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Valeriana wallichii extract inhibits tert-BOOH induced oxidative damage and cytotoxicity

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1. ABSTRACT

*Oxidative s*tress is well known to be involved in pathophysiology of several disorders. *Valeriana wallichii* (VW) root extracts is known for its expedient activities but its antioxidant and cytoprotective efficacy need to be explored further. In present study tertiary-butyl hydroperoxide (tert-BOOH) was used to induce oxidative stress in C6 glioma cells. Antioxidant activity of the VW root extracts were evaluated by chemical assays *i.e*. DPPH, ABTS and FRAP assay. Further effect of VW on tert-BOOH induced oxidative stress and mitochondrial damage was studied. Result of present study revealed that exposure of cells to tert-BOOH resulted in increase in cytotoxicity, reactive oxygen species (ROS) production and decrease in mitochondrial membrane potential, super oxide dismutase, catalase, glutathione peroxidase and glutathione reductase. On the other hand, pretreatment of cells with VW extracts ameliorated these damaging effects. Additionally, HPLC analysis revealed hesperidin as an active ingredient and concentration of heavy metal was found within the maximum permissible limits prescribed by WHO. In conclusion present study revealed the antioxidant and cytoprotective property of VW against oxidative stress.

2. INTRODUCTION

Plants are naturally gifted for the synthesis of medicinal compounds and their use for medical purpose has a long history throughout the world. In recent years there has been a renaissance of interest in natural or herbal remedies worldwide. Lately, it has been realized that the modern medicine is not capable of providing a cure-all solution against human diseases and that the presence of unwanted side effect is almost unavoidable. The key factor in the widespread acceptance of natural or alternative therapies in the international community involves the standardization and quality control of herbal material using modern science and technology (1).

Valeriana wallichii commonly known as Indian valerian is one of the important plant species of commerce, which belongs to the family *Valerianaceae*. It is a small perennial herb of 15–45 cm height, with root stock, thick branching stem, sharply pointed leaves, white or pink flowers in clusters and hairy fruit. This plant is widely known for its use for the treatment of anxiety, epilepsy, failing reflexes, neurosis, sciatica, etc. It is also considered useful as potent tranquilizer, emmenagogue, diuretic and hepatoprotective (2)

A number of clinical trials confirm that the use of valerian extract decreases sleep latency, improves sleep quality and hence considered useful in treating anxiety and insomnia (3). Valepotriates isolated from the roots are reported as potent anxiolytic, cytotoxic and sedative agents (4-5). The hesperidin is also known as anxiolytic, sedative (6) and antioxidant (7).

In recent years, there has been a wide interest in substituting synthetic antioxidants commonly used in foods such as butylated hydroxytoluene and butylated hydroxyanisole, which have been shown to have one or the other side effects (8). In this sense, some studies have reported the interest of herbs and spices, commonly employed as food ingredients to flavour different types of food preparations, since they contain a wide variety of compounds that can have beneficial health effects (9). Also, several studies have revealed that plants have potent antioxidants in the form of vitamins, flavonoids, and other phenolic compounds that act as scavengers of free radicals and inhibitors of lipid peroxidation (10-11). Nevertheless, to the best of our knowledge, there is no report available on the comparative evaluation of antioxidant activity, chemical composition in terms of total flavonoid and phenol content and also about the heavy metal contamination in *Valeriana wallichii*.

Antioxidant capacity of drugs can be evaluated using chemical methods, which are easy to execute and have high reproducibility. Nevertheless, such methods do not represent what happens *in vivo*. Assays using living cells have proven to be very useful in the routine testing of various products, being fast, sensitive, reproducible, as well as producing reliable results in terms of the identification of biological and antioxidant activity (12).

In view of this, in the present study, antioxidant activity of the VW extracts were evaluated in the chemical and biological systems. Simultaneously one of the phenolic constituent, hesperidin was determined with the help of reverse-phase high-performance liquid chromatography (RP-HPLC) to correlate the antioxidant activity. In addition, since heavy metal contamination in the herbal preparations is of major concern (Saggu *et al.*, 2006), the concentration of heavy metals (As, Pb, Hg, Cd, Cu and Cr) in the extracts was analyzed by atomic absorption spectrophotometer and compared with the World health organization (WHO) prescribed permissible limit (WHO 2005).

3. MATERIALS AND METHODS

3.1. Apparatus

Atomic absorption spectrophotometer (AAS), GBC model No.932; High performance liquid chromatography (HPLC), Waters; Spectrophotometer, Bio-Rad SmartSpec 3000; Nitrogen digestion unit (Kel Plus, Kes-12, Pelican, Chennai, India), ELISA reader (Molecular Devices, USA), Spectrofluorimeter (Varian, USA).

3.2. Chemicals and reagents

2,2′-Diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2, 4, 6-tripyridyl-s-triazine(TPTZ), Trolox, Ascorbic acid, Gallic acid, Rutin, Folin–Ciocalteu reagent, aluminium chloride, acetonitrile, methanol, Hesperidin, from Sigma. HNO_3, standard stock solutions of 100ppm lead(II), mercury(II), cadmium(II), zinc(II), copper(II), chromium(II), from E Merck and arsenic(III) standard stock solution of 1ppm from National Physical Laboratory, Delhi, India.

3.3. *In vitro* cell culture model

C6 glioma cell line was obtained from National Centre of Cell Sciences (NCCS) Pune, India. Cells were propagated in DMEM supplemented with 10% FBS, 100µg/ml ampicillin and 100µg/ml streptomycin. They were maintained at 37 ºC in a humidified CO_2 incubator. The cells were grown to a density of $1X10^4$ cells per well in 96 well plates (Greiner, Germany) for determination of cytotoxicity, mitochondrial membrane potential, reactive oxygen species (ROS) levels and antioxidant enzymes activity (super oxide dismutase, catalase, glutathione peroxidase and glutathione reductase). The cells were grown in 24 well plates (Falcon make) to a density of about $1X10^5$ per well for determination of anti-oxidants levels.

3.4. Extraction of plant extract

Valeriana wallichii root material was procured from Numero-uno Natural Herbs, Delhi, India. 100g of powdered *Valeriana wallichii* (VW) root sample was soaked separately in 500ml of water and 70% ethanol at room temperature. After 24 h, supernatant was decanted and the residue was resoaked in the fresh solvents. The process was repeated for four times for the sufficiently complete extraction. The supernatants were pooled, filtered through muslin cloth and stored in amber colored bottle. The solution was centrifuged at 8000rpm for 10 min. Alcoholic content of the hydro alcoholic extract was evaporated using Rota vapor at 40ºC. Finally, the supernatant solutions were lyophilized and the dried extracts were stored at 5ºC for the further analyses (13).

3.5. Determination of total phenol content

Total phenolic content of extracts was determined by the Folin– Ciocalteu method (14). 150

µL of extract, 2400 µL of nanopure water and 150 µL of 0.2.5 N Folin–Ciocalteu reagent were combined and then mixed well. The mixture was allowed to react for 3 min then 300 µL of 1N Na_2CO_3 solution was added and mixed well. The solution was incubated at room temperature in the dark for 2 h. The absorbance was measured at 725nm using a spectrophotometer and the results were expressed in milligram of gallic acid equivalents per 1 gram of extract.

3.6. Determination of total flavonoid content

1.0. ml aliquot of appropriately diluted VW sample solution was mixed with 2 mL of distilled water and subsequently with 0.1.5 mL of a 5% $NaNO_2$ solution. After 6 min, 0.1.5 mL of a 10% $AlCl_3$ solution was added and allowed to stand for 6 min, then 2 mL of 4% NaOH solution was added to the mixture. Then the mixture was thoroughly mixed and allowed to stand for another 15 min. Absorbance of the mixture was measured at 510 nm versus prepared blank (15). Rutin was used as standard compound for the quantification of total flavonoid. All values were expressed as milligram of rutin equivalents per 1 gram of extract.

3.7. Determination of antioxidant activity

3.7.1. ABTS assay

For Anitoxidant activity measurement, 7.4.mM ABTS solution and 2.6.mM potassium persulfate solution were used as stock solutions. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1mL ABTS+· solution with 60mL methanol to obtain an absorbance of 1.1.0±0.0.2 units at 734 nm using the spectrophotometer. 150 µL of VW root extract solution was allowed to react with 2850 µL of the ABTS+· solution for 2 h in a dark condition. Then the absorbance was measured at 734nm using the spectrophotometer. The standard curve was linear between 25 and 150 ppm Trolox. Results are expressed in mg Trolox equivalents (TE)/g of extract.

3.7.2. DPPH assay

Stock solution was prepared by dissolving 24 mg DPPH with 100mL methanol and then stored at -20ºC until required. The working solution was prepared by mixing 10mL stock solution with 45mL methanol to obtain an absorbance of 1.1.0±0.0.2 units at 515 nm using the spectrophotometer. 150 µL of VW root extract solution was allowed to react with 2850 µL of the DPPH solution for 2 h in the dark. Then the absorbance was taken at 515 nm. The standard curve was linear between 25 and 200 ppm Trolox. Results are expressed in mg TE/g of extract.

3.7.3. FRAP assay

The stock solutions included 300mM acetate buffer pH 3.6., 10mM TPTZ (2, 4, 6- tripyridyl-s-triazine) solution in 40mM HCl, and 20mM $FeCl_3.6H_2O$ solution. The fresh working solution was prepared by mixing 25mL acetate buffer, 2.5.mL TPTZ solution, and 2.5.mL $FeCl_3.6H_2O$ solution and then warmed at 37°C before using. 150 µL of VW root extract solution was allowed to react with 2850 µL of the FRAP solution for 30 min in the dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) were then taken at 593 nm. The standard curve was linear between 25 and 150 ppm Trolox. Results are expressed in mg TE/g of extract.

3.8. Determination of reducing power

1.0. mL of VW root extract solution (0.2.-1.0.mg/ mL) was mixed with 2.5. mL of a 0.2. M phosphate buffer (pH 6.6.) and 2.5. mL of a 1% (w/v) solution of potassium ferricyanide. The mixture was incubated in a water bath at 50ºC for 20 min. Afterward, 2.5. mL of a 10% (w/v) trichloroacetic acid solution was added, and the mixture was then centrifuged at 3000 rpm for 10 min. A 2.5.-mL aliquot of the upper layer was mixed with 2.5. mL of distilled water and 0.5. mL of a 0.1.% (w/v) solution of ferric chloride, and the absorbance was measured spectrophotometrically at 700 nm (15).

3.9. Determination of cytotoxicity and antioxidant activity in cell culture model

The optimal incubation time and concentration of tert-BOOH required to produce cytotoxicity on C6 glioma cells were determined. To determine the efficacy of various antioxidants the cells were supplemented with antioxidants before tert-BOOH addition. The lowest concentration of antioxidant that provided the maximum protection against tert-BOOH induced cytotoxicity was used in the present study. Raw wells were incubated with 100µM tert-BOOH for 1h in the presence and absence of different antioxidant for determination of cytotoxicity and ROS. For measuring GSH levels the cells were exposed to tert-BOOH for 2hr.

3.10. Determination of cytotoxicity

Cytotoxicity was studied by using neutral red uptake (16), a supra-vital dye, that is selectively taken by the live cells. Briefly, 10µl of neutral red dye (0.1.%) was added to the cells and incubated at 37ºC in CO_2 incubator for 45min. Later the cells were washed with PBS followed by the addition of 200µl ethanol-acetic acid (50:1) solution. The absorbance was measured at 570nm using Elisa reader (Molecular Devices).

3.11. Determination of mitochondrial transmembrane potential

Mitochondrial membrane potential (MMP) was determined using fluorescent probe Rhodamine123 (29). After the cells were exposed to tert-BOOH, 10µl Rhodamine 123 (10µ/ml) was added to cells and incubated for 30 min. The cells were washed three times with PBS and fluorescence was measured using spectrofluorimeter (Spectra Max M2 Molecular Devices) with an excitation of 485nm and emission at 531nm.

3.12. Measurement of reactive oxygen species (ROS)

It was measured (17) using fluorescent probe 2, 7 dichlorofluorescein diacetate (DCFHDA). Briefly after incubation, 10µl of DCFHDA stock solution (200µM in DMSO) was added to 190µl of medium in 96 well plate to get final concentration of 10µM. The cells were incubated at 37 ºC for 30min in CO_2 incubator. The cells were washed thrice with PBS and fluorescence was measured by mutliwell spectrofluorimeter (Molecular Devices) Elisa reader at 485nm and emission at 530nm. Alternately radical scavenging activity of the VW extracts were determined in cultured cells using fluorescent probe DCFHDA. Briefly, after incubation, 10µl of DCFHDA stock solution (200µM in DMSO) was added to cells ($1X10^6$ cells). The cells were incubated at 37 ºC for 30 min in CO_2 incubator. The cells were washed twice with PBS. The ROS production was monitored by Flowcytometer equipped with cell quest software (Beckton Dickinton, USA).

3.13. Determination of reduced glutathione levels

After incubation, the cells were lysed by adding 200µl of lysis buffer (100µM Tris, 20mM EDTA, 0.2.5% Triton X-100 pH 8.0.). The reduced and oxidized glutathione (GSSG) levels in the cells were determined fluorimetrically (18).

3.14. Determination of antioxidant enzymes activity

The SOD (EC1.1.5.1.1.), catalase, glutathione peroxidase and glutathione reductase activity (EC 1.1.1.1.9.) were determined spectrophotometrically using commercially available kits (RANDOX Laboratories, Crumlin, UK).

The one unit activity of SOD was determined by monitoring the 50% inhibition of the rate of reduction of 2-(4-Iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium under the conditions of the assay as mentioned in kits protocol.

One unit of catalase activity was defined as the amount of enzyme required to decompose 1µ mol of hydrogen peroxide (H_2O_2) per minute. Catalase activity was expressed as moles of hydrogen peroxide reduced / min / mg protein.

GPX activity was expressed as µmol NADPH oxidized / min / mg protein and GR activity was expressed as 1n mole of NADPH oxidized / min / mg protein.

3.15. HPLC Analysis

Waters HPLC system (Waters Corporation, USA) equipped with Waters 515 HPLC pump, Waters 717 plus autosampler and Waters 2487 UV detector was used. The separation was performed on a Symmetry C18 250 X 4.6.mm ID; 5µm column (Waters, USA) by maintaining the gradient flow rate 0.7.5ml/min of the mobile phase (Solution A; Water:O-Phosphoric acid 99.7.:0.3. and Solution B; Acetonitrile:Methanol 75:25) and peaks were detected at 285 nm. Identification of compound was performed on the basis of the co injections and retention time matching with standard. For the preparation of the calibration curve, standard stock solution of hesperidin (1 mg/2 mL) was prepared in 70% ethanol, filtered through 0.2.2 *µ*m filters (Millipore), and appropriately diluted (0.0.1–100 *µ*g/mL) to obtain the desired concentrations in the quantification range. The calibration graph was plotted after linear regression of the peak areas versus concentrations.

3.16. Digestion of plant samples for heavy metal analysis

0.5. g of plant extract was transferred to a 100ml Nessler tube and 6ml of concentrated nitric acid was added and heated at 150±10°C using nitrogen digestion unit. The samples were digested until the clear solution was obtained. The digested solutions were cooled and made to the final volume of 25 ml with de-ionized distilled water. Reagent blank was also prepared for analysis.

The digested samples were analyzed three times using flame atomic absorption spectrophotometer for Pb(II), Cd(II), Cu(II) and Cr(III) and using hydride generation technique for As(III) & Hg(II) (13). The percentage of relative standard deviation (%RSD) in the concentration replicates was ±2%. All the measurements made by using spectral lines and under optimized parameters as mentioned in table 1. The heavy metal content in the various extracts were expressed in milligram of metal per kilogram (ppm) of extract and compared with the prescribed maximum permissible limits by WHO.

3.17. Statistical analysis

Each analysis was done three times from the same extract in order to determine their reproducibility.

Table 1. Spectral lines and instrument conditions of GBC atomic absorption spectrophotometer used in emission measurements and the instrumental detection limit for the elements measured

Metal	Cd	Cr	Cu	Pb	Zn	As	Hg
Wave length (nm)	228.8.	357.9.	324.7.	217.0.	213.9.	193.7.	253.7.
Slit Width (nm)	0.5.	0.2.	0.5.	1.0.	0.5.	1.0.	3.0.
Lamp current(mA)	3.0.	6.0.	3.0.	5.0.	5.0.	8.0.	3.0.
Calibration range(ppm)	0.5.-1.5.	0.5.-2.0.	1.0.-5.0.	0.5.-5.0.	0.5.-1.5.	0.0.1-0.0.5	0.0.25-0.1.
Detection limit (ppm)	0.0.2	0.0.2	0.0.2	0.0.2	0.0.2	0.0.004	0.0.005
Flame composition	Air:C_2H_2	Air:C_2H_2	Air:C_2H_2	Air:C_2H_2	Air:C_2H_2	Air:C_2H_2	Air:C_2H_2
Atomizer	Standard burner	Standard	burner	Standard	burner	Standard	burner
Measurement mode	Concentration least square	Concentration least square	Concentration least square	Concentration least square	Concentration least square	Concentration least square	Concentration least square

Results are expressed as mean ± SD. Statistical comparisons were made by one-way analysis of variance (ANNOVA). Differences were considered to be significant when the p values were < 0.0.5.

4. RESULTS AND DISCUSSION

4.1. Total Phenol and Flavonoid contents

As a part of chemical composition analysis, total flavonoid and total phenol content of V W root extracts were determined by colorimetric method as described in the materials and methods section. The results as given in the table 2 indicate the presence of higher total phenolic and flavonoid content in the hydroalcoholic extract than aqueous extract. Phenolics are the major plant compounds with having antioxidant activity. This activity is believed to be mainly due to redox properties, which play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (19). Results obtained in the present study revealed the presence of substantial quantity of these phenolic compounds in the VW root extracts. Some of the reported pharmacological effects of VW could be attributed to the presence of these valuable constituents.

Flavonoids, a class of benzo-γ-pyrone derivatives which include flavones, flavanes, flavonols, anthocyanidines and catechins are known to possess a wide spectrum of biological activities such as anticancer, antibacterial, antifungal, antiviral, hypoglycaemic, antihistaminic and radioprotective potential (20-23). Some of these properties are derived from the free radical-scavenging activities of flavonoids. There are several reports relating to the reactivity of flavonoids with active oxygen species and the potential health benefits provided eventually from their antioxidant activity.

4.2. Antioxidant Activity of VW in chemical system

In order to study the effectiveness of antioxidant activity, it has recently been recommended to employ at least two different *in vitro* models because of the differences between various free-radical scavenging assay systems (8). Therefore, the extracts were subjected to three different antioxidant bioassays employing DPPH, ABTS and FRAP.

The free radical scavenging activity of VW was studied by its ability to bleach the stable radical DPPH. This assay provides information about the reactivity of compounds with a stable free radical. Because of the odd electron, DPPH shows a strong absorption band at 517 nm in visible spectroscopy. In the presence of free radical scavenger, the absorption band vanishes, and the resultant decolorisation of solution is stoichiometric with respect to the number of electrons taken up. From the DPPH assay results it may be postulated that VW reduces the radical to the corresponding hydrazine on reacting with the hydrogen donors in VW and is sufficiently effective in scavenging DPPH radicals.

ABTS is also a relatively stable free radical. The reduction of free radicals by the test compound using ABTS was measured at 734 nm. The chemistry of ABTS involves direct generation of ABTS radial mono cation with non involvement of any intermediary radical and is also a decolorization assay. In this case, the radical cation is formed prior to the addition of antioxidant test system, rather than the generation of the radical taking place continuously in the presence of antioxidant activity and is applicable to both lipophilic

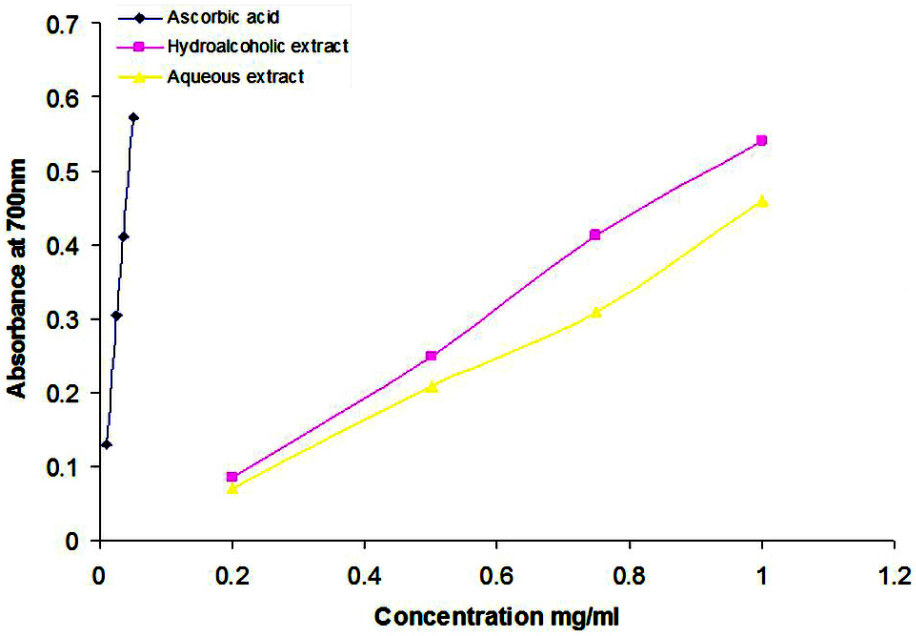

Figure 1. Reducing power of ascorbic acid, aqueous and hydroalcoholic extract of *Valeriana wallichii.*

and hydrophilic antioxidants (24). The results of the study clearly indicate that the activity of Valeriana Wallichii may be either inhibiting or scavenging the ABTS radicals.

The antioxidant potential of the aqueous and alcoholic extracts of the Valeriana Wallichi was determined by FRAP assay, based on the ability of the extracts to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II). Antioxidant activity VW was found to be directly proportional to the phenol content which appears to be the trend in many plant species (25). The results of ABTS, FRAP and DPPH• assays for both the extracts of VW are presented in table 3, clearly indicate the higher antioxidant activity of hydroalcoholic extract than the aqueous extract.

4.3. Reducing power of VW extract

The reducing power of VW extracts, which may also serve as a significant reflection of the antioxidant activity, was determined using a modified iron (III) to iron (II) reduction assay. In this assay, the yellow color of the test solution changes to various shades of green and blue depending upon the reducing power of extracts or compounds. The reducing agents in the solution reduces Fe^{3+}/Ferricyanide complex to the ferrous form and the resultant Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm (15).

fig1. depicts the reducing power of VW extracts in comparison to ascorbic acid. Both the extracts have shown some degree of reducing power; however, as anticipated, their reducing power was inferior to ascorbic acid, which is known to be a strong reducing agent. Like the antioxidant activity, the reducing power of extract increased with increasing amount of extract; the equation of reducing power (y) and amount of extract (x) was $y = 0.4.761x - 0.0.301$ ($r^2 = 0.9.93$) for aqueous extract and y= 0.5.749x – 0.0.301(r^2= 0.9.98) for hydroalcoholic extract, indicating that reducing ability correlated well with amount of extract. Reducing power of the extracts increased with increasing amount of extract indicating that reducing ability correlated well with amount of the extracts. The reducing power of ascorbic acid, and *Valeriana Wallichi* extracts followed the following order: Ascorbic acid > Hydro alcoholic extract > Aqueous extract.

It will be relevant to mention here that earlier paper (8) have demonstrated the correlation between the phenolic content of plants to their antioxidant power. In this study also, a good correlation was observed between the phenolic content and the antioxidant power of both the extracts. The results given in the table 2 clearly showed the higher value of total phenolic content in the hydro alcoholic extract compared to the aqueous extract, which is evidently in accordance with the observed antioxidant activity.

4.4. Evaluation of cytotoxicity and antioxidant activity of VW extracts in cell culture

4.4.1. Evaluation of Cytotoxicity

Cytotoxicity of tert-BOOH by exposing C6 glioma cells to various concentration of tert-BOOH

Table 2. Analysis of total phenol, total flavonoid and hesperidin contents

Sample	Total Phenolic content(±SD)[1] mg gallic acid /g of extract	Total Flavonoid content(±SD)[1] mg rutin/g of extract	Hesperidin (±SD)[1] mg/g of extract
Aqueous extract of *Valeriana wallichii*	56.1.5±1.3.1	102.5.1±3.5.3	6.1.8 ±0.2.6
70% alcoholic extract of *Valeriana wallichii*	64.6.3±2.0.8	119.5.3±4.2.5	9.8.3±0.0.7

[1]Average of three determinations

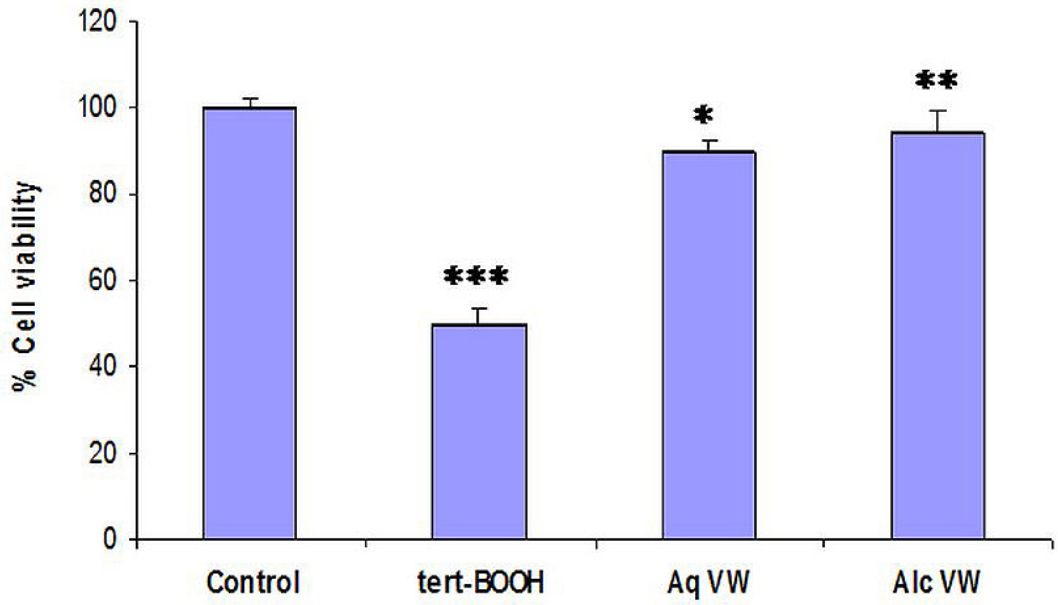

Figure 2. Cytotoxicity analysis was done using neutral red dye. Cells were grown and divided into different groups i.e. Control, tBOOH, tBOOH + VW (aqueous extract) and VW (alcoholic extract). Data represents Mean ± SEM. * represents p < 0.0.1 and ** represents p < 0.0.5 when compared to tBOOH group and *** represents p<0.0.01 when compared to control group.

(10-500 μM) was assessed. The LD_{50} was observed at 100μM concentration of tert-BOOH. Various concentration of VW (10μg to 500μg) was also tested to determine the optimum dose and cytotoxicity, if any towards C6 glioma cells. We found that upto100μg / ml of VW drug had no cytotoxicity. However, a concentration as low as 100μg/ml was effective and showed viability >95% that was maintained through the test period. Addition of tert-BOOH (100μM) to C6 glioma cells resulted in significant increase in cytotoxicity (55%) as revealed by the fall in neutral red uptake as compared to control cells. Results shown in fig 2 indicate that pretreatment of cells with extracts of VW significantly attenuated cells viability (P<0.0.1) compared to cells treated with tert- BOOH alone.

4.4.2. Reactive Oxygen Species

The free radical quenching activity of VW was evaluated in cultured cells against tert-BOOH induced oxidative stress as measured by fluorescent probe 2, 7 dichlorofluorescein diacetate. There was significant increase by about 2.1. times in cells exposed to tert-BOOH relative to control cells indicating the generation of intracellular ROS. Pretreatment of cells with VW extract appreciably inhibited the ROS generation induced by tert-BOOH. Results shown in the fig3(A) indicate that hydroalcoholic extract was found to possess better free radical quenching activity than aqueous extract.

4.4.3. Mitochondrial transmembrane potential

Addition of tert-BOOH (100μM) to C6 glioma cells resulted in significant decrease in mitochondrial transmembrane potential by about 3.5. times in cells exposed to tert-BOOH. Results shown in the fig3(B) indicate that pretreatment of cells exposed to VW restored the mitochondrial transmembrane potential similar to that of normal cells.

4.4.4. Antioxidant status

The GSH level was determined both during 1hr and 2hr of tert-BOOH treatment to understand the dynamics of GSH metabolism in the C6 glioma

Table 3. Antioxidant activity determination

Sample	DPPH(±SD)[1] mg Trolox/g of extract	ABTS(±SD)[1] mg Trolox/g of extract	FRAP(±SD)[1] (mg Trolox/g of extract)
Aqueous extract of *Valeriana wallichii*	112.8.1±4.6.	123.9.6±4.3.	110.4.5±3.1.
70% alcoholic extract of *Valeriana wallichii*	136.0.5±5.1.	143.8.3±5.3.	127.7.8±4.6.

[1]Average of three determinations

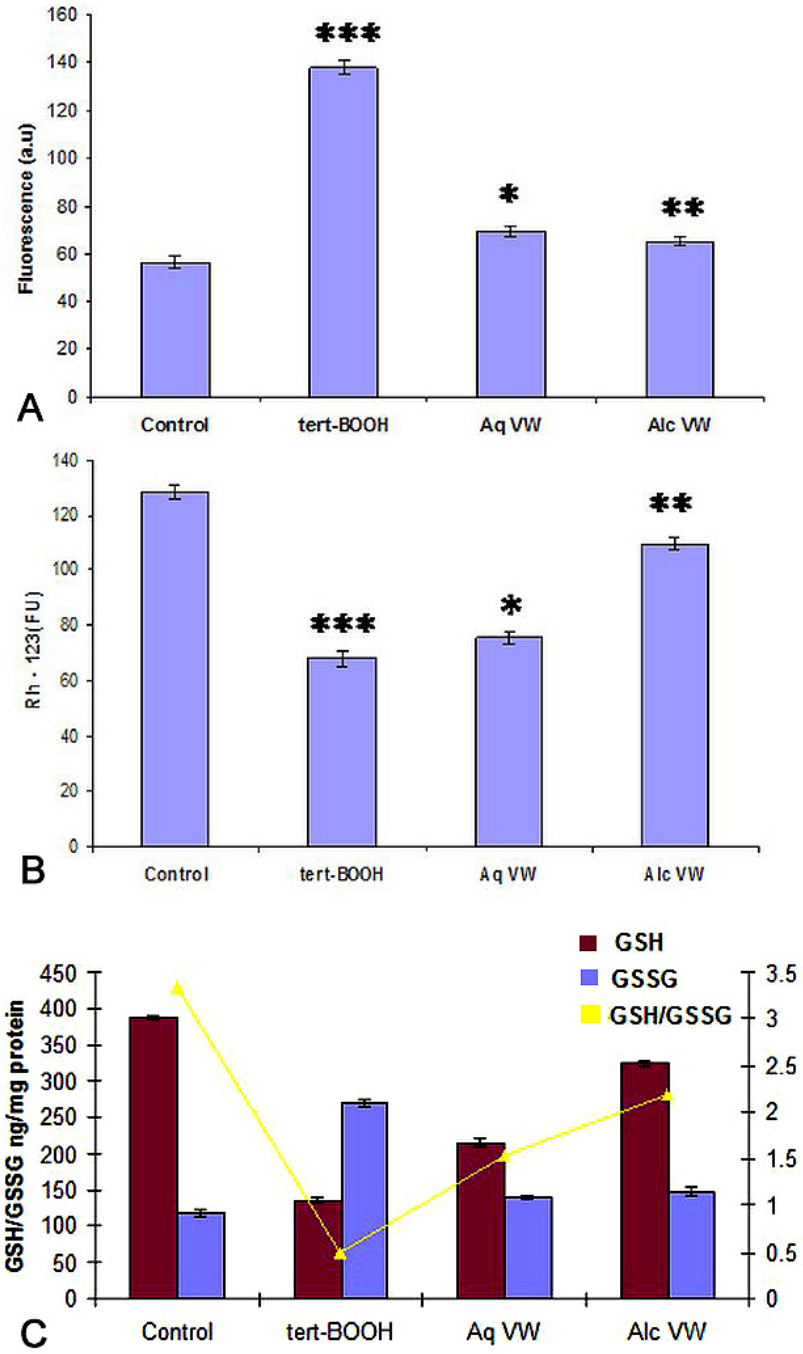

Figure 3. Oxidative status was assessed in different groups by estimating Reactive Oxygen Species (A), Mitochondrial membrane potential (B), Glutathione levels (C). Data represents Mean ± SEM. * represents $p < 0.0.1$ and ** represents $p < 0.0.5$ when compared to tBOOH group and *** represents $p<0.0.01$ when compared to control group.

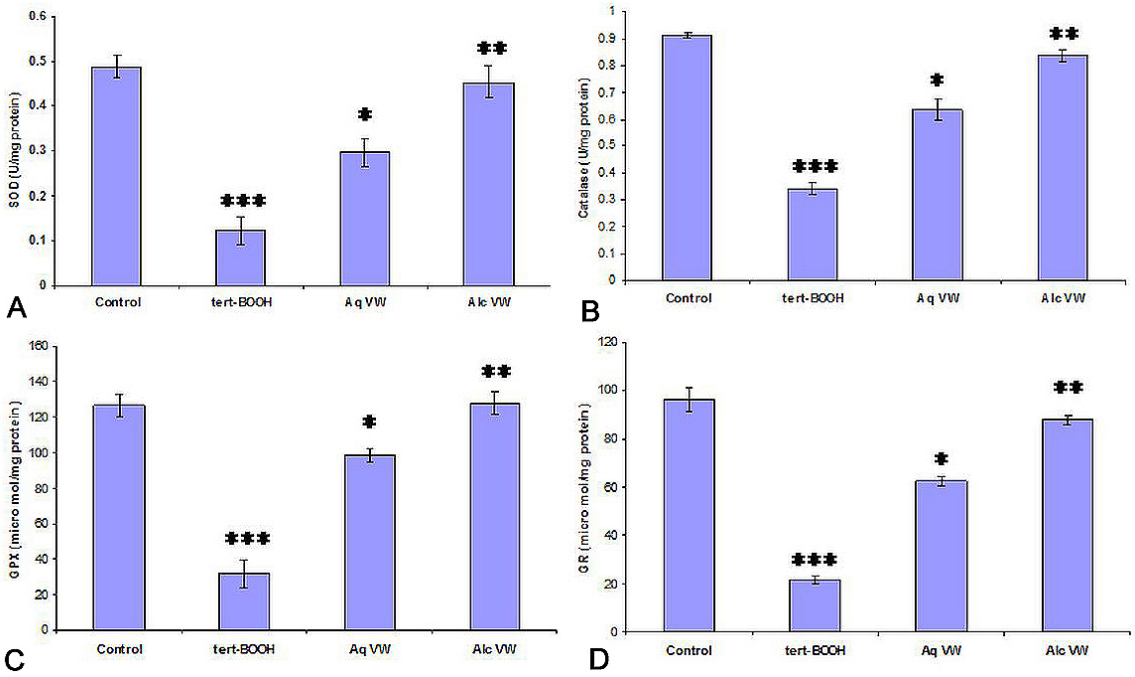

Figure 4. Antioxidant status in different groups was assessed by evaluating Super oxide dismutase (A), Catalase (B), Glutathione peroxidase (C) and Glutathione Reductase (D). Data represents Mean ± SEM. * represents p < 0.0.1 and ** represents p < 0.0.5 when compared to tBOOH group and *** represents p<0.0.01 when compared to control group.

cell line. Since no significant change in GSH level was observed at 1h, the data at 1hr was not shown. Exposure of cells to tert-BOOH for 2hr resulted in considerable fall in intracellular reduced glutathione (GSH) by about 23% and an increase in oxidized glutathione (GSSG) by about 2.9. fold in C6 glioma cell line as compared to control cells. Further there was a considerable fall in GSH/GSSG ratio from 3.6. in control cells to 0.9.6 in cells incubated with tert-BOOH. Results shown in fig3(C) demonstrate that, supplementation of VW extract resulted in significant increase in GSH and decrease in GSSG levels. The GSH/GSSG ratio in VW treated cells was increased significantly, when compared to control cells. However GSH level dropped to further 40% of the control cells after 3hr incubation in presence of tert-BOOH.

4.4.5. Antioxidant enzymes activities

SOD has an antitoxic effect against the superoxide anion. It is known that SOD converts superoxide anion into H_2O_2 and O_2, whereas CAT and GPX reduce H_2O_2 to H_20, resulting in the detoxification of free radicals. The GSH/GSSG ratio is maintained by enzymatic activities of GR and GPx. GR converts GSSG to GSH in presence of NADPH (26). In the present study, the antioxidant enzyme (SOD, Catalase, GPX and GSH Reductase) activities were significantly decreased (P<0.0.1) in oxidant treated group of cells and the antioxidant enzyme activities are maintained or restored almost similar to that of control in the VW extract treated group. The obtained results shown in fig4(A) –fig4(D).

4.5. Identification and quantification of hesperidin by RP-HPLC

A simple and gradient elution-based RP-HPLC method was developed for the quantitative analysis of hesperidin (fig5A), in the extracts. For the development of an effective mobile phase, various solvent systems, including different combinations of acetonitrile, methanol and water with ortho phosphoric acid were tried. Finally, a solvent system consisting of 0.3.% ortho phosphoric acid in water and acetonitrile: methanol (75:25) was proved successful because it allows for the separation of maximum compounds with good resolution. Heperidin, which might contribute to the antioxidant behavior of the plant, was identified in extracts, as shown in fig5(B) & fig5(C). Identification of compounds was performed on the basis of the co injections and retention time matching with standard. The results as given in the table 2 indicate the presence of higher hesperidin content in hydro alcoholic extract than the aqueous extract.

4.6. Heavy metals analyses

The practices of most herbal medicine include the use of crude or raw herbs that are collected from the wild or from cultivated fields to prepare herbal products. Toxic contaminants especially heavy metals

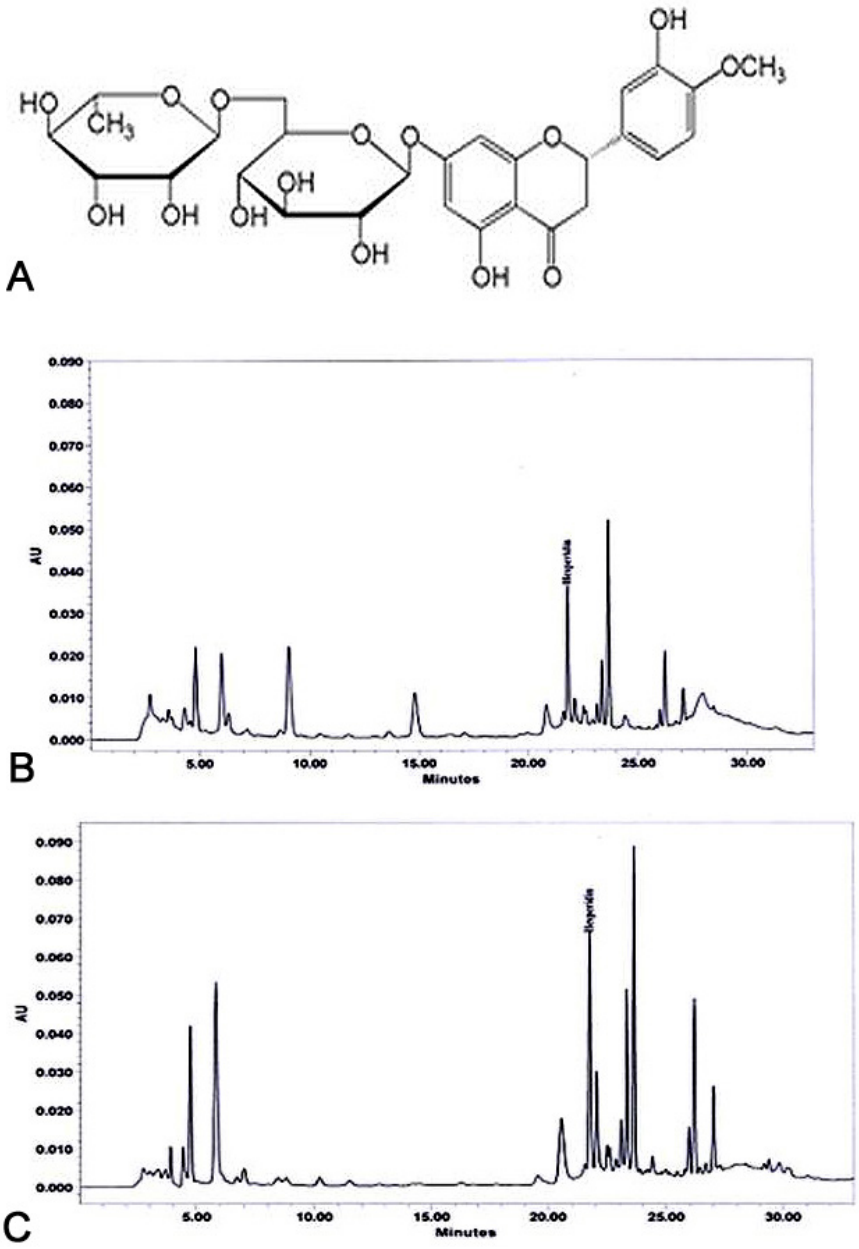

Figure 5. represents detailed structure of Hesperidin (A), HPLC chromatogram of aqueous extract (B) and hydro-alcoholic extract (C) of *Valeriana wallichii*.

may add from environment and the soil in which medicinal plants are grown, collected, dried, processed, stored, transported and during manufacturing process. Toxic metal contamination may also result from direct atmospheric deposition, geological weathering or through the water sources due to solubility of metal ions. Living organisms surely require trace amounts of some metals; however their excessive levels can be detrimental to their health. Heavy metals contamination including mercury was reported in the Indian Ayurvedic products sold in market and put users at risk of metal poisoning (27). Therefore, WHO has prescribed the limits for the heavy metals in the herbal products (28).

Concentration of heavy metals, Pb(II), Cd(II), Cu(II) , Cr(III), As(III) & Hg(II) in both the extracts was determined using atomic absorption spectrophotometer(AAS). The results as given in the table 4 indicate that, concentrations of heavy metals in both the extracts are within the prescribed limit of WHO.

Table 4. Heavy metal analysis of *Valeriana wallichii* extract

Metal	As	Pb	Hg	Cd	Cu	Cr
WHO permissible limit(ppm)	5.0.	10.0.	0.5.0	0.3.0	20.0.	2.0.
Aqueous extract of *Valeriana wallichii*	<MDL	<MDL	0.2.4	0.1.5	3.1.	<MDL
70% alcoholic extract of *Valeriana wallichii*	<MDL	<MDL	0.1.9	<MDL	1.8.5	<MDL

MDL = Minimum determinable limits

5. CONCLUSION

The present study concludes the presence of sufficient amount of total phenol and flavonoid content in the *Valeriana wallichii* root, which is evidently in accordance with the observed antioxidant activity. The present study also reports the cytoprotective and antioxidant properties of VW against tert-BOOH induced oxidative stress in C6 glioma cells. VW extracts are capable of inhibited cytotoxicity, ROS production and maintained antioxidant enzymes (SOD, CAT, GPx and GR) levels similar to that of control cells.

In addition, extracts were standardized based on the marker compound by RP-HPLC and concentrations of heavy metals (As, Pb, Hg, Cd, Cu and Cr) are with in the maximum permissible limits prescribed by WHO. The results are promising and demonstrate the practical feasibility of standardized *Valeriana wallichii* to be used as nutraceutical-rich formulations.

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