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Research Article

# ANTIOXIDANT ACTIVITY OF N-PHENYLBENZOHYDROXAMIC ACID

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**ABSTRACT**

Antioxidant potential of the parent compound of N-arylhydroxamic acids series, N-Phenylbenzhydroxamic acid trivially named as PBHA,C6H5NOH.C6H5C=O, is evaluated for the first time following the three procedure, (i) 2,2-diphenyl-1-picrylhydrazyl (DPPH), (ii) β-Carotene-Linoleate thiocyanate bleaching (iii) DNA cleavage methods. The result revealed that decrease in absorbance spectra of DPPH, increase inhibition of autoxidation of linoelic acids and inhibition of DNA cleavage activity shows antioxidant behaviour of PBHA. The $EC_{50}$ value is obtained 225mM. The percentage inhibition of lipid peroxidation is 74.48% ± 1.18, Protection of DNA from cleavage by peroxide, shows antioxidant potential of PBHA. The effect of concentration on percentage inhibition of lipid peroxidation, scavenging activity DPPH and DNA cleavage activities were discussed. The antioxidant activity mechanism of PBHA due to conjugation of electron pair on nitrogen with carbonyl group.

**Keywords:** Antioxidant, PBHA, DPPH,

## INTRODUCTION

Antioxidants are believed to play a very important role in the body defense system against reactive oxygen species (ROS), which are the harmful byproducts generated during normal cell aerobic respiration. Furthermore, due to oxidative stress resulted organisms and cells damages protected by antioxidants are extensively studied part of research [1]. Oxygen is absolutely essential for the life of aerobic organism but it may become toxic if supplied at higher concentrations. Dioxygen in its ground state is relatively unreactive, its partial reduction gives rise to active oxygen species (AOS). Which are sometimes called Reactive oxygen species (ROS) [2-3]. ROS containing chemical species, such as superoxide ($O^{\bullet}_2$) and hydroxyl radicals ($HO^{\bullet}$), that contain an unpaired electron and non-radical molecule hydrogen peroxide, ($H_2O_2$) with reactive chemical properties. In biological systems, ROS are constantly generated through a variety of pathways, including both enzyme-catalyzed reactions and non-enzyme reactions.[4-7] The oxidation induced by ROS can resulted cell membrane disintegration, membrane protein damage and DNA mutation. Which can further initiate or propagate the development of many diseases, such as cancer, inflammatory, and neurodegenerative diseases [8-11].ROS, along with reactive nitrogen species, (RNS) may play an vital role in the pathogenesis and progress of many diseases, including cancer, diabetes and sickle cell [12].

The imbalanced state of oxidant and antioxidant in organism, initiated by ROS/ RNS production is termed as oxidative stress and responsible for many pathological conditions. These oxidants can, react with substances such as nitric oxide (NO), and converted into peroxynitrite and hydroxyl radicals with pronounced reactivity. These molecules are capable of attacking sensitive cellular targets like lipids, proteins and nucleic acids causing their inhibition, acceleration and degradation. [13-15]

N-Aryl hydroxamic acids of general formulae, R1NOH.R2C=O (where, R1 and R2 are phenyl or substituted phenyl group) are reported as antitumor and anticancer, and antioxidant, agents [16-17]. The pharmacophoric part of hydroxamic acids fig .1 make it more potent in therapeutic use as, it contains one hydrogen bond donor (HBD), with three hydrogen bond acceptor (HBA), sites[18]. The antioxidant nature of this functionality is due to the presence of oxygen and nitrogen atom in core functional group. The reported hydroxyl urea and alginic acid structurally similar to hydroxamic acid are already used as antioxidant [19-20].

The aim of present study is to evaluate the antioxidant activity of PBHA by different methods as it is investigated as potent antitumor agent.

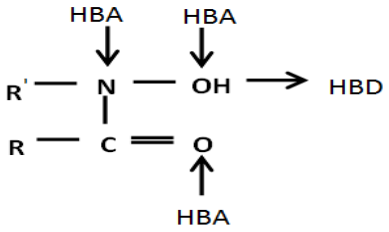



Fig 1

## MATERIALS AND METHODS

### Chemicals and apparatus

PBHA was prepared following the reported standard procedure [21].The purity of synthesized compound (Fig.2) was ascertained by determining its melting point, and elementary analysis. M.P observed 121°C and reported 122°C .Elementary analysis for C=74.68, N=5.28 and O=15.14.and the values obtained are 74.58, 5.21, and 15.14 respectively.

The 0.001M stock solution of PBHA was prepared in ethanol as it is very sparingly soluble in water.

**Fig 2: Optimized Geometry structures of PBHA**

Ammonium thiocyanate, DPPH, ferrous sulfate and linoleic acid, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hydrogen peroxide (33%) was from Merck Chemical Industry (Osaka, Japan). Ct-DNA (Sigma Aldrich Chemical.Co.USA) was dissolved in double distilled deionised water at final concentration $2.55x10^{-4}$ $molL^{-1}$ and stored at 4°C. Other chemicals and reagents were from Sigma Chemical Co. (St. Louis, MO, USA).

**Apparatus**

The measurement of spectrum was thermostatically controlled by attached temperature controller Gl-635.The absorption spectra were measured on Biospectrum BL-198(Elico India) by using quartz cells of 1.0cm. The Gel electrophoresis was performed on Genei. pH measurements were carried out with Cyber510 digital pH meter .

**Measurement of Antioxidant property**

**Scavenging activity of DPPH radicals**

The free radical scavenging activity of the PBHA was evaluated according to a method described in literature.[ 22] In brief, a solution of the PBHA (50 µL) in ethanol and a solution of DPPH (0.1 mM) in ethanol with Tris-HCl buffer (50 mM) (pH 7.4) was mixed gently. After incubation of 30 min at room temperature, the absorpation spectra of the DPPH free radical was measured at 517 nm with visible spectrophotometer, using ethanol as negative control. The degree of discolouration indicates the scavenging potential of the antioxidant extract.

The percent inhibition was calculated from the following equation,

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

and represented in fig.3

**β-Carotene-Linoleate thiocyanate bleaching**

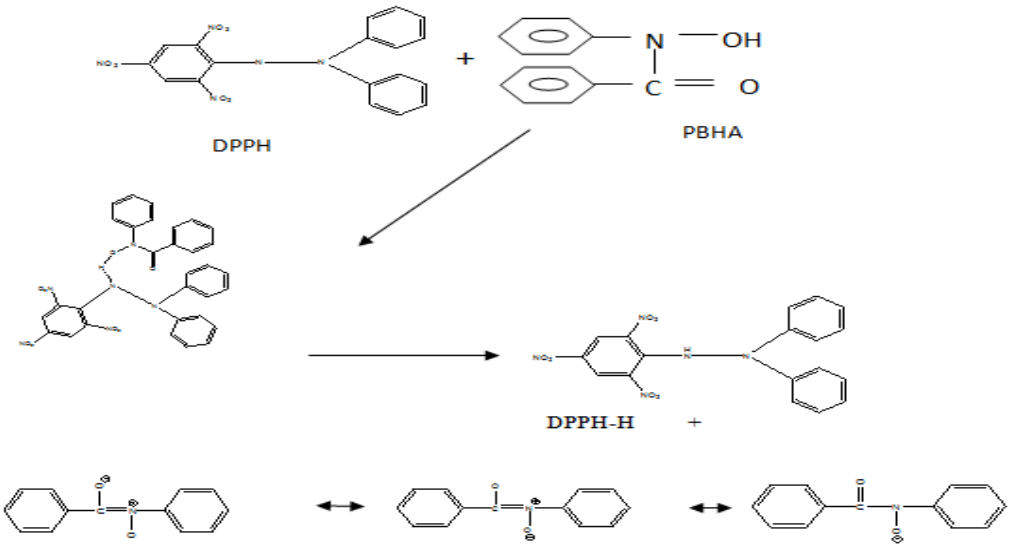
The ethanol solution of PBHA was added to a reaction mixture in a screw cap vial. Each reaction mixture in screw cap vial consists of 2.51% β-carotene-linoleic acid in ethanol and 9 ml of 40 mM phosphate buffer (pH 7.0). The vial was placed in oven at 40 °C in the dark. At intervals of ten hrs during incubation, 0.5% aliquot of the mixture was diluted with 75% ethanol. This was followed by adding 30% ammonium thiocyanate. Precisely 3 min after the addition of 20mM ferrous chloride (in 3.5% HCl) to the reaction mixture, then the absorbance of red color was measured at 500 nm.

**Protective effect of PBHA on $H_2O_2$ induced DNA damage**

The extent of DNA oxidation by $H_2O_2$ was analyzed on 0.8% submarine agrose gel [23]. 25 µg calf thymus DNA was treated with 1mM with or without PBHA in 100ul final volume of 20mM potassium phosphate buffer at 7.4 pH. The reaction mixture was mixed with 10µl of loading buffer and then reaction mixture was incubated at 37°C for 30 min.25µl DNA reaction mixture with ethidium bromide dye were loaded in well of agarose gel for electrophoreses for 1hr at100 V.

**RESULTS AND DISCUSSION**

The reactive oxygen species and free radical-mediated reactions have been reported to involve in diseases like cancer, cardiovascular diseases, Alzheimer's disease and tuberculosis [24-25]. DPPH antioxidant assay is based on the ability of DPPH a stable free radical, to decolorize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 517 nm and also for a visible deep purple color. DPPH is characterized as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecules do not dimeris, as would be the case with most other free radicals. The delocalization also gives rise to the deep violet colour, characterized by an absorption band in ethanol solution centered at about 517 nm[26]. When a solution of DPPH is mixed with substance that can donate a hydrogen atom, then its violet colour decolorized due to arise of reduced form in solution by accepting an electron donated by an antioxidant compound, the decolorized DPPH can be quantitatively measured from the changes in absorbance.




**Scheme 1**

From Scheme I DPPH radical absorbs at 517 nm, but the absorption decreases upon reaction with PBHA due to the formation of its non-radical form, DPPH-H [27]. The radical scavenging activity of PBHA was concentration dependent and increased with increase in concentration. The DPPH radical scavenging activity of PBHA is given in Fig. 3. This absorbance section of DPPH shows satisfactory decrease with increasing concentration of PBHA. From graph 1, it is indicated that percentage inhibition of DPPH increases with increase in concentration of PBHA. The activity test was calculated as the concentration exhibiting 50 % of activity ($EC_{50}$). Hence, a lower $EC_{50}$ value indicated higher activity, $EC_{50}$ of PBHA obtained is 225mM.

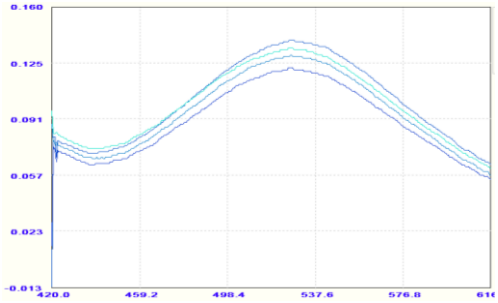
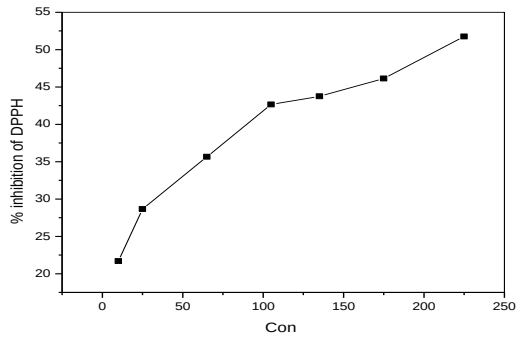



**Fig 3: Absorption spectra DPPH at 517 nm in absence and presence (a-e) of PBHA**




**Graph 1 : % inhibition of DPPH at 517 nm in presence (10-225mM) of PBHA**

**β-Carotene-Linoleate thiocyanate bleaching**

In this method, linoleic acid free radical reacts with conjugated double bonds of β-carotene, causing a rapid degradation and discoloration[28]. Thus, by simulation of the oxidation of the membrane lipid components in the presence of antioxidants, this test gives an insight of the inhibitory effect of substance on the lipid peroxidation. This method was used to measure the amount of peroxide at the beginning of the lipid peroxidation and to know which peroxide reacts with ferrous chloride and form ferric ion. The ferric ion then combines with ammonium thiocyanate and produce ferric thiocyanate a reddish pigment [29]. Hydrogen donating antioxidants can react with lipid peroxyl radicals and break the cycle of generation of new radicals. The substance investigated in this study was able to reduce the rate of degradation of β-carotene significantly. Antioxidant activity was measured as the percentage of inhibition of lipid peroxidation in the β-carotene-linoleic acid system [29], normally antioxidant activity at 60 and 120 minutes of incubation (AA60 and AA90) reflects the antioxidant activity more accurately.

The rate was calculated according to first-order kinetics [1]:

$$R = 1/t \times \ln A_0/A_t \quad \ldots\ldots\ldots 1$$

Where

R = rate of β-carotene bleaching, t is time in minutes

$A_0$ = initial absorbance

$A_t$ =absorbance after time interval, t (t = 15, 30, 45 minutes…etc.)

Furthermore, the antioxidant activity was calculated from the absolute changes in absorbance at t = 60 and 90 min (AA60 and AA90, respectively). The results normalized using both controls: negative, no protection against oxidation of β-carotene and positive, offers maximum protection. The antioxidant activity of the compound was expressed as,

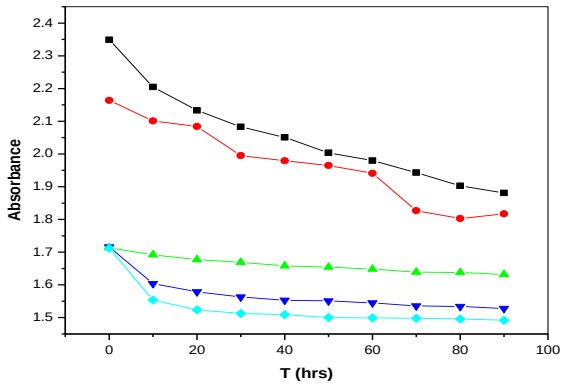
$$AA = \left[1 - \frac{R_t}{R_0}\right] \times 100 \quad \ldots\ldots\ldots 2$$

Where, AA = % inhibition after 60 and 90 min

$R_t$ = rate of inhibition with sample

$R_0$ = control rate of inhibition

This might be explained by the fact that at higher concentrations PBHA acts as a hydrogen donating agent for preventing the oxidation of linoleic acid. The percentage of inhibition of lipid per oxidation was exhibited by PBHA in ethanolic extract at $AA_{60}$=68.95% and $AA_{90}$=78.56%. Significantly lower in absorbance shown in graph.2 as compared to control indicate that PBHA has greater antioxidant activity. The antioxidant activity of PBHA is calculated 74.48% ± 1.18.




**Graph 2: Lipid Peroxidation at 470 nm in presence of PBHA**
**Protective Effect of PBHA on DNA Damage.**

Free radicals could damage macromolecules in cells, such as DNA, protein and lipids of membranes, resulted in various degenerative diseases .The extremely reactive OH radical derived from $O_2^-$ and $H_2O_2$ is considered a cause of DNA strand scission in cellular damage. [30-31] When calf thymus DNA was treated with
$H_2O_2$ for 30 min, extensive DNA uncoiling occur due to oxidation of DNA by hydroxyl radicals produced by $H_2O_2$ in UV light that resulted fast movement of DNA as shown (lane1)in Figure 4. The presence of PBHA at 35-75 mM concentration (Lane 2, 3, 4) suppressed the uncoiling of calf thymus DNA.

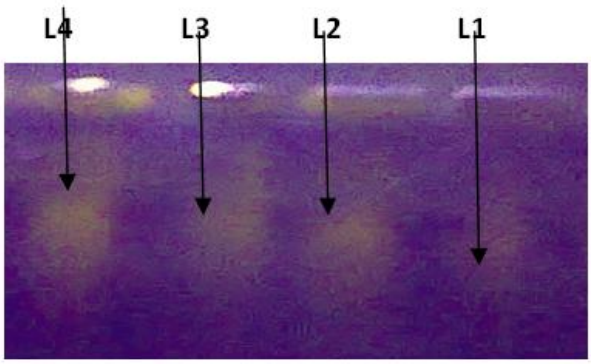



**Fig.4: DNA Protection against cleavage in presence of PBHA**

**CONCLUSION**

In the present investigation the antioxidant property of PBHA is reported first time. From the results, it is evident that PBHA showed the value of percent inhibition of DPPH (Ec 50%) at concentration of 225 mM. , The value of inhibition of lipid peroxidation for PBHA (74.48% ± 1.18). Thus it is concluded that PBHA exhibit antioxidant property.

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