidal alkaloid has been found mainly in Solanaceae family of the plants. In the

current investigation the free radical scavenging ability of the Solanidine was carried out using different independent *in-vitro* assay system. Amusingly, Solanidine demonstrated significant free radical scavenging activity at a minimum concentration and the result is in agreement with the presence of various antioxidant molecules .

Authentication of the obtained *in-vitro* anti-oxidant assay results in *in-vivo* tumor model is very important as it provides the physiological microenvironment [17]. Most of the cancers are comparatively solid tumor and treating the same by effective pharmacophore is important in cancer therapeutics, in order to imitate the solid tumor microenvironment we have induced solid tumour in mouse model using Dalton's Lymphoma cells. DLA tumor are used frequently for tumors introduction in mice, for assess the anti-cancer action of small molecules *in vivo*. Solanidine successfully attenuated the *in-vivo* murine solid tumor by activating important apoptotic candidate genes such as Bax, Bad and inducing nucleosomal damage.

Overall the current investigation postulates the antioxidant and anti-proliferative activity of the natural steroidal alkaloid which could be effectively translated to novel phamacophore.

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CONFLICT OF INTEREST

Authors exhibit no conflict of interest.

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