



## THE *IN VITRO* ANTIOXIDANT ACTIVITY AND TOTAL PHENOLIC CONTENT OF FOUR INDIAN MEDICINAL PLANTS

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### ABSTRACT

Methanolic extracts of *Plumbago zeylanica* (Root), *Acorus calamus* (Rhizome), *Hemidesmus indicus* (Stem) and *Holarrhena antidysenterica* (Bark), used in Ayurvedic medicines for number of ailments were evaluated for their antioxidant activity by ferric thiocyanate (FTC) assay and compared with thiobarbituric acid (TBA) method. The order of antioxidant potential according to FTC assay was found to be highest in *Plumbago zeylanica* followed by *Holarrhena antidysenterica*, *Acorus calamus* and *Hemidesmus indicus*. Whereas there is slightly difference in activities as measured by TBA method. The antioxidant activity of medicinal plants was at par with the commercial antioxidant butylated hydroxy toluene (BHT), L-Ascorbic acid and  $\alpha$ -tocopherol. Further, the radical-scavenging activity of the extracts was measured as decolourizing activity followed by the trapping of the unpaired electron of DPPH. The percentage decrease of 1, 1-diphenyl-2-picryl hydrazyl radical (DPPH) standard solution was recorded maximum for *Hemidesmus indicus* (77.0%) followed by *Plumbago zeylanica* (73.41%), *Acorus calamus* (20.88%) and *Holarrhena antidysenterica* (20.06%) extracts at a concentration of 100  $\mu$ g/ml. Phytochemical analysis revealed the presence of major phytochemicals like alkaloids, glycosides, phenolics and saponins. Moreover, total phenolics concentration equivalents to gallic acid was found in the range of 59.50 to 109.0 mg/g of plant extracts, which correlated with antioxidant activity. The findings indicated promising antioxidant activity of crude extracts of the above plants and needs further exploration for their effective use in both modern and traditional system of medicines.

**Keywords:** Antioxidant activity, Medicinal plants, DPPH, FTC assay, TBA method

### INTRODUCTION

Reactive oxygen species (ROS) including superoxide radicals, hydroxyl radicals, singlet oxygen and hydrogen peroxide are often generated as by products of biological reaction or from exogenous factors<sup>1</sup>. *In vivo*, some of these ROS play an important role in cell metabolism including energy production, phagocytosis and intercellular signaling<sup>2</sup>. However, these ROS produced by sunlight, ultraviolet light, ionizing radiation, chemical reactions and metabolic processes have a wide variety of pathological effects such as DNA damage, carcinogenesis and various degenerative disorders such as cardiovascular diseases, aging and neuro-degenerative diseases<sup>3,4,5</sup>. A potent broad spectrum

scavenger of these species may serve as a possible preventive intervention for free radical mediated cellular damage and diseases<sup>6</sup>. Antioxidant based drugs and formulations for the prevention and treatment of complex diseases like Alzheimer's disease and cancer have appeared during last three decades<sup>7</sup>. Recent studies have shown that a number of plant products including polyphenols, terpenes and various plant extracts exerted an antioxidant action<sup>8-11</sup>. There is also a considerable amount of evidence revealing an association between individuals who have a diet rich in fresh fruits and vegetables and the decreased risk of cardiovascular diseases and certain forms of cancer<sup>12,13</sup>. There is currently immense

interest in natural antioxidants and their role in human health and nutrition<sup>14</sup>. Considerable amount of data have been generated on antioxidant properties of food plants around the globe<sup>15,16</sup>. However, traditionally used medicinal plants awaits such screening. On the other hand, the medicinal properties of plants have also been investigated in the light of recent scientific developments through out the world, due to their potent pharmacological activities, low toxicity and economic viability<sup>17</sup>. Several medicinal plants (Rasayana) have also been extensively used in the Indian traditional (Ayurveda) system of medicine for the treatment of number of diseases<sup>18</sup>. Some of these plants have shown potent antioxidant activity<sup>16,7</sup>. However, majority of plants have not yet been screened for such activity. So, in order to contribute further to the knowledge of Indian traditional plants, our present study is focussed on four plants namely *Acorus calamus*, *Hemidesmus indicus*, *Holarrhena antidysenterica* and *Plumbago zeylanica* to determine their antioxidant and free radical scavenging properties. The literature survey showed scanty information available on these plants and thus prompted us to analyze these common ayurvedic plants. Further an attempt has also been made to find the relationship between phenolic content and antioxidant activity of these plants.

#### **MATERIALS AND METHODS**

Four authenticated plant samples were kindly provided by Dr. S. Farooq, Director, The Himalaya Drug Company, Dehradun, India. All the plant materials were further identified in the Department of Botany, AMU, Aligarh (India). The specimens have been deposited

in the Department of Agricultural Microbiology, Faculty of Agricultural Sciences, AMU, Aligarh, India.

#### **Chemical reagents**

The solvents used were of HPLC grade. The standards (BHT,  $\alpha$ -tocopherol, L-Ascorbic acid and gallic acid) and chemicals used were obtained from Hi-Media lab. Ltd, Mumbai, India. 1,1-diphenyl-2-picryl hydrazyl (DPPH) radicals were purchased from Sigma Chemical Co, St. Louis, MO, USA.

#### **Preparation of extracts**

The plant extracts were prepared as described earlier<sup>19</sup> with little modification. Hundred (100) grams of dry plant powder were soaked in 1 litre of 97% methanol for 3-5 days with intermittent shaking. At the end of extraction, it was passed through Whatman filter paper No.1 (Whatman Ltd., England). This methanolic filtrate was concentrated under reduced pressure on rotary evaporator at 40 °C and then stored at 4 °C for further use. The filtrate was reconstituted in known amount of DMSO to obtain methanol extract of known concentration.

#### **Antioxidant assay**

The antioxidant activity of the plant extracts was tested using two methods: ferric thiocyanate (FTC) and thiobarbituric acid (TBA) methods. The FTC method was used to measure the amount of peroxide at the beginning of the lipid peroxidation, in which peroxide reacts with ferrous chloride and form ferric ion. The ferric ion then combines with ammonium thiocyanate and produce ferric thiocyanate. The substance is red in colour. The thicker the colour, the higher the absorbance. Whereas the TBA methods measures free radicals present after peroxide oxidation.

### **Ferric thiocyanate (FTC) method**

The standard method as described by (Kikuzaki and Nakatani, 1993) was used<sup>1</sup>. A mixture of 4.0 mg plant extract in 4ml absolute ethanol, 4.1 ml of 2.5% linolenic acid in absolute ethanol, 8.0 ml of 0.05M phosphate buffer (pH 7.0) and 3.9 ml of water was placed in a vial with a screw cap and then placed in an oven at 40 °C in the dark. To 0.1 ml of this solution was added 9.7 ml of 75% ethanol and 0.1 ml of 30% ammonium thiocyanate. Precisely 3 min after addition of 0.1 ml of 0.02M ferrous chloride in 3.5% HCl to the reaction mixture, the absorbance of red colour was measured at 500nm each 24 hr until the day after absorbance of control reached maximum. BHT and  $\alpha$ -tocopherol were used as positive controls while the mixture without plant sample was used as the negative control.

### **Thiobarbituric acid (TBA) method**

The method of (Ottolenghi, 1959)<sup>2</sup> was referred. Two ml of 20% trichloroacetic acid and 2 ml of 0.67% 2-thiobarbituric acid was added to 1 ml of sample solution, as prepared in FTC method. The mixture was placed in a boiling water bath and, after cooling, was centrifuged at 3000 rpm for 20 min. Absorbance of supernatant was measured at 552 nm. Antioxidant activity was based on the absorbance on the final day of FTC method.

### **DPPH radical scavenging activity**

The free radical scavenging activity by different plant extracts was done according to the method reported by (Gyamfi et al, 2002)<sup>3</sup>. Fifty micro liters of the plant extract in methanol, yielding 100 $\mu$ g/ml respectively in each reaction was mixed with 1ml of 0.1mM DPPH in methanol solution and 450 $\mu$ l of 50mM Tris-HCl buffer (pH 7.4). Methanol (50 $\mu$ l) only was used as control of experiment. After 30 min of incubation at

room temperature the reduction of the DPPH free radical was measured reading the absorbance at 517nm.

L-Ascorbic acid and BHT used as controls. The percent inhibition was calculated from the following equation:

$$\% \text{ Inhibition} = \left[ \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}} \right] \times 100$$

### **Determination of major phytochemicals and total phenolic content**

Major groups of phytochemicals were detected by standard colour tests as described earlier<sup>20</sup>. The amount of total phenolics in methanolic plant extracts was determined with the Folin-Ciocalteu reagent using the method of Spanos and Wrolstad (1990)<sup>21</sup>, as modified by Lister and Wilson (2001)<sup>22</sup>. To 0.50 ml of each sample (three replicates), 2.5 ml of 1/10 dilution of Folin-Ciocalteu's reagent and 2 ml of Na<sub>2</sub>CO<sub>3</sub> (7.5%, w/v) were added and incubated at 45 °C for 15 min. The absorbance of all samples was measured at 765 nm using a Spectronic 20D+ spectrophotometer. Results were expressed as milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g dw).

### **Statistical analysis**

The results are presented as the average and standard error of three experiments. The data was analysed by using Sigma plot 10.0.

## **RESULTS AND DISCUSSION**

There is an increasing evidence that indigenous antioxidants may be useful in preventing the deleterious consequences of oxidative stress and there is increasing interest in the protective biochemical functions of natural antioxidants contained in spices, herbs and medicinal plants<sup>4,5</sup>. The details of four medicinal plants selected in this study is described in table 1.

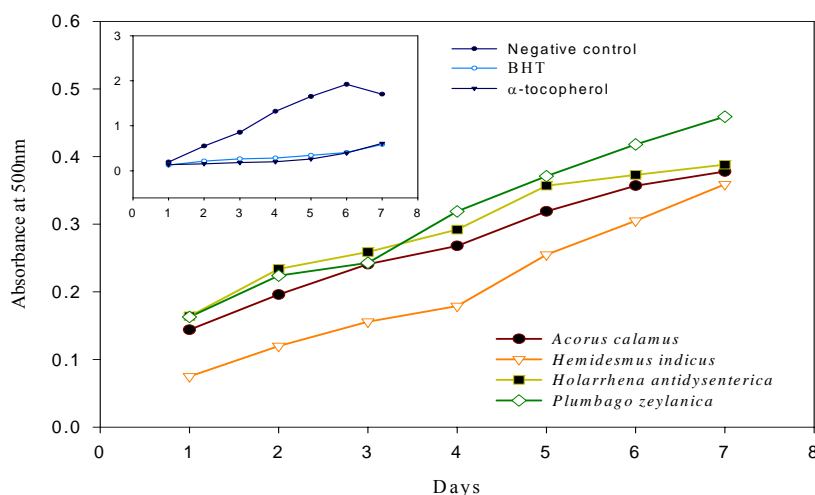
**Table 1: Ethnobotanical details of the selected Indian medicinal plants**

Plant name (Family)/ Voucher specimen	Common Name	Part Used	Traditional Uses
<i>Acorus calamus</i> L. (Acoraceae) HDCO-166/297	Bach	Rhizome	Emetic, Stomachic, dyspepsia, colic, remittent fevers, nerve tonic, in bronchitis, dysentery in children, insectifuge and in snake bite <sup>18</sup> .
<i>Hemidesmus indicus</i> R. Br. (Asclepiadaceae) HDCO-204/7	Ananthamul	Stem	Demulcent, diuretic, in skin diseases, blood purifier, syphilis, rheumatism, scorpion sting, snake bite <sup>18</sup> .
<i>Holarrhena antidysenterica</i> Wall. (Apocynaceae) HDCO-120/ 235	Kurchi	Bark	Used in dysentery, dropsy, fever, diarrhoea and intestinal worm infections <sup>18</sup> .
<i>Plumbago zeylanica</i> L. (Plumbaginaceae) HDCO 43/64	Chitra	Root	Used in paralytic affection, secondary syphilis, leprosy & Ophthalmia <sup>18</sup> .

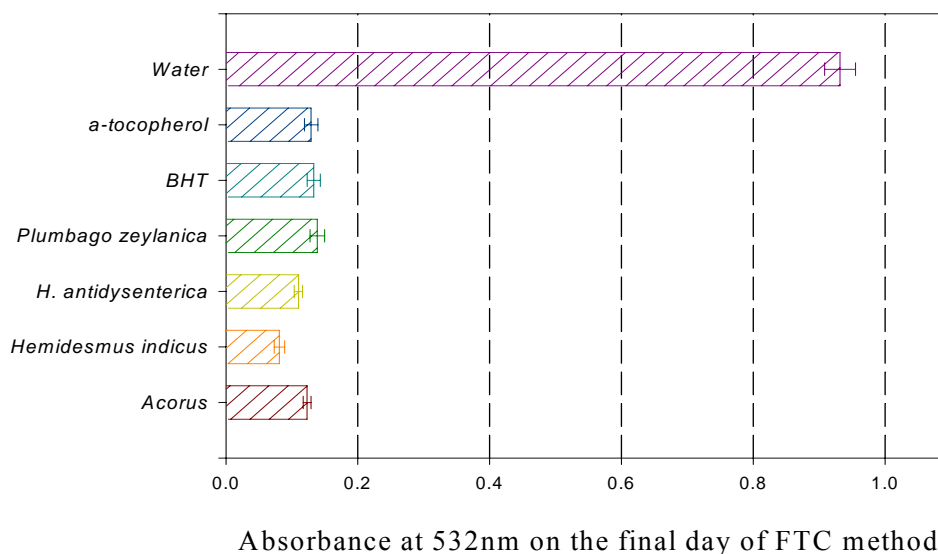
Antioxidant activity of methanolic extracts is measured to inhibit lipid peroxidation (LPO) by FTC and TBA methods (Fig.1 & 2). The tested plant extracts showed strong antioxidant activity or differential capacity to inhibit LPO by FTC and TBA method which is indicated by their low absorbance values. The FTC method measures the amount of peroxide produced during the initial stages of lipid oxidation. Subsequently, at a later stage of lipid oxidation, peroxide decomposes to form carbonyl compounds that are measured by the TBA method<sup>23</sup>. So, at a given

concentration, the relatively higher activity was recorded in the extracts of *H. indicus* followed by *H. antidysenterica*, *A. calamus* and *P. zeylanica*, surpassing the activity of the standard commercial antioxidants,  $\alpha$ -tocopherol and butylated hydroxy toluene. In general, the antioxidant by TBA method is higher than that of FTC method. This might suggest that the amount of peroxide in the initial stage of lipid per oxidation is less than the amount of peroxide in the secondary stage. Furthermore, the secondary product is much more stable for a period of time<sup>1</sup>.

**Fig 1. Antioxidant properties of plant extracts by FTC method**



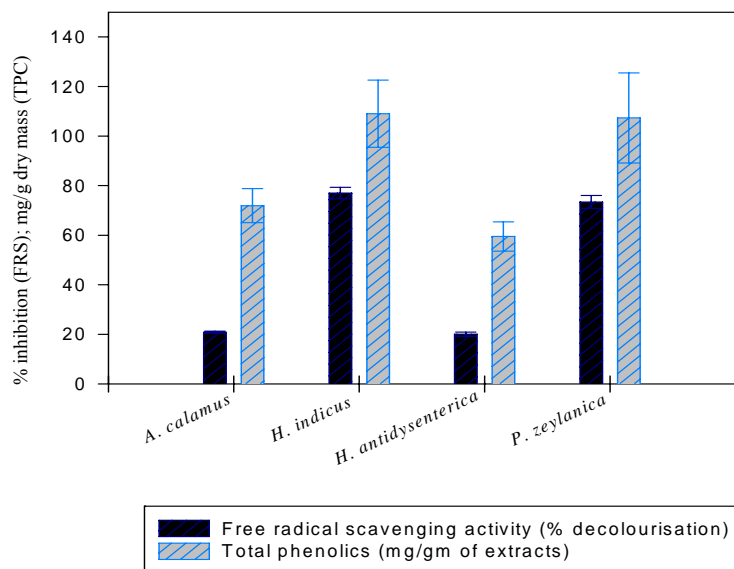
**Fig 2. Antioxidant activities of plant extracts by TBA method**



There are several other mechanisms by which antioxidants can act. One of them is by scavenging of reactive oxygen and nitrogen free radicals. There are many different experimental methods by which the free radical scavenging activity can be estimated. One such method, by which total free radical scavenging can be evaluated, is by determining their efficiency to scavenge DPPH radicals. This method is based on the reduction of DPPH, a stable free radical and any molecule that can donate an electron or hydrogen to DPPH can react with it and thereby bleach the DPPH absorption. Because of its odd electron, DPPH gives a strong absorption maximum at 517nm by visible spectroscopy (purple colour). As the odd electron of the radical becomes paired off in the presence of a hydrogen donor, that is, a free radical scavenging antioxidant, the absorption strength is decreased and the

resulting decolorization is stoichiometric with respect to the number of electrons captured<sup>24</sup>. therefore when the above extracts were tested for the DPPH free radical scavenging ability, the methanolic extract of *H. indicus* and *P. zeylanica* at 100µg/ml showed strong radical scavenging activity with percentage decrease of 77.0% and 73.41% whereas *A. calamus* and *H. antidysenterica* showed relatively poor free radical scavenging activity of 20.88% and 6.32% respectively. The order of scavenging activity was maximum in *H. indicus* followed by *P. zeylanica*, *A. calamus* and *H. antidysenterica*. The values are also comparable with commercial antioxidant L-ascorbic acid (90.0%) and butylated hydroxy toluene (83%) at the same concentration (Fig. 3). This suggested that *H. indicus* and *P. zeylanica* contain compounds such as polyphenolics that can donate electron/hydrogen easily.

**Fig 3. Free radical scavenging activity (FRS) and total phenolics concentration (TPC) of methanolic plant extracts**



To further confirm this, total phenolics concentration equivalents of gallic acid was estimated. Gallic acid being the most important polyphenol in natural products was used to determine the phenolics of tested plants which are found to be in the range of 59.50 mg/g to 109.0 mg/g . From all these observations it can be concluded that the plant extracts with high level of polyphenolic compounds such as *H. indicus* and *P. zeylanica* show excellent antioxidant activity *in vitro* systems. However, *A. calamus* and *H. antidysenterica* showed less free radical scavenging activity which might be due to their low phenolic constituents as compared to above two extracts.

Our findings on *H. indicus* are in agreement with the reports of (Ravishanker et al., 2002; Mary et al., 2003)<sup>25,26</sup>. They have reported varying levels of antioxidant and free radical scavenging properties of root bark extracts of this plant. However, we have tested the stem with bark showing similar activity. The antioxidant activity of *A. calamus* extract is

partly expected due to its one of the active constituents,  $\alpha$ -asarone<sup>27</sup>. Some variations in the extent of extract antioxidant activity were observed for each type of assay used in this study. The extract of *P. zeylanica* exhibited good DPPH radical scavenging activity, but low lipid peroxidation while the extracts of *A. calamus* and *H. antidysenterica* showed relatively fair antioxidant potential according to FTC and TBA method but comparatively low DPPH radical scavenging activity. These differences might be due to their different antioxidant mechanisms or variations in their ability to scavenge free radicals. A fair correlation between total phenolic content and antioxidant activity was also observed. These observations clearly indicated a cross linkage between phenolics and antioxidant activity. However a large number of phytochemical groups are implicated for antioxidant activity<sup>28</sup>. Many authors have also correlated antioxidant activity with their polyphenolic or phenolic contents<sup>29,16</sup>.

This study supports the contention that traditional medicines remain a valuable source in the potential discovery of natural product pharmaceuticals. Significant antioxidant activity showed by *A. calamus*, *H. indicus*, *H. antidysenterica* and *P. zeylanica*, provide a scientific validation for the traditional use of these plants. Further work on isolation and identification of active compounds and its efficacy needs to be done.

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