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Research Article

ANTIOXIDANT AND FREE RADICAL SCAVENGING POTENTIAL OF ETHANOLIC FRACTION OF *ADHATODA VASICA* IN A549 CELL LINE

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ABSTRACT

Objective: To characterize and standardize the ethanolic extract of Adhatoda vasica (AV) and evaluate its antioxidant potential *in vitro*, and to study its free radical scavenging effect in A549 cell line.

Methods: Antioxidant activity of AVE through DPPH, ABTS, superoxide radical scavenging activity, H2O2, NO and Lipid peroxidation assays were carried out.

Results: In A549 cell line, the NO scavenging ability of AVE was analysed through NO production measurement. IC_{50} values of ethanolic extract through DPPH, ABTS, superoxide radical scavenging activity, H_2O_2, NO and lipid peroxidation assays were found to be 64, 200, 54, 62, 40 and 340 µg/ml, respectively. The reducing power was found to increase in a concentration dependent manner. AVE (1 - 2µg/ml) scavenged NO radicals (11% and 28% in comparison to control).

Conclusion: The present study showed the ethanolic extract of AV to be a good oxygen radical scavenger and a natural source of antioxidants. AVE showed significant nitric oxide radical scavenging activity in A549 cell line.

Keywords: *Adhatoda vasica*, Phytochemicals, Flavonoids, Tannins, Antioxidant potential.

INTRODUCTION

The importance of oxygen-derived free radicals, commonly named reactive oxygen and nitrogen species (ROS and RNS) in health and disease is now recognized by every branch of medicine and biological science [1]. The most common ROS include superoxide anion (O_2–), hydrogen peroxide (H_2O_2), peroxyl radicals (ROO•), and RNS such as nitric oxide (•NO) and peroxynitrite (ONOO–), which is chemically unstable or highly reactive [2]. ROS and RNS play an important role in cell signaling for energy production, phagocytosis, and intercellular signaling. Various internal and external stressors increase the levels of ROS and RNS significantly, thereby troubling the equilibrium between ROS/RNS production and causing oxidative stress [3]. These excessively produced ROS/RNS can injure cellular biomolecules such as proteins, carbohydrates, nucleic acids, and lipids causing cellular and tissue damage [4,5]. This damage has shown to be associated with an increased risk of carcinogenesis and various degenerative disorders such as respiratory diseases, cardiovascular diseases, aging and neuro-degenerative diseases, atherosclerosis and rheumatoid arthritis, etc.

Antioxidants are compounds that trap free radicals (ROS and RNS), thus inhibiting the oxidative mechanisms that lead to degenerative diseases. These antioxidants could be either synthetic (butylated hydroxyanisole and butylated hydroxytoluene [BHT], etc.) or natural (plant secondary metabolites such as polyphenols and flavonoids), and the latter is in focus as an herbal remedy since they are obtained from medicinal plants [6-8]. These natural antioxidants have been reported to protect the human body from free radicals and have been shown to retard the progress of many chronic diseases.

The plant for the present study is *Adhatoda vasica* (AV) (Family: Acanthaceae); an evergreen shrub found all over India. It is an Ayurvedic medicinal plant which is a home remedy for several diseases [9]. AV is traditionally used for the treatment of respiratory disorders such as asthma, acute and chronic cough, and bronchitis. The different parts of the plant are also used for the treatment of joint pain, lumber pain and sprains, eczema, malaria, rheumatism, swellings, venereal diseases, etc. [10-12]. It is also used as an expectorant in the treatment of acute and chronic bronchial catarrh and pulmonary diseases. It is an official drug and is mentioned in the India Pharmacopeia [13]. In homeopathy also, the preparation from AV has been used in the treatment of cold, cough, pneumonia, spitting of blood, fever, jaundice, catarrh, whooping cough, and asthma. Limited information exists on antioxidant activity of AV. Karthikeyan *et al.* examined the phytochemical properties of ethanolic extracts of AV (AVE) but the extraction method was different, and no findings are recorded for the antioxidant activity of this extract [14]. In spite of its various uses and mechanism of action reported until date, the present study is aimed to evaluate the preliminary phytochemical screening, antioxidant and ROS/RNS scavenging activity of AVE in a cell-free system as well as its NO radical scavenging activity in human lung alveolar (A549) cell line.

METHODS

All the analytical grade reagents *viz*. Sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_2$), Sodium hydroxide (NaOH), Folin–Ciocalteu reagent, Sodium acetate, ethylenediaminetetraacetic acid (EDTA), potassium acetate, ferric thiocyanate (FTC), ammonium thiocyanate, trichloroacetic acid (TCA), ferric chloride ($FeCl_3$), glacial acetic acid, acetonitrile, and riboflavin were purchased from Central Drug House (P) Ltd., New Delhi. Other reagents such as aluminum chloride ($AlCl_3$), TCA, potassium ferricyanide $\{K_3Fe(CN)_6\}$, 2-thiobarbituric acid (TBA), potassium bromide (KBr), and hydrogen peroxide (H_2O_2) were purchased from Qualigens Fine Chemicals, Mumbai. The nitroblue tetrazolium (NBT) and ascorbic acid were procured from Himedia Laboratories, Mumbai; Sodium nitroprusside from SRL Limited, New Delhi; Griess reagent, Linoleic acid, BHT, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic) acid (ABTS), phenazine methosulfate, potassium persulfate, and gallic acid from Sigma-Aldrich Co., India.

Plant material

AV dried leaf powder was purchased from Jeevan Herbs, Madhya Pradesh, India. AVE extract was prepared exhaustively extracted with 90% ethanol in Soxhlet's apparatus for 72 hrs. The AVE thus obtained was collected and concentrated using rotary evaporator under reduced pressure at <40°C. Then the extract was stored in aliquots at –80°C (stock) until used for further study.

Fluorescence characteristics of powdered drug under visible and ultra-violet light

Powdered drug was screened for fluorescence characteristic with and without chemical treatment. The observations pertaining to the color in daylight and under ultra-violet light (short and long) were noticed [15].

Antioxidant activities of AVE leaves

DPPH scavenging activity

DPPH scavenging activity of AV extract was determined by the method of Liyana-Pathirana and Shadidi [16]. 1 ml of DPPH (0.135 mM) was mixed with 1 ml of different concentrations of AVE (0-100 µg/ml). The reaction mixture was vortexed thoroughly and left in dark at room temperature for 30 minutes. Ascorbic acid was used as reference standard while ethanol was used as a control. Change in color from deep-violet to light-yellow was measured at 517 nm. The percentage inhibition of free radical DPPH was calculated from the following equation:

$$\% \text{ inhibition} = \frac{A_{control} - A_{test}}{A_{control}} \times 100$$

All the tests were carried out in triplicates. Though the activity is expressed as 50% inhibitory concentration, IC_{50} was calculated based on the percentage of DPPH radicals scavenged. The lower the IC_{50} value, the higher is the antioxidant activity.

ABTS scavenging activity

The ABTS scavenging activity of AV was determined by the method of Brand–Williams *et al.* (1995) [17]. 7 mM ABTS solution and 2.4 mM potassium persulfate solution was added in equal volume and incubated for 12-16 hrs at room temperature in dark. 1 ml of freshly prepared ABTS**+** solution was added to the resulting mixture. The samples of AVE and standard (BHT) were mixed individually with the resulting mixture in 1:1 ratio. After 10 minutes of incubation, the absorbance was measured at 734 nm. The quenching or inhibition capacity of the extract for ABTS·+ and standard BHT was calculated. The percentage of scavenging capacity of the extract was calculated by the equation as mentioned above.

Superoxide anion scavenging activity

The superoxide scavenging activity of AVE was determined by the riboflavin-NBT assay as adapted from Lai *et al*., 2008 [18]. Briefly, AVE in distilled water (0.1 ml) at different concentrations was first mixed with 2.9 ml of 20 mM phosphate buffer (pH 7.2) containing 0.2 ml of 0.1 M EDTA and 0.1 ml of 1.5 mM NBT (prepared in 0.1 M phosphate buffer, pH 7.4). After incubation at 37°C for 10 minutes, 0.05 ml of 1.2 mM riboflavin (prepared in 0.1 M phosphate buffer, pH 7.4) was added. The reaction mixture was illuminated with a 25 W light tube for 15 minutes in a foil-lined box. The absorbance was measured at 560 nm. The percent of superoxide scavenging capacity of the extracts was calculated individually using the above-mentioned equation.

H_2O_2 radical scavenging activity

Scavenging of hydrogen peroxide by AVE was determined by the method of Ruch *et al.* (1989) with minor variation [19]. AVE at various concentrations (50-800 µg/ml) in distilled water were mixed with 4 mM H_2O_2 solution (prepared in 0.1 M phosphate buffer, pH 7.4) and incubated for 10 minutes at room temperature. Ascorbic acid was used as standard, and the absorbance of the solutions were taken at 230 nm.

NO scavenging activity

The NO radical scavenging activity of AVE was determined by the method of Garrat [20]. 2 ml of 10 mM sodium nitroprusside (prepared in 0.1 M phosphate buffer, pH 7.4) was mixed with 0.5 ml of AVE at various concentrations (5-100 µg/ml) and incubated for 2 hrs at 27°C. The resulting solution was further mixed with 100 µl Griess reagent and incubated at room temperature for 20 minutes. The absorbance of the chromophore was measured at 540 nm. The percentage NO radical scavenging activity of AVE and standard (ascorbic acid) was calculated using the formula mentioned in the case of DPPH scavenging activity.

Reducing power assay

The ferric reducing power of AVE was determined using the FRAP method [21,22]. Different concentrations of the plant extract (0-150 µg/ml) were mixed with phosphate buffer (0.2M, pH 6.6) to make up the volume to 350 µl, and then 250 µl of 1% $K_3Fe(CN)_6$ was added. The reaction mixture was incubated for 20 minutes at 50°C followed by rapid cooling of the solution. 250 µl of 10% TCA was added to this mixture to stop the reaction. The mixture was vortexed and centrifuged at 500 × *g* for 5 minutes. 250 µl of the above layer was taken and to it were added 250 µl of distilled water and 50 µl of 0.1% $FeCl_3$ followed by incubation of the reaction mixture for 30 minutes at room temperature, for the development of blue color. The absorbance was measured at 700 nm, and the activity was reported as OD values in comparison to the reference standard BHT.

Lipid peroxidation assays

The antioxidant activity of the extracts against lipid peroxidation was determined using FTC and TBA methods. The FTC method was used to evaluate the peroxides at the initiation of lipid peroxidation, and TBA method was used to analyze free radicals after the oxidation of peroxides. The inhibition of lipid peroxidation was estimated by the % inhibition formula as mention before.

FTC method

The standard method described by Kikuzaki *et al.* (1991) [23] was used for FTC determination. A mixture of 4 mg of sample (final concentration of 0.02% w/v) in 4 ml of 99.5% ethanol, 4.1 ml of 2.5% linoleic acid in 99.5% ethanol, 8 ml of 0.02 M phosphate buffer (pH 7.0), and 3.9 ml of distilled water contained in screw cap vial was placed in an oven at 40°C in the dark.

To measure the extent of antioxidant activity, 0.1 ml of the reaction mixture was transferred to a test tube and, to it 9.7 ml of 75% (v/v) aqueous ethanol, followed by 0.1 ml of 30% aqueous ammonium thiocyanate and 0.1 ml of 0.02 M ferrous chloride in 3.5% hydrochloric acid were added. 3 minutes after the addition of ferrous chloride to the reaction mixture, the absorbance of the resulting mixture (red) was measured at 500 nm after 24 hrs until the absorbance of the control reached its maximum. BHT (final concentration of 0.02% w/v) was used as positive control while the mixture without the plant extract was used as the negative control.

TBA method

The method of Ottolenghi modified by Kikuzaki and Nakatani was used for determination of free radical scavenging activity of ethanolic leaf powder extract [23,24]. The final sample concentration of 0.02% w/v from the same samples prepared for FTC assay was used. 2 ml of 20% TCA and 2 ml of 0.67% of TBA were added to 1 ml of sample solution from the FTC method. The mixture was boiled for 10 minutes, cooled and centrifuged at 400 × *g* for 20 minutes. The absorbance activity of the supernatant was measured at 552 nm, and recorded after it has reached its maximum.

Measurement of NO production in A549 cell line

A549 cells (human alveolar Type II basal epithelial cell line) were obtained from the National Centre for Cell Science, Pune, India and cultured by a standard method [25]. The production of NO was estimated spectrophotometrically as nitrite formed in cell-free culture supernatants of differently treated samples as per standard protocol [26].

RESULTS

Fluorescence analysis

Fluorescence analysis of AV leaves powder is summarized in Table 1.

The phytochemical screening of AVE was discussed in our previous articles in which the presence of alkaloids, steroids, flavonoids, and reducing sugars, etc. were found in AVE.

DPPH radical scavenging activity of AVE

The plant extract exhibited a dose-dependent DPPH radical scavenging activity (Fig. 1). The IC_{50} value of AVE required to scavenge 50% of DPPH• was 64 µg/ml.

ABTS radical scavenging activity of AVE

In ABTS assay, the plant extract exhibited a dose-dependent ABTS radical scavenging activity (Fig. 2). The IC_{50} value of AVE required to scavenge 50% of ABTS• was 200 µg/ml.

Superoxide anion scavenging activity

As shown in Fig. 3, AVE at a concentration of 0-100 µg/ml has shown a dose-dependent superoxide radical scavenging activity using NBT reduction method. The 50% inhibition of NBT reduction by the AVE has been found to be 54 µg/ml.

H_2O_2 scavenging activity

The H_2O_2 scavenging activity of both AVE and standard decreased with increasing concentration (Fig. 4). The IC_{50} value was found to be 62 µg/ml for AV and 30 µg/ml for ascorbic acid.

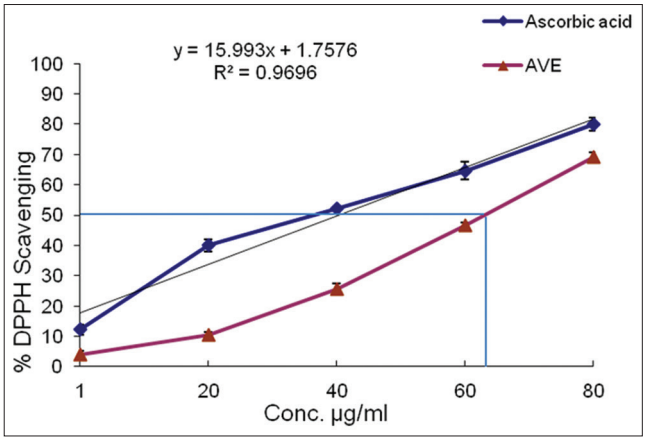

Fig. 1: 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity of ethanolic extract of *Adhatoda vasica* in comparison ascorbic acid

NO scavenging activity

The AVE showed a concentration-dependent increase in NO scavenging activity (Fig. 5). The IC_{50} value was found to be 40 µg/ml for AV and 28 µg/ml for ascorbic acid.

Reducing power assay

A dose-dependent increase in reducing power of the tested sample (as indicated by the absorbance at 700 nm) was observed, as shown in Fig. 6. The reducing ability of plant extract was lower than that of BHT.

FTC assay

AVE showed significant inhibition of peroxides with increasing concentrations. The IC_{50} value for AVE based on FTC assay was found to be 0.340 mg/ml as compared to 0.300 mg/ml for BHT (Fig. 7a).

TBA reactive substances (TBARS) assay

AVE could also reduce TBARS when compared with a standard anti-oxidant BHT. The IC_{50} value for BHT and AVE was found to be 0.280 mg/ml and 0.430 mg/ml, respectively. The anti-lipid peroxidation activity of the AVE was comparable to that of BHT standard (Fig. 7b).

Effect of treatment with AVE on NO production in A549 cell line

AVE (1-2 µg/ml) treatment showed a decrease in NO level (11 and 28%, respectively, wherein 2 µg/ml showed a significant decrease in NO level) as compared to control (untreated cells) in cells (Fig. 8). This result clearly showed that AVE scavenges free radicals and act as antioxidant endogenously in A549 cells.

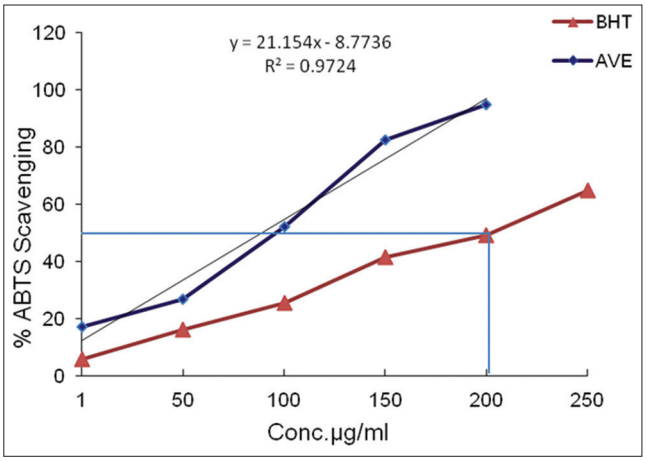

Fig. 2: 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic) radical scavenging activity of ethanolic extract of *Adhatoda vasica* in comparison to butylated hydroxytoluene

Table 1: Fluorescence analysis of AV leaves powder

S. no.	Solvents treatment	Visible light	Short UV (252 nm)	Long UV (366 nm)
1.	Drug as such	Olive	Green	Olive dark
2.	Drug ammonia soln.	Violet brown	Dark green	Green yellow
3.	Drug+picric acid	Olive	Olive dark	Dark gray
4.	Drug+$FeCl_3$ 5% soln.	Dark brown	Dark green	Black
5.	Drug+iodine soln. (5%)	Dark gold	Dark green	Invisible
6.	Drug+1 M NaOH in water	Dark brown	Lawn green	Dark green
7.	Drug+petroleum ether	Brown	Green	Lawn green
8.	Drug+chloroform	Dark olive green	Green	Olive dark
9.	Drug+ethyl acetate	Dark olive green	Green	Olive dark
10.	Drug+1 M H_2SO_4	Dark goldenrod	Forest green	Dark brown
11.	Drug+1 M HCl	Brown dark	Green	Olive dark
12.	Drug+ethanol (99%)	Olive dark	Green	Olive dark
13.	Drug+methaznol (99%)	Olive dark	Green	Olive dark

AV: *Adhatoda vasica*

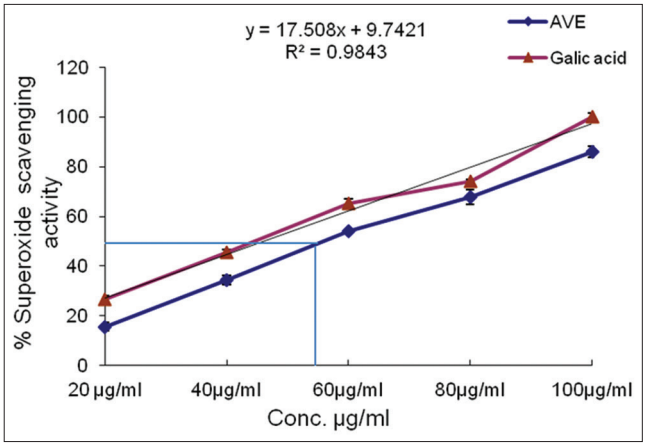

Fig. 3: Percentage inhibition of superoxide anion by ethanolic extract of *Adhatoda vasica* and Gallic acid

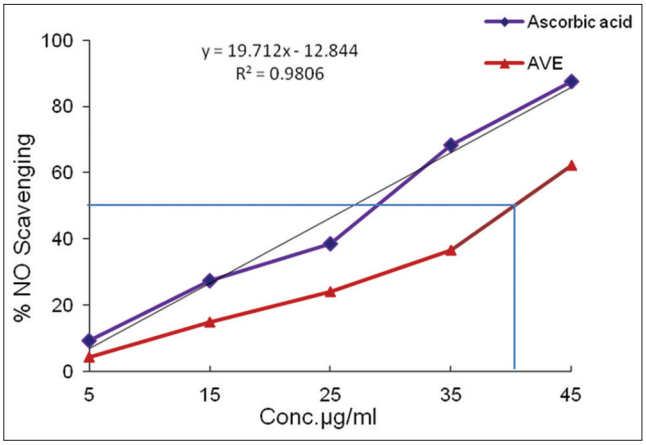

Fig. 5: Nitric oxide radical scavenging activity of ethanolic extract of *Adhatoda vasica* in comparison ascorbic acid

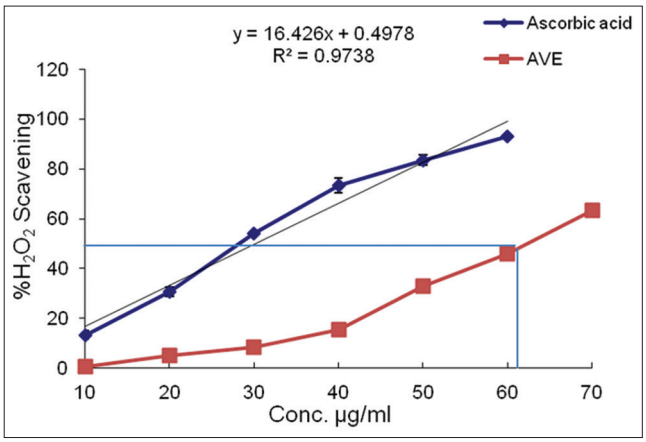

Fig. 4: Percentage inhibition of hydrogen peroxide scavenging activity of ethanolic extract of *Adhatoda vasica* in comparison to ascorbic acid

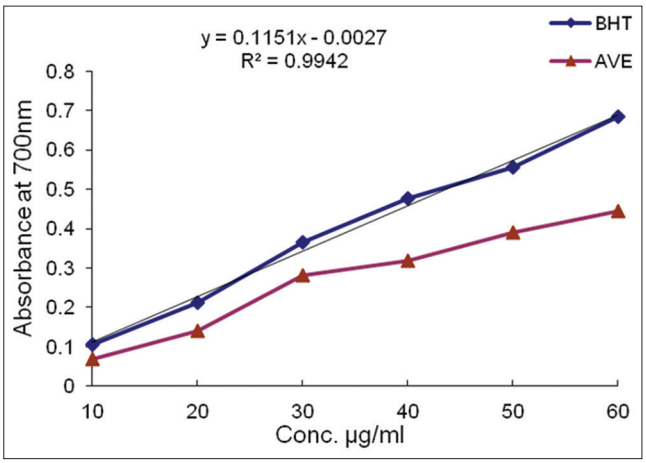

Fig. 6: Reducing power of ethanolic extract of *Adhatoda vasica* in comparison to butylated hydroxytoluene

Statistical analysis

For quantitative analysis, all data in triplicates were expressed as mean ± standard deviation. The significance of differences in the data was evaluated by one-way ANOVA test followed by Tukey test. All the results were significant at $p<0.01$.

DISCUSSION

The fluorescence analysis of AV leaf powder showed color change under visible and UV light, thus indicating the presence of organic and inorganic compounds with fluorescence properties. The preliminary phytochemical studies of AV revealed the presence of various phytochemicals such as alkaloids, glycoside, tannins, saponin, steroids, tri-terpenoid and flavonoids, etc. [14]. The addition of AVE to the pre-formed radical cations (DPPH and ABTS) reduced it to DPPH and ABTS in a concentration-dependent manner. The IC_{50} value of DPPH and ABTS radical scavenging by AVE was found to be was 64 µg/ml and 200 µg ml, respectively. Besides this, the H_2O_2 scavenging activity of the plant extract was more (62 µg/ml) than that of the standard ascorbic acid (30 µg/ml).

NO is an unstable species whose toxicity greatly increases when it reacts with superoxide radical, forming the highly reactive peroxynitrite anion (ONOO–) [27]. AVE might be responsible for inhibition of nitrite formation by directly/indirectly competing with oxygen in the reaction with NO.

Superoxide radicals are also very harmful to cellular components [28]. Robak and Glyglewski have been reported that flavonoids are effective antioxidants because they mostly scavenge superoxide anions [29]. The superoxide radical scavenging activity of AVE and the reference compound were increased markedly with increasing concentrations. The results suggest that the plant extract is a potent scavenger of superoxide radical.

We also investigated the ability of AVE to prevent peroxidation of lipids by TBARS assay, which is based on the acid-catalyzed decomposition of lipid peroxides. The analysis of data confirms that increasing concentrations of AVE significantly inhibited the peroxides formation.

This study thus, showed that AVE possesses a considerable amount of polyphenols which might be contributing to its strong antioxidant property, as evident by a similar study by Karthikeyan *et al.*, 2009 [14]. This antioxidant activity is mainly due to its redox property, hydrogen donating ability, and singlet oxygen quenching, which can play an important role in adsorbing and neutralizing free radicals [30].

NO is an unstable species under aerobic condition and play an important role in many physiological as well as pathological mechanisms such as vasodilatation, inflammation, and malignant transformation [31]. The role of NO in lung carcinogenesis, including initiation, promotion, and malignant progression, has been extensively studied. Our previous study showed 1-2 µg/ml of AVE to be the optimum safe dose for A549 [32]. In the present study, we have observed that NO production was significantly scavenged by 1-2 µg/ml AVE in A549 cells. Thus, the present study proved that AVE might provide protection against RNS production inside cells through its free radical scavenging activity.

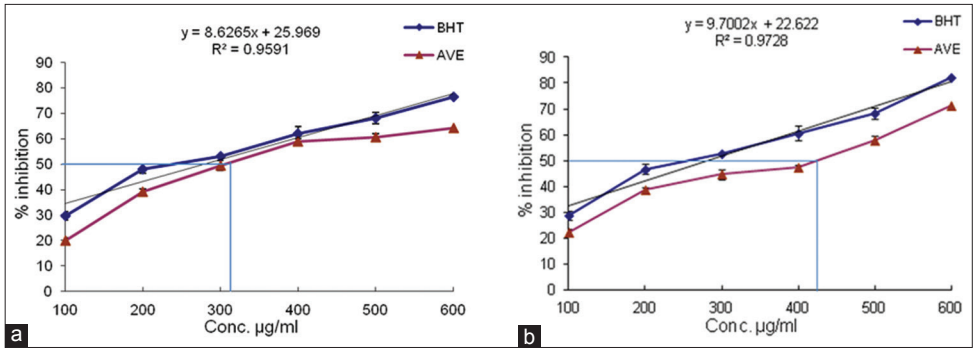

Fig. 7: (a) Antioxidant properties ethanolic extract of *Adhatoda vasica* (AVE) compared to the standards (butylated hydroxytoluene [BHT]) as determined with the ferric thiocyanate (500 nm) method, (b) Antioxidant properties AVE compared to the standards (BHT) as determined with the 2-thiobarbituric acid (552 nm) method

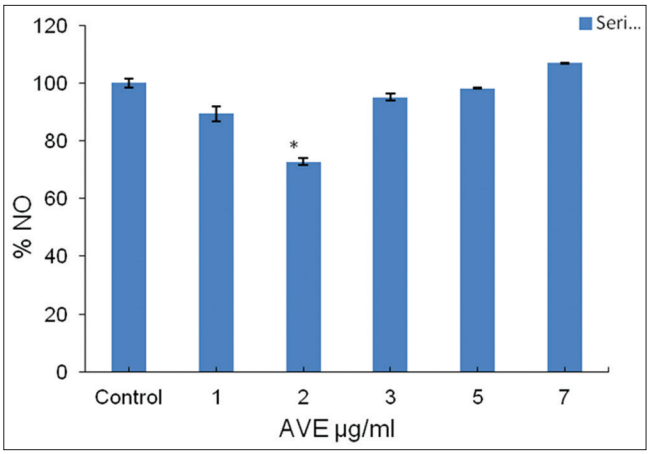

Fig. 8: Effect of different concentrations of ethanolic extract of Adhatoda vasica (AVE) (1-7 µg/ml for 3 hrs) on A549 cell line on NO level. NO level was decreased until 2 µg/ml after NO level was increased once the AVE concentration exceeded 2 µg/ml. For statistical evaluations, one-way ANOVA analysis followed by the Tukey test was used. *p<0.01

CONCLUSION

On the basis of the results obtained in the present study, it can be concluded that an AVE leaves contain a large amount of flavonoids and phenolic compounds that contribute to its good antioxidant and free radical scavenging activities. These *in vitro* assays indicate that this plant extract is a significant source of natural antioxidant, which might be helpful in preventing the progress of oxidative stress in various diseased conditions. Therefore, further investigation is needed to isolate and identify the antioxidant compounds present in the plant extract. Furthermore, the *in vivo* antioxidant activity of this extract requires to be assessed prior to clinical use.

REFERENCES

1. Oliveira I, Coelho V, Baltasar R, Pereira JA, Baptista P. Scavenging capacity of strawberry tree (*Arbutus unedo* L.) leaves on free radicals. Food Chem Toxicol 2009;47(7):1507-11.
2. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 2007;39(1):44-84.
3. Blokhina O, Virolainen E, Fagerstedt KV. Antioxidants, oxidative damage and oxygen deprivation stress: A review. Ann Bot 2003;91:179-94.
4. Aruoma OI. Assessment of potential pro-oxidant and antioxidant actions. J Am Oil Chem Soc 1996;73:1617-24.
5. Pulido R, Bravo L, Calixto S. Antioxidant of dietary polyphenols as determined by a modified ferric reducing antioxidant power assay. J Agric Food Chem 2000;46:3396-402.
6. Madhavi DL, Deshpande SS, Salunkhe DK. Toxicological Aspects of Food Antioxidants. New York: Dekker; 1995. p. 267.
7. Bursal E, Gulcin I. Polyphenolcontents and *in vitro* antioxidant activities of lyophilised aqueous extract of kiwifruit (*Actinidia deliciosa*). Food Res Int 2011;44:1482-9.
8. Ebrahimzadeh MA, Nabavi SM, Nabavi SF, Bahramian F, Bekhradnia AR. Antioxidant and free radical scavenging activity of *H. officinalis* L. *Var. angustifolius, V. odorata, B. hyrcana* and *C. speciosum*. Pak J Pharm Sci 2010;23(1):29-34.
9. Rachana, Basu S, Pant M, Kumar MP, Saluja S. Review and future perspectives of using vasicine and related compounds. Indo Glob J Pharm Sci 2011;1:85-98.
10. Kirtikar KR, Basu BD, Basu LM. Indian Medicinal Plants. 2nd ed., Vol. III. Allahadad, India: Lalit Mohan Basu; 1935. p. 1593-2393.
11. Chopra RN, Nayer SL, Chopra IC. Glossary of Indian Medicinal Plants. Vth ed., Vol. 12. New Delhi: CSIR; 1956. p. 157.
12. Rastogi S, Mehrotra BN, Kulshreshtha DK. Proceedings of IV International Congress of Ethnobiology. New Delhi: Deep Publications; 1994. p. 93.
13. Anonymous. Pharmacopoeia of India. 2nd ed. New Delhi: Controller of Publications, Government of India; 1966. p. 792.
14. Karthikeyan A, Shanthi V, Nagasathaya A. Preliminary phytochemical and antibacterial screening of crude extract of the leaf of *Adhatoda vasica*. L. Int J Green Pharm 2009;3:78-80.
15. Kokashi CJ, Kokashi RJ, Sharma M. Fluorescence of powdered vegetable drugs in ultra-violet radiation. J Am Pharm Assoc 1958;47:715-7.
16. Liyana-Pathirana CM, Shahidi F. Antioxidant activity of commercial soft and hard wheat (*Triticum aestivum* L.) as affected by gastric pH conditions. J Agric Food Chem 2005;53(7):2433-40.
17. Brand-Williams W, Cuvelier ME, Berset C. Use of a radical method to evaluate antioxidant activity. Lebenson Wiss Technol 1995;28:25-30.
18. Lai LS, Chang PC, Chang CT. Isolation and characterization of superoxide dismutase from wheat seedlings. J Agric Food Chem 2008;56(17):8121-9.
19. Ruch RJ, Cheng SJ, Klaunig JE. Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. Carcinogenesis 1989;10(6):1003-8.
20. Garrat DC. The Quantitative Analysis of Drugs. 3rd ed. Japan: Chapman and Hall; 1964.
21. Amir M, Khan A, Mujeeb M, Ahmad A, Usmani S, Akhtar M. Phytochemical analysis and *in vitro* antioxidant activity of *Zingiber officinale*. Free Radic Antioxid 2011;1:75-81.
22. Sivakumar V, Rajan MS, Riyazullah MS. Preliminary phytochemical screening and evaluation of free radical scavenging activity of *Tinospora cordifolia*. Int J Pharm Pharm Sci 2010;2:186-8.
23. Kikuzaki H, Usuguchi J, Nakatani N. Constituents of Zingiberaceae I. Diarylheptanoid from the rhizomes of ginger (*Zingiber officinale Roscoe*). Chem Pharm Bull 1991;39:120-2.
24. Ottolenghi A. Interaction of ascorbic acid and mitochondria lipids. Arch Biochem Biophys 1959;79:355-63.
25. Cushion MT, Walzer PD. Growth and serial passage of *Pneumocystis carinii* in the A549 cell line. Infect Immun 1984;44(2):245-51.
26. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. Anal Biochem 1982;126(1):131-8.

27. Huie RE, Padmaja S. The reaction of no with superoxide. Free Radic Res Commun 1993;18(4):195-9.
28. Korycka-Dahl M, Richardson T. Photogeneration of superoxide anion in serum of bovine milk and in model systems containing riboflavin and amino acids. J Dairy Sci 1978;61:400-7.
29. Robak J, Gryglewski RJ. Flavonoids are scavengers of superoxide anions. Biochem Pharmacol 1988;37(5):837-41.
30. Sahaa MR, Hasana SM, Aktera R, Hossaina MM, Alamb MS, Alam MA, *et al*. *In vitro* free radical scavenging activity of methanol extract of the leaves of *Mimusops elengi* Linn. Bangladesh J Vet Med 2008;6(2):197-202.
31. Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev 2007;87(1):315-424.
32. Mamta P, Sujata B, Rachana. Anti-oxidant activity and cytoprotective potential of ethanolic extract of *Adhatoda vasica*. Int J PharmTech Res 2013;5(2):501-10.