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Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

J P P Journal of Pharmacognosy and Phytochemistry

ISSN 2278-4136
ISSN 2349-8234
JPP 2014; 2 (5): 55-59

Received: 20-11-2013
Accepted: 04-12-2013

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GC-MS profiling and *in vitro* radical scavenging effect of *Adhatoda beddomei*

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ABSTRACT

To investigate the *in vitro* radical scavenging assays and phytochemical identification of an ethanol leaf extract of *Adhatoda beddomei* (AB). The fresh leaves of AB (1000 gm) were shade dried at room temperature for 30 days and the dried leaves was made into a fine powder. The phytochemical screening studies identified fourteen chemical constituents present in the leaf extract of AB. The ethanol extract of AB exhibited a significant dose dependent inhibition of *in vitro* radical scavenging assays and their corresponding IC_{50} values as follows such as Hydroxyl radical (48.10±0.49 µg/mL), Superoxide anion (34.50±0.49 µg/mL), Nitric oxide radical (49.11±0.49 µg/mL), DPPH radical (50.11±0.50 µg/mL) and Reducing power (16.80±0.16 µg/mL). Thus, our results show that AB exhibits various *in vitro* radical scavenging assays and possess important bioactive phytocompounds such as squalene and vitamin E, campesterol, stigmasterol, *β*-Sitosterol and lupeol.

Keywords: *Adhatoda beddomei*, Antioxidants, Oxidative stress, Ayurveda, Superoxide radical, DPPH assay.

1. Introduction

Oxidative stress is characterized by an imbalance between reactive species and endogenous antioxidants that disrupts the intracellular reduction-oxidation (redox) balance, which results in oxidative damage to biological molecules and impairment in signaling pathways. It has been widely implicated in a number of diseases, such as cardiovascular, pulmonary conditions, diabetes, eye diseases, aging and cancer [1].

Adhatoda beddomei (AB) is an evergreen herb, growing to a height of 80 cm. The plant is a member of Acanthaceae family. It is a common shrub widely distributed throughout India [2]. In Ayurveda, the ancient system of Indian medicine is commonly known as Vasa [3]. The medicinal properties of plants are widely used for the treatment of leprosy, blood disorders, heart troubles, thrist asthma, fever, vomiting, loss cough, asthma[4], diseases of eyes, bleeding diarrhea, dysentery, bronchitis, inflammation, jaundice, tumors, mouth-troubles, sore-eye, fever, gonorrhoea, tuberculosis, hemorrhage and haemorrhoids [5]. Its leaves, bark, the root-bark, the fruit and flowers are useful in the removal of intestinal parasites [6]. Thus the aim of our present study was to investigate the phytochemical identification by GC-MS analysis and *in vitro* radical scavenging assays.

2. Materials and Methods

2.1 Chemicals

2,2'azobis-3-ethylbenzthiazolie-6-sulfonic-acid (ABTS), 1,1-diphenyl-2-picryl hydrazil (DPPH), phenazine methosulphate (PMS), nitroblue tetrazolium (NBT), 5′,5′-dithio(bis)-2-nitrobenzoic acid (DTNB), nicotin amide adenine dinucleotide (NAD) and ascorbic acid were purchased from M/s. Sigma Chemical Co., St Louis, USA. All other chemicals and solvents were of analytical grade and obtained from Himedia, Mumbai, India.

2.2 Plant collection and preparation of the extract

Fresh leaves of *Adhatoda beddomei* (AB) were collected from Trivandrum district, Kerala, India. The plant specimen was authenticated by Mrs. Padmaja, an expert in the field of Botany and the Herbarium voucher specimen (Accession No-1065, Field book No-6097) was deposited in Ayurveda Research Institute for Mother and Child Health Care (ARIMCHC), Trivandrum.

The fresh leaves of AB (1000 gm) were shade dried at room temperature (28±2 ºC) for 30 days and the dried leaves was made into a fine powder (particle size-0.25 mm) by using an electric blender. About 250 g of dried leaf powder were extracted with 750 mL ethanol (60–80 ºC) for 2–3 h in a Soxhlet apparatus. The ethanol extract obtained was dried and used for *in vitro* radical scavenging and phytochemical identification.

2.3 Sample preparation for GC-MS

20 gm of the powdered leaves was soaked in absolute ethanol for 12 h. The extract was then filtered through Whatman filter paper No. 41 along with 2 gm sodium sulphate to remove the sediments and traces of water in the filtrate. Before filtering, the filter paper along with sodium sulphate was wetted with absolute ethanol. The filtrate was then concentrated by bubbling nitrogen gas into the solution. The extract contained both polar and non-polar phytocomponents of the plant extract was used. 2 µL of crude extract of these solutions was employed for GC/MS analysis [7].

2.4 GC-MS analysis

GC-MS analysis was carried out at Indian Institute of Crop Processing Technology (IICPT), Thanjavur, India, GC Clarus 500 Perkin Elmer system and gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions: Column Elite-1 fused silica capillary column (30 mm x 0.25 mm ID x 1 µmdf, composed of 100% Dimethylpolysiloxane), operating in electron impact mode at 70 ev; Helium (99.999%) was used as carrier gas at a constant flow of 1 mL/min and an injection volume of 2 µL was employed (Split ratio of 10:1); Injector temperature 250 ºC; Ion-source temperature 280 ºC. The oven temperature was programmed from 110 ºC (isothermal for 2 min) with an increase of 10 °C/min, to 200 °C, then 5 ºC/min to 280 °C, ending with a 9 min isothermal at 280 °C. Mass spectra were taken at 70 ev; a scan interval of 0.5 seconds and fragments from 45 to 450 Da. Total GC running time was 36 min.

2.5 Identification of components

Interpretation of mass spectrum GC-MS was conducted using the database of National Institute Standard and Techniques (NIST). WILEY 8 and FAME having more than 65,000 patterns. The spectrum of the unknown components stored in the NISTO8s, WILEY8 and FAME library. The name, molecular weight, molecular formula and structure of the component of the test material was ascertained [8]. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Software adopted to handle mass spectra and chromatograms was a GC-MS solution Ver.2.53.

2.6 Preparation of plant extract for biochemical assays

Ethanol extract (100 mg/mL) was dissolved in 0.05% dimethyl sulfoxide (DMSO) and was used as the stock solution. The stock solution was then diluted with sterile distilled water (Milli-Q water) to arrive at a final concentration of 20, 40, 60, 80, 100 µg/mL. 0.05% DMSO was used as a sham control.

2.7 *In vitro* radical scavenging assay

The antioxidant activity of the plant extract was determined in terms of hydrogen donating or radical scavenging ability, using the stable radical DPPH, according to the method of Bilios [9]. A measurement of superoxide anion scavenging activity of the extract was performed based on the method described by Nishimiki *et al.* [10]. Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and the ethanol extract for hydroxyl radical generated by Fe^{3+}-Ascorbate-EDTA-H_2O_2 system (Fenton reaction) according to the method described by Kunchandy and Rao[11]. The nitric oxide radical inhibition activities of the extracts were measured by the method described by Garrat [12]. The reducing power of plant extracts was determined according to the method of Oyaizu [13]. The percentage of inhibition was calculated by comparing the absorbance values of control and test samples. All the tests were performed for six times and the graph was plotted with the average of six observations.

2.8 Statistical analysis

Statistical analysis was performed using one way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT) by using statistical package of social science version 10.0 for windows. The values are expressed as mean ± SD for six experiments in each group and the P values <0.05 was considered as level of significance.

3. Results and Discussion

The phytochemical constituents identified and their pharmacological activities of ethanol leaf extract of AB was presented in Table 1. The GC-MS analysis of plant extract revealed the presence of fourteen chemical compounds (Phytochemical constituents) that could contribute the medicinal properties of the plant. The identification of the active principles present in the leaf extract was confirmed based on the peak area, retention time, molecular formula, molecular weight and peak area in percentage were shown in Table 1 and Fig. 1. The first compound identified with less retention time (8.10 min) was phosphoric acid, diethyl pentyl ester, whereas lupeol was the last compound which took longest retention time (33.42 min) to identify.

Table 1: Phytocomponents identified in the ethanol leaf extract of *Adhatoda beddomei* by GC-MS analysis

S. No	RT	Name of the compound	Molecular formula	MW	Peak area %
1	8.10	Phosphoric acid, diethyl pentyl ester	$C_9H_{21}O_4P$	224	0.18
2	11.36	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296	7.83
3	13.14	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	284	0.35
4	14.63	Phytol	$C_{20}H_{40}O$	296	19.44
5	15.36	9,12,15-Octadecatrienal	$C_{18}H_{30}O$	262	0.26
6	19.43	O-Ethyl S-2-dimethylaminoethyl methylphosphonothiolate	$C_7H_{18}NO_2PS$	211	0.79
7	20.45	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl ester)	$C_{16}H_{22}O_4$	278	1.14
8	24.27	Squalene	$C_{30}H_50$	410	9.76
9	25.20	7-Octadecyne, 2-methyl	$C_{19}H_{36}$	264	8.97
10	28.52	Vitamin E	$C_{29}H_{50}O_2$	430	2.37
11	29.99	Campesterol	$C_{28}H_{40}O$	400	5.28
12	30.48	Stigmasterol	$C_{29}H_{48}O$	412	19.17
13	31.65	β-Sitosterol	$C_{29}H_{50}O$	414	13.63
14	33.42	Lupeol	$C_{30}H_{50}O$	426	10.82

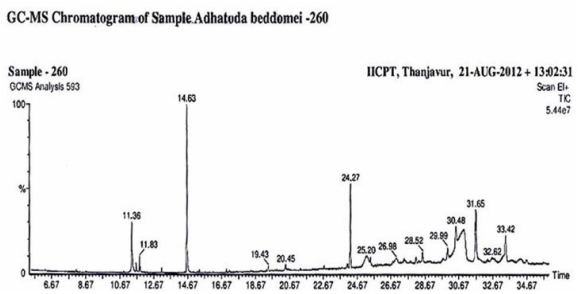

Fig 1: GC-MS analysis of ethanol leaf extract of *Adhatoda beddomei*

The antioxidant effect of the ethanol leaf extract of AB was studied using standard biochemical assays. Corresponding to different levels of antioxidant protection. All experiments were carried out with different concentration of AB to investigate whether an increase in concentration influences the antioxidant activity and the IC_{50} values were calculated and compared with that of the standard ascorbic acid for all experiments and is shown in Table 2.

Table 2: IC_{50} values of *Adhatoda beddomei* compared with that of standard Ascorbic acid

S.NO	Radical scavenging assays	Ascorbic acid (µg/mL)	*Adhatoda beddomei* (µg/mL)
1.	DPPH radical scavenging assay	50.11 ± 0.50^{c}	60.25 ± 0.50^{c}
2.	Hydroxyl radical scavenging assay	48.10 ± 0.48^{c}	50.25 ± 0.50^{c}
3.	Superoxide anion radical scavenging assay	34.50 ± 0.34^{b}	42.50 ± 0.42^{b}
4.	Nitric oxide radical scavenging assay	49.11 ± 0.49^{c}	55.25 ± 0.55^{c}
5.	Evaluation of reducing power assay	16.80 ± 0.16^{a}	15.18 ± 0.15^{a}

The results obtained by DPPH scavenging assay are shown in Fig. 2. The DPPH radical scavenging assay is based on the ability of DPPH, a stable free radical, to decolorize the presence of antioxidants. The DPPH radical contains an odd electron that is responsible for the absorbance at 540 nm and also in the visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized which can be quantitatively measured from the changes in absorbance. The ethanol extract of AB exhibited a significant dose dependent inhibition of DPPH activity, with a 50 % inhibition (IC_{50}) at a concentration of 60.25 µg/mL as compared with the standard ascorbic acid (50.11 µg/mL).

Superoxide anion indirectly initiated lipid peroxidation as a result of superoxide and hydrogen peroxide serve as a precursor of singlet oxygen and hydroxyl radicals. As shown in Fig. 3, AB was found to possess good scavenging activity on superoxide radicals and at 100 µg/mL ($p<0.05$), it exerts optimum scavenging effects. Since the optimum dose was calculated as 100 µg/mL, further increase in the concentration of AB beyond the optimum dose did not produce any significant increasing scavenging effect and this may be due to the saturation of the molecule in the system. The IC_{50} value of AB on superoxide anion scavenging effect was found to be 42.50 µg/mL, which was found to be compared with that of standard ascorbic acid (34.50 µg/mL). The results were found to be statistically significant ($p<0.05$).

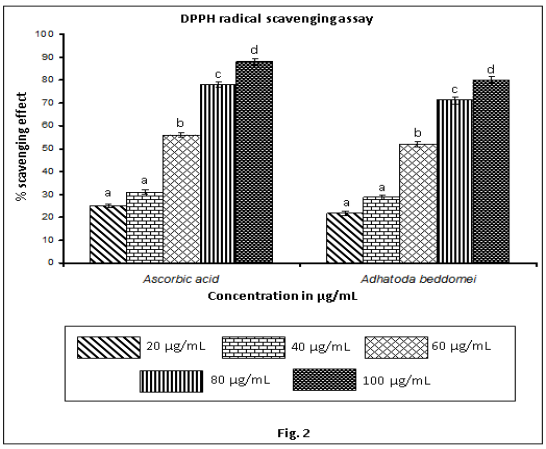

Fig 2: DPPH radical scavenging effect of *Adhatoda beddomei* with different concentrations in comparison with standard ascorbic acid. Values are given as mean ± SD of six experiments in each group. Bar values are sharing a common superscript (a, b, c, d, e) differ significantly at P<0.05 Duncan's Multiple Range Test (DMRT).

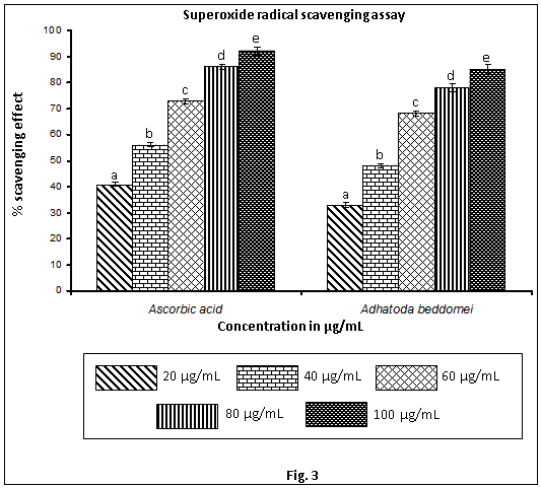

Fig 3: Superoxide radical scavenging effect of *Adhatoda beddomei* with different concentrations in comparison with standard ascorbic acid. Values are given as mean ± SD of six experiments in each group. Bar values are sharing a common superscript (a, b, c, d, e) differ significantly at P<0.05 Duncan's Multiple Range Test (DMRT).

Fig. 4 shows the % scavenging effects on hydroxyl radical of AB of ethanol extract. Hydroxyl radicals are the major active oxygen species causing lipid oxidation and enormous biological damage. Ferric-EDTA was incubated with H_2O_2 and ascorbic acid at pH 7.4. Hydroxyl radicals were formed in free solution and detected by their ability to degrade 2-deoxy-2-ribose into fragments that on heating with TBA at low pH from a pink chromogen. When ethanol extract of AB and ascorbic acid were added to the reaction mixture, they neutralized the hydroxyl radicals from the sugar and prevented degradation. As shown in Figure 4, AB was capable of reducing hydroxyl radical formation at all concentrations and at 100 µg/mL ($p<0.05$), it exerts optimum scavenging effects. Since the optimum dose was calculated as 100 µg/mL, further increase in the concentration of AB beyond the optimum dose did not produce any significant increasing scavenging effect and this may be due to saturation of the molecule in the system. The IC_{50} value of AB of the hydroxyl

radical scavenging assay was found to be 50 µg/mL, which was found to be compared with that of standard ascorbic acid (48 µg/mL). The results were found to be statistically significant ($p<0.05$).

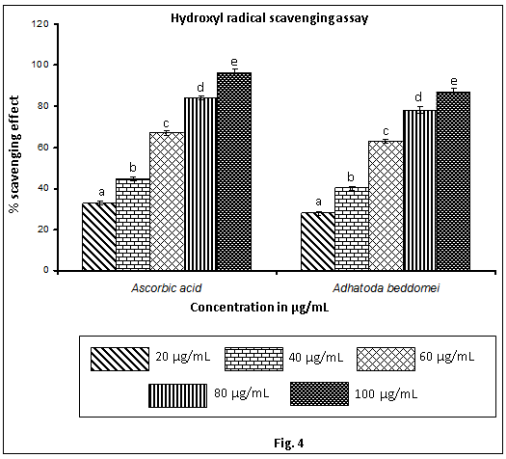

Fig 4: Hydroxyl radical scavenging effect of *Adhatoda beddomei* with different concentrations in comparison with standard ascorbic acid. Values are given as mean ± SD of six experiments in each group. Bar values are sharing a common superscript (a, b, c, d, e) differ significantly at P<0.05 Duncan's Multiple Range Test (DMRT).

Fig. 5 shows the % scavenging effect on nitric oxide radical of AB of ethanol extract. It is well known that nitric oxide has an important role in various types of inflammatory processes in the animal body. From Figure 5, it was clear that the scavenging of nitric oxide by AB was found to be concentration dependent and at 100 µg/mL ($p<0.05$), it exerts its optimum scavenging effect. Since the optimum dose was calculated as 100 µg/mL, further increase in the concentration of AB beyond the optimum dose did not produce any significant increasing scavenging effect and this may be due to the saturation of the molecule in the system. The IC_{50} value of AB on nitric oxide radical scavenging assay was found to be 55 µg/mL, which was found to be compared with that of standard ascorbic acid (49 µg/mL). The results were found to be statistically significant ($p<0.05$).

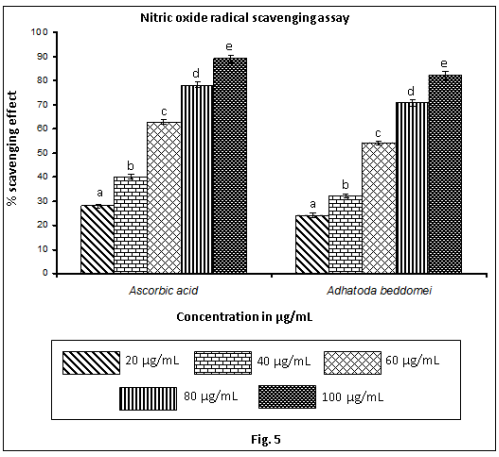

Fig 5: Nitric oxide radical scavenging effect of *Adhatoda beddomei* with different concentrations in comparison with standard ascorbic acid. Values are given as mean ± SD of six experiments in each group. Bar values are sharing a common superscript (a, b, c, d, e) differ significantly at P < 0.05 Duncan's Multiple Range Test (DMRT).

The reducing capacity of a compound may serve as a significant indicator of its potent antioxidant activity. The increased absorbance of the reaction mixture indicates increasing reducing power. Like the antioxidant activity, reducing power of AB increases with increasing concentration. As shown in Fig. 6, our data on reducing power suggest that AB contributes significantly to the observed antioxidant effect and at 100 µg/mL ($p<0.05$), it exerts optimum scavenging compared with that of standard ascorbic acid. Since the optimum dose was calculated as 100 µg/mL, further increase in the concentration of AB beyond the optimum dose did not produce any significant increasing scavenging effect and this may be due to the saturation of the molecule in the system. The IC_{50} value of AB on reductive ability was found to be 15.18 µg/mL, which was found to be compared with that of standard ascorbic acid (16.80 µg/mL). The results were found to be statistically significant ($p<0.05$).

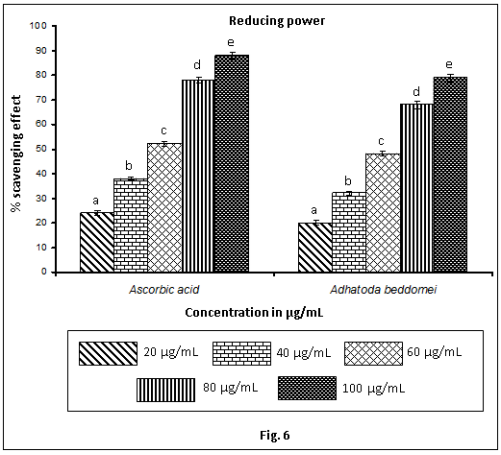

Fig 6: Free radical scavenging activity of *Adhatoda beddomei* with different concentrations in comparison with standard ascorbic acid as measured in reducing power assay. Values are given as mean ± SD of six experiments in each group. Bar values are sharing a common superscript (a, b, c, d, e) differ significantly at P < 0.05 Duncan's Multiple Range Test (DMRT).

The results of our studies indicated that 100 µg/mL concentration of AB showed optimum protection against free radical induced oxidative damage. The hydroxyl, superoxide, nitric oxide and DPPH radical scavenging activity of AB can be attributed to the presence of vitamin E, phytol, stigmasterol, squalene, lupeol and β-sitosterol which donates hydrogen and an electron to hydroxyl radicals, stabilizing them and giving rise to a relatively stable radical. Thus, the free hydroxyl group on the aromatic ring is responsible for the antioxidant properties. Our data on the reducing power of the plant extract suggest that it was likely to contribute significantly toward the observed antioxidant effect. However, the reducing properties of triterpenes and phytosterols are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom. Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation [14].

4. Conclusion

Thus, from the results obtained we observed that AB showed significant antioxidant potential in terms of scavenging free radicals produced by various *in vitro* assays and phytochemical screening studies have been identified the presence of lupeol, stigmasterol, β-sitosterol, campesterol, vitamin-E, squalene and phytol. These compounds possess important biological activity such as hepatoprotective, anti-malarial, anti-arthritic, anti-

carcinogenic, immunomodulatory, hypoglycemic, antimicrobial, anti-inflammatory, anticancer and antioxidant effect.

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