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Anti-oxidant activity and cytoprotective potential of ethanolic extract of Adhatoda vasica

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Abstract : *Adhatoda vasica* L. Nees (Acanthaceae) is an evergreen shrub found in several parts of India. It has proven medicinal property against respiratory ailments and possesses abortifacient, hepatoprotective, antiinflammatory effects as well. To investigate the antioxidant potential and cytoprotective role of ethanolic extract of *Adhatoda vasica* (AVE, leaves) against Tobacco Smoke Extract (TSE) induced cell death in A549 (human lung carcinoma) cell line.

The antioxidant potential of AVE was established through *in vitro* assays. Various concentrations of AVE and TSE were further used to establish the cytoprotective role of AVE through MTT assay and microscopic analysis.Total phenolic and flavonoid content in AVE was found to be 88.77 mg/g (w/w) and 55.28% mg/g (w/w), equivalent to Gallic acid and Rutin, respectively. IC_{50} of AVE in DPPH assay was 68 µg/ml. A dose dependent increase in reducing power of AVE was observed. Treatment of A549 with 1-3 µg/ml AVE for 3 hrs maintained cell viability, as confirmed through MTT assay and microscopic analysis. Pre-treatment of A549 with AVE showed cytoprotective effect against toxicity induced by TSE (0.5, 1, 3, 5 and 10%) for 24 hrs. Approximately 50% cell death was observed at 5% TSE on 24 hrs exposure while, 2 µg/ml AVE could retain the cell viability to near control.

This study showed that AVE has a great potential in protecting lung cells from TSE induced toxicity and it might have implications in the treatment or prevention of tobacco induced respiratory diseases.

Key Words: *Adhatoda vasica*, Tobacco smoke, alveolar (A549) cell line, Cytotoxicity, Antioxidants, Total phenolic and flavonoids content, radical scavenging activity.

Introduction

Antioxidants are endowed with the property of scavenging and inhibiting the production of free radicals in the cytoplasm thus, conferring protection against degenerative and inflammatory diseases. Tobacco smoking has been reported to have link with various degenerative and inflammatory diseases of the lungs.[1] According to research carried out by the Toronto-based Centre for Global Health Research (CGHR) in partnership with Mumbai's Tata Memorial Hospital, over 120,000 deaths have been reported across India in 2010 due to tobacco use.[2] TS is an extremely complex mixture of multiple components known for their carcinogenicity, mutagenicity and other harmful effects.[3] It is known to induce oxidative stress in the body via increased production of Reactive Oxygen Species (ROS).[4] Oxygen derived free radicals or Reactive Oxygen Species (ROS) includes superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), nitric oxide (NO), hydroxyl (OH^-) and peroxyl radicals (ROO^-). ROS are also generated naturally in biological system but, they are maintained at normal levels by the endogenous enzymes such as catalase, superoxide dismutase (SOD), glutathione

peroxidase system and non-enzymatic antioxidative mechanisms.[5,6] However, the activities of these endogenous defense systems may not be sufficient enough to mop up the excess built-up of free radicals during abnormal conditions in the body. Several studies have shown that, administration of exogenous compounds (antioxidants) helps in managing/lowering down these free radicals and thus, possess great potential in ameliorating many inflammatory diseases.[7,8]

Adhatoda vasica L. Nees (Acanthaceae) is an evergreen shrub found perennially from the open fields of Narthamalai, Pudukkottai District, Tamil Nadu, and several other parts of India. It is also grown in the tropical regions of Southeast Asia. Its immense therapeutic value has been recognized since thousands of years as reported in several official monographs of indigenous systems of medicine.[9] The most explored active constituent of this plant is vasicine, a quinazoline alkaloid which is believed to be responsible for most of its pharmacological activities.[10] Other chemical ingredients of *Adhatoda vasica* as reported in several studies are given in table 1.

Table 1: Few alkaloids of *Adhatoda vasica*

Name of the Sample	Appearance
Vasicol, 2'-hydroxy-4-glucosyloxychalcone,deoxyvasicinone	Leaves
Vasicinone	Leaves, stem and roots
vasicinone, maiontone, and adhatonine	Leaves and roots
Vasicinol	Stem and roots
Steroid like daucosterol	Leaves and roots
Triterpenes like α-amyrin	Flowers
Flavonoids like apigenin, astragalin, kaempferol, quercetine	Leaves and roots

A. vasica is valued for its proven medicinal properties against a broad array of diseases specially respiratory ailments like dry cough, asthma, bronchitis, common cold, smoker's cough; menstrual disorders, eye infections, skin diseases, sore throat, bleeding diarrhoea, etc. [11,12] It has also been reported to be abortifacient, hepatoprotective, sedative, antiulcer, antispasmodic, antiallergic, antiinflammatory, antitubercular, and anthelmintic etc..[13-19] Inspite of possessing such a wide array of pharmacological significance, the data available pertaining to *in vitro* antioxidant property of this plant extract is still insufficient in order to scientifically validate its wide range of pharmacological activities related to its antioxidant property. Hence, the present study is aimed to evaluate the *in vitro* antioxidant and ROS scavenging capacity of AVE and its cytoprotective activity in tobacco smoke treated alveolar (A549) cell line.

Materials And Methods

Chemicals

The principle reagents used in this study and their sources are as follows. Gallic acid, 1, 1'- diphenyl-2-picrylhydrazyl ($DPPH^{\cdot}$), dimethylsulfoxide (DMSO), penicillin, streptomycin, ascorbic acid and potassium ferricyanide were purchased from Sigma-Aldrich (St Louis, MO, USA). Folin-Ciocalteu, sodium carbonate anhydrous, sodium hydroxide, and aluminum chloride hexahydrate were sourced from CDH (New Delhi). Fetal bovine serum (FBS), RPMI-1640 and 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) from Himedia, Mumbai. All other reagents were of analytical grade. A549 cell line was obtained from the National Centre for Cell Science (NCCS) Pune, India.

Preparation of the ethanolic extract of *Adhatoda vasica*

The powdered leaves of *A. vasica* were exhaustively extracted with 90% ethanol in a Soxhlet's apparatus.[20] The ethanolic extracts of *A. vasica* (AVE) thus obtained, were collected and concentrated using rotary evaporator under reduced pressure at less than 40°C. Then the extracts were stored in aliquots at -80°C (stock) until used for further study.

Estimation of total phenolics content

The amount of phenols in the AVE was determined by Folin-Ciocalteu reagent using the method of Spanos.[21] The absorbance of the reaction mixture was measured at 765 nm using UV/visible light. Results were expressed as micrograms of Gallic acid.

Antioxidant activity (DPPH & FRAP assay)

Antioxidant activity was determined by DPPH radical scavenging activity and Ferric-Reducing Antioxidant Power (FRAP) assay.[22,23] Absorbances of the reaction mixture were measured at 517 & 700 nm, respectively.

Cell Culture & cell viability assay

A549 cells (carcinomic human alveolar type II basal epithelial cell line) were obtained from the National Centre for Cell Science (NCCS), Pune, India and cultured by standard method.[24] Cell viability was measured by the MTT method.[25]

Tobacco Smoke Extract (TSE)-induced toxicity

In order to evaluate the toxicity caused by TSE, TSE was prepared by burning three bidis (Indian tobacco rolls) in 3 ml of incomplete media and was considered as 100% TSE (stock). Cells were seeded into 96-well plates ($3x10^5$) and incubated for 24 hrs. Then the cells were treated with respective percentage doses of TS extract (1, 2, 3, 5 & 10%) for two time periods (4 hrs & 24 hrs) in separate sets experiments. For further experiments, treatment of cells with 1, 2, 3, 5 & 10% concentrations of TSE for 24 hrs were used.

Treatment with AVE

To investigate the cyto-protective activity of AVE, cells (3×10^5cells/well) were seeded into 96-well plates. After 24 hrs of seeding, cells were pretreated with AVE (1 - 10 µg/ml) for 3 hrs. This was followed by addition of TSE (5%) to each well and further incubated for 24 hrs. Cell viability was determined by MTT assay.

Microscopic analysis

Cells ($1x10^4$) were seeded onto six well plate and grown overnight in DMEM media with 10% FBS. Treatments of the cells were given according to three experimental sets mentioned in MTT assay. After treatment, the cells were washed with PBS (pH 7.4, 20 mM) and then fixed with methanol. After fixing, the cells were again washed with PBS buffer and the plate was observed under inverted microscope at 40X.

Statistical analysis

Statistical analysis for MTT assay was performed using the one way ANOVA test. $P < 0.05$ and $P < 0.01$ were considered to be statistically significant as shown in the figure legends.

Results:

Estimation of total phenolics content

The total phenolics content of ethanolic extract of *Adhatoda vasica* was found to be 88.77 mg/g (w/w), and equivalent to Gallic acid as depicted in Figure 1.

Estimation of total flavonoids content

The total flavonoids content of AVE was found to be 55.28 mg/g (w/w), and equivalent to Rutin as shown in Figure 2.

DPPH radical scavenging activity of AVE

The plant extract exhibited a dose dependent free radical scavenging activity against the stable radical (DPPH•) (Figure 3). The IC_{50} value of the AVE required to scavenge 50% of DPPH• was 68 µg/ml.

The reducing power potentials of AVE

AVE at a concentration of 10 – 125 µg/ml has shown a dose dependent reducing power (r^2: 0.995) using the Potassium Ferricyanide Reduction method. The reducing power of the ethanolic extract of the plant was less than the standard Gallic acid as shown in Figure 4.

Dose optimization for AVE

On incubating the cells with AVE with a dose range of 0, 1 - 5 µg/ml for 3 hrs, 1- 3 µg/ml AVE was proved to be safe dose range for the cells (Figure 5). Significant cytotoxic effect was observed when the dose exceeded 3 µg/ml. As a result, 1 – 3 µg/ml range of AVE was taken up for further experiments.

Effect of TSE induced toxicity on the viability of A549

Treatment of cells with TSE at a dose range of 1 - 10% for 24 hrs showed that 5% TSE (Figure 6) brought about almost 50% cell death toxic its toxic effect on the cells.

Study of protective role of AVE against TSE induced toxicity

To study the cyto-protective effects of AVE, A549 cells were pretreated for 3 hrs with AVE (1 -3 µg/ml) and then exposed to 5% TSE for 24 hrs. Figure 6 shows ~50% reduction in cell viability in TSE- treated A549 cells as compared to non-treated cells. However, AVE pretreated cells seemed to be more viable (16.56%, 33.37% and 32.71%, for 1, 2 and 3 µg/ml, respectively) (Figure 7) than the negative control. These results indicate that, pretreatment of AVE protects A549 cells against TSE-induced toxicity.

Microscopic analysis

On treating the cells with 5% TSE, significant change in cell shape, number, and size were observed under light microscope, as compared to control (Figure 8a and 8b). Cell shrinkage was also observed. But, when cells were pre-treated with AVE followed by 5% TSE, there was a significant increase in cell number as compared to TSE treated cells but, the cell size was almost similar to negative control (Figure 8c and 8d).

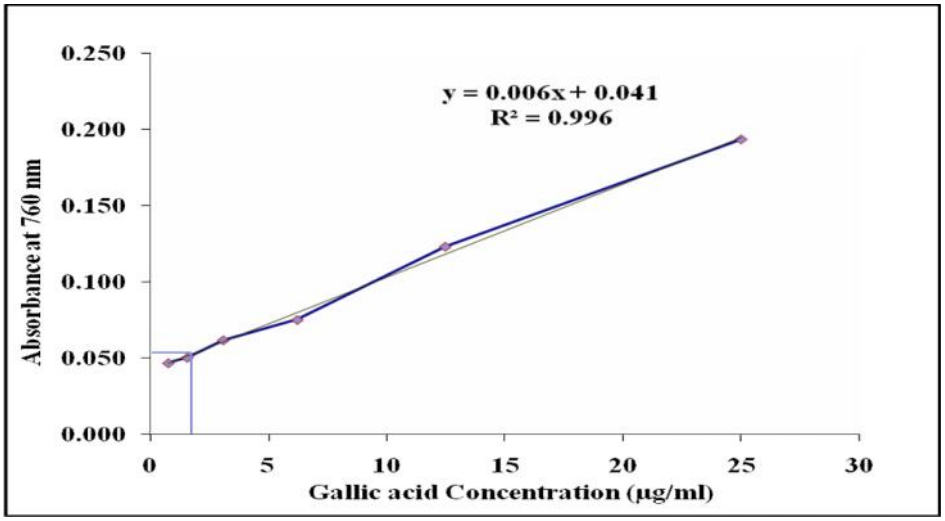

Figure 1: Calibration curve for Gallic acid

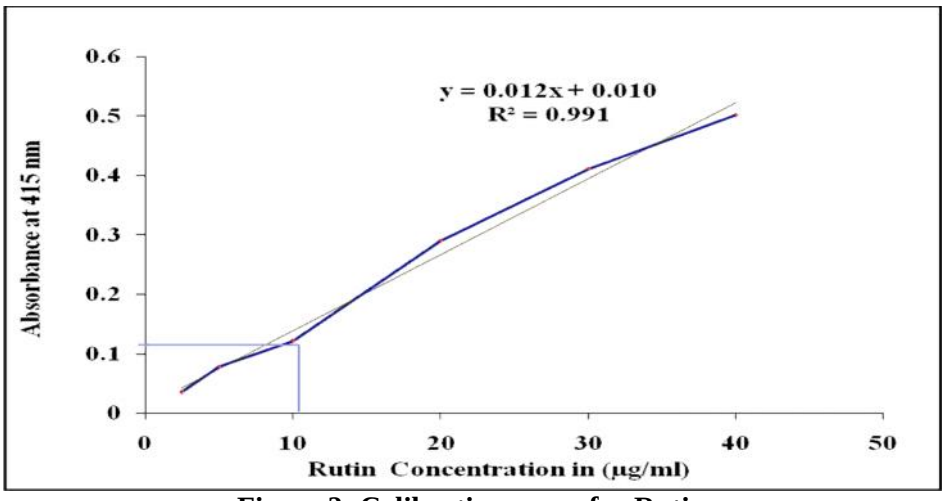

Figure 2: Calibration curve for Rutin

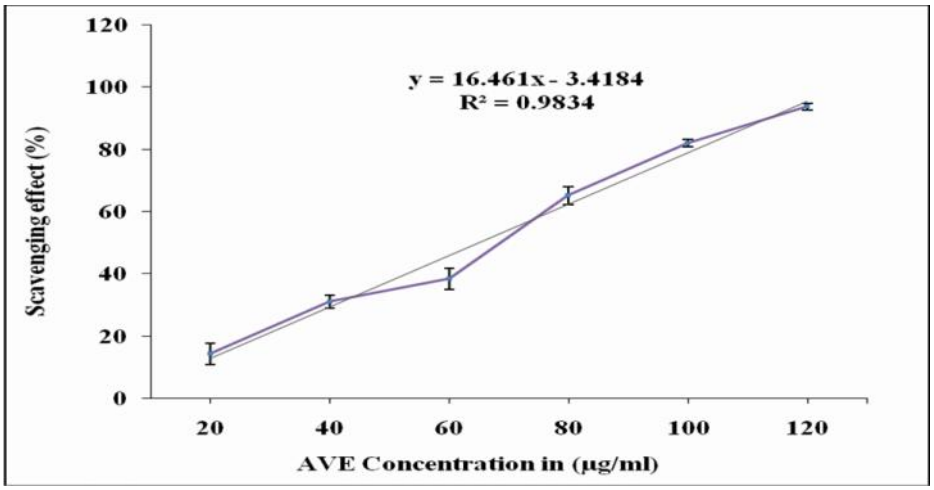

Figure 3: DPPH Radical Scavenging Activity of AVE

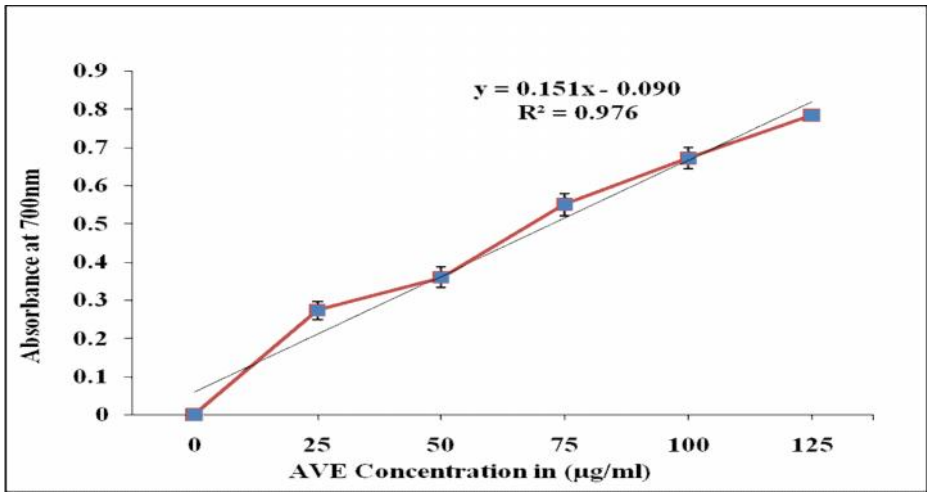

Figure 4: Reducing Power Potential of AVE

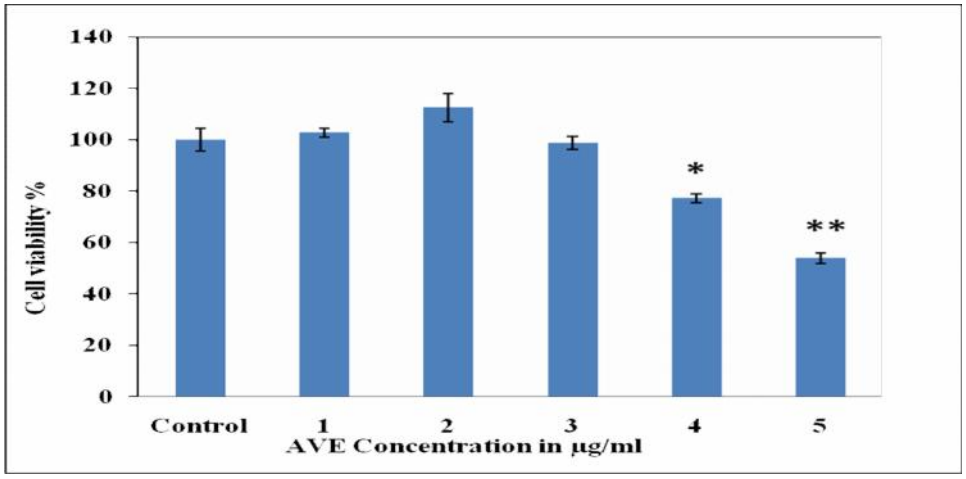

Figure 5: Effects of different concentrations of AVE on human alveolar cell line (A549).Concentration of 2 µg/ml is showing increase in cell viability. Concentrations more than 3 µg/ml seem to be toxic for the cells. For statistical evaluations, one-way ANOVA analysis followed by a Dunnett Multiple Comparisons Test were used. *, P < 0.05; **, P < 0.01

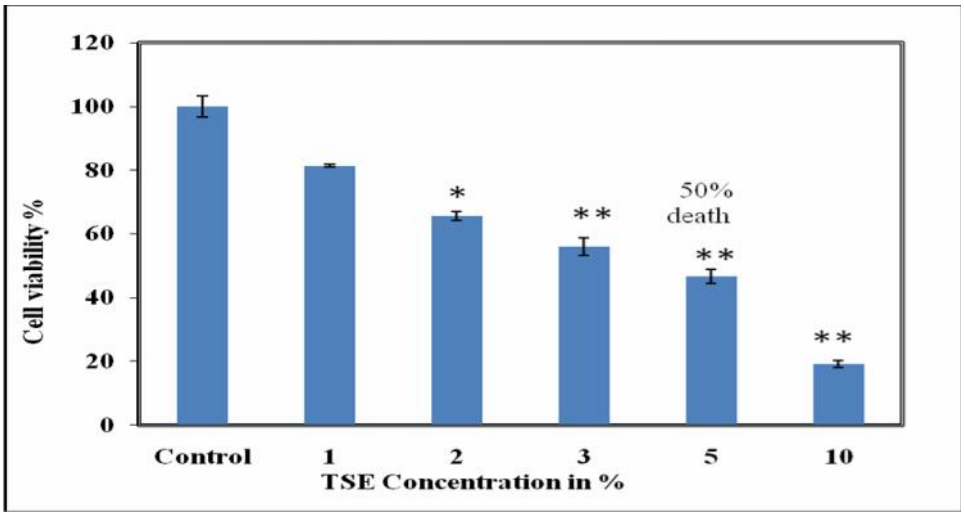

Figure 6: Effects of different concentrations of TSE on cell viability. 5% TSE caused almost 50% cell death as confirmed through MTT assay. Statistical data evaluations done using one-way ANOVA analysis, followed by a Dunnett Multiple Comparisons Test., *, P < 0.05; **, P < 0.01. Results were compared with untreated A549 cells as assessed by three independent experiments

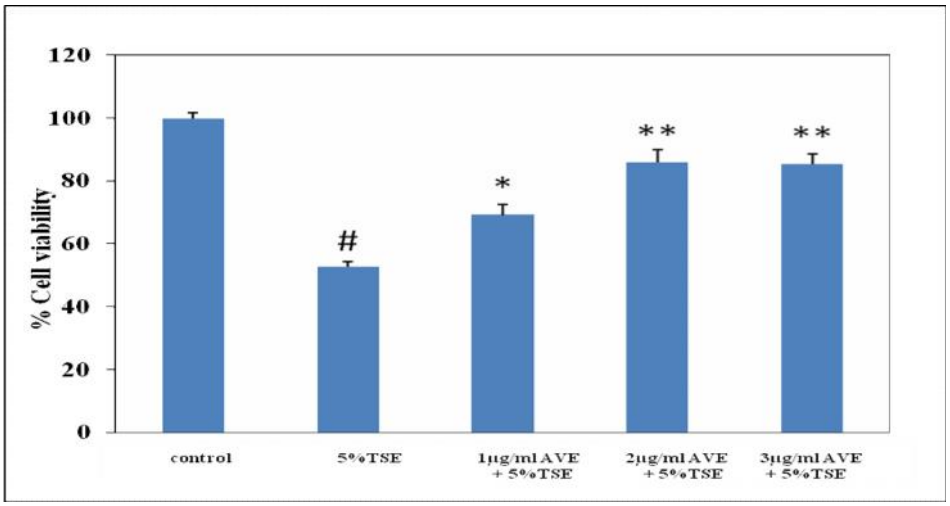

Figure 7: Effects of AVE (1 – 3 µg/ml) on TSE (5%) induced toxicity in A549 cells. The first bar depicts control group; followed by 5% TSE; pre-treated AVE (at 1 µg/ml, 2 µg/ml, and 3 µg/ml) + 5% TSE respectively. In the last three bars, the extract caused an increase in cell viability of 16.56 ± 3.36%, 33.37 ± 3.36% and 32.71 ± 3.38% for successive AVE concentrations as compared to 5% TSE treated cells (negative control) and untreated cells (positive control)

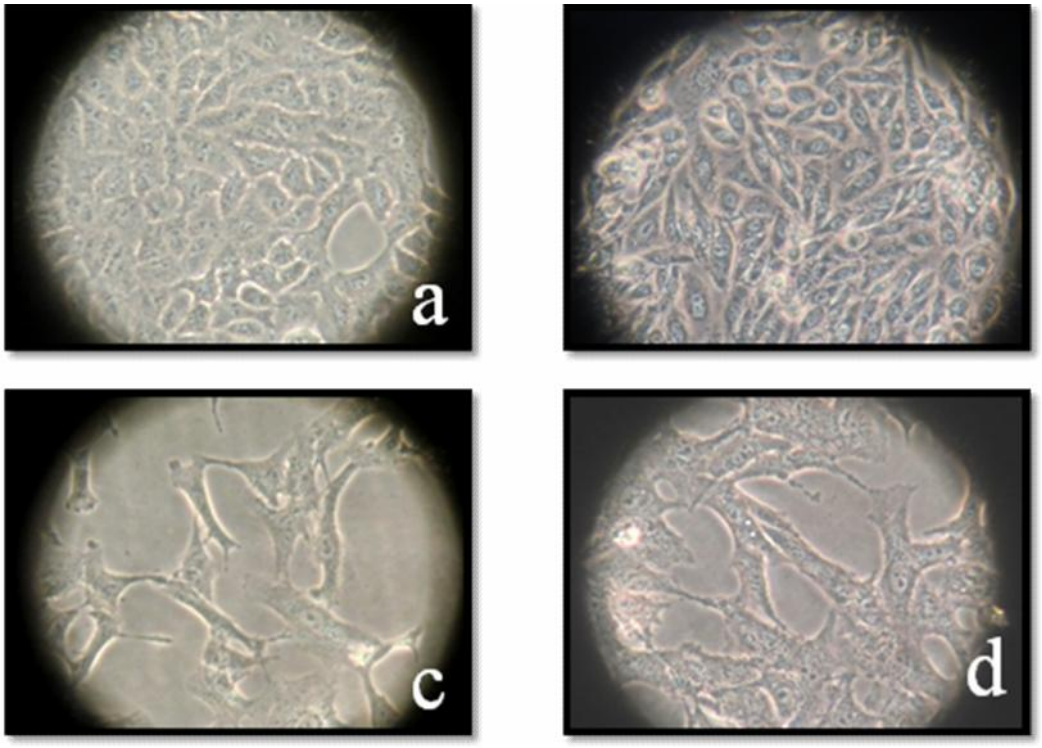

Figure 8: Microscopic analysis of A549 cells (a) control (untreated cells) (b) treated with 2 µg/ml AVE (c) 5% TSE treated and (d) 5%TSE treated cells which were pre-treated with 2 µg/ml AVE.

Discussion

Free radicals and oxidants are constantly generated in living systems and they are intimately associated with causation of extensive damage to tissues.[26] Normally, endogenous defense mechanisms play a key role in combating the harmful effects of ROS. But, in various conditions like exposure to various kinds of stresses or under pathological conditions, the increase in oxidants and a decrease in antioxidants impair the physiological homeostasis. Subsequent induction of oxidative stress initiates toxic effects in cells and tissues, which has been implicated in several human diseases.[27] Therefore, targeting to overcome the systemic and local oxidative stress with antioxidants/redox modulating agents, or enhancing the endogenous levels of antioxidants is foreseen as a novel pharmacological approach in the treatment/management of these disease conditions. Many synthetic drugs effectively alleviate oxidative injury but, the adverse side effects associated with them limits their widespread clinical use. Instead, antioxidants from traditional medicines could be considered owing to the fact that they are comparatively safer and commonly available.[28] Furthermore, due to the wide acceptance of traditional medicines among the population, phytopharmaceuticals with proven antioxidant property could become a suitable therapeutic alternative to current medication. They can also be used as adjuvants for treatment of disorders where oxidative stress is the underlying factor. But, validation of traditionally used herbal medicines through recent pharmacological investigations before using them clinically is essential. Antioxidants isolated from different plant materials have been found to protect the cells against the detrimental effects of ROS[29-31] thus, establishing an inverse relationship between the consumption of antioxidant rich plants and the incidence of human diseases.[32]

Presence of many compounds like; phenols and flavonoids have been reported to be present in traditional herbal medicine system those are efficient free radical scavengers.[33] Phenolic compounds and flavonoids are the major constituents in *A.vasica* also. Phenolic compounds and flavonoids are known for their hydrogen or electron donating and metal ion chelating properties.[5, 34-36] This study showed that *A. vasica* has a good amount of phenolic component (88.77mg/g, w/w) and flavonoids (55.28 mg/g, w/w) as well as an IC_{50} value of 68 µg/ml in DPPH assay as compared to previous study (phenolic component - 81.51 mg/g, flavonoids- 46.49 mg/g, IC_{50}value - 337 µg/ml).[37,38] This might be attributed to the difference in region of collection of sample as well as, the harvesting time of the plant.

In the present study two main procedures have been used to evaluate the antioxidant potential of AVE. The DPPH free radical scavenging assay provides an easy, rapid and sensitive method to evaluate capacity of plant extract to donate hydrogen or to scavenge free radicals.[39,40] The IC_{50} value is a parameter used to quantify antioxidative activity and it is defined as the extract concentration required for 50% quenching of DPPH radicals under experimental condition employed. The second method used was FRAP assay. Unlike DPPH assay, the FRAP assay does not involve any free radicals but the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) is monitored. In the present study, the reductive ability of the extract observed in FRAP assay reflected that the antioxidants contained in the extracts acted as reductants in this redox-linked colorimetric reaction.[41] The reducing capacity of AVE may serve as a significant indicator of the potential antioxidant activity of *A. vasica* which could have manifested due to the contribution of different active principles present in the extract. This report is in agreement with several reports that showed a close relationship between total phenolics content and high antioxidant activity.[41-43] This study, therefore, suggests that the antioxidant potential of the *A. vasica* extract may be envisaged as its curative ability by adsorbing and neutralizing free radicals or by chelating metal ions, subsequently preventing the deleterious oxidative processes. Also, significant correlation between the antioxidant capacities was achieved as shown by the DPPH and FRAP assay because both of these assays share the same principle of single electron transfer.

Exposure to various toxic compounds may lead to elevate ROS level in a biological system. ROS are responsible for cytotoxicity and natural antioxidant is very useful for balancing the level of ROS. In this *in vitro* study, it was found that a dose of 5% TSE induces significant (50% death) extent of toxicity in A549 cells.

As reported above and in several old studies, *Adhatoda vasica* has numerous phytocompounds which are responsible for antioxidant activity.[18] The present study proves that pre-exposure of the A549 cells to AVE (1 – 3 µg/ml), can overcome the toxic effect caused by TSE. It can thus be proposed that AVE might provide protection to the oxidatively stressed cells by increasing the activity of succinate dehydrogenase in the mitochondria. In line/ agreement with this is the microscopic analysis that also revealed the cytoprotective action of AVE supporting the finding in MTT assays. Therefore, future extensive studies are warranted to investigate the antioxidant activity of this extract at cellular level as these *in vitro* results can enable assessment of potential *in vivo* efficacy of AVE.

On the basis of the results obtained in the present study, it can be concluded that an ethanolic extract of *A. vasica* leaf powder exhibits high antioxidant and free radical scavenging activities which may be accounted for the high phenolics and flavonoids content. Its ability to chelate iron and high reducing power and/or free radical scavenging property could be ascribed to the observed effects. The cytoprotective effect of AVE was also evidenced in A549 cell culture owing to attenuation of cell death in presence of AVE. Taken together, these *in vitro* assays clearly revealed that, this plant extract is a promising source of natural antioxidants, and it can reasonably be anticipated that the extract could have useful applications in preventing the progression of conditions where oxidative stress plays a pivotal role. Therefore, it merits further investigation to isolate and identify the antioxidant compounds present in the plant extract. Furthermore, the proof of bio-efficacy of this extract must emanate from *in vivo* models prior to clinical use.

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