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FREE RADICAL SCAVENGING ACTIVITY OF 4-HYDROXYPROPIOPHENONE BY *IN VITRO* ASSAYS

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ABSTRACT

The present study concentrated on deciding the free radical scavenging activity of 4-hydroxypropiophenone (4-HPPP) through *in vitro* free radical scavenging tests. 4-HPPP was determined by using, 1,1-diphenyl-2-picryl-hydrazyl(DPPH), 2'-Azinobis-(3-Ethylbenzothia zoline-6-Sulfonic Acid) ($ABTS^{+}$), Ferric reducing antioxidant power (FRAP), nitric oxide scavenging assay (NO), reducing power, superoxide anion radical scavenging (SOD), hydroxyl radical scavenging assay (HRSA), metal chelating activity, phosphomolybdeneum and hydrogen peroxide assay. A score of bioavailability and medication resemblance properties of 4-HPPP has a decent score exclusively. Free radical scavenging potentiality of DPPH (37.74µg/ml) compared to standard drug Ascorbic acid (AA) (35.62µg/ml) respectively, ABTS (62.31µg µg/ml) and AA (57.74µg/ml), FRAP (42.79µg/ml) and AA(39.74µg/ml.), NO (45.28µg/ml) and AA (49.74µg/ml), reducing power (39.09µg/ml) and AA (34.74µg/ml), hydroxyl (36.57µg/ml) and (31.74µg/ml), superoxide (54.48µg/ml) and AA (50.74µg/ml), and hydrogen peroxide (68.74µg/ml) and AA(74.02µg/ml), metal chelating(37.90µg/ml) and AA (55.74µg/ml) and also phosphomolybdneum (43.35µg/ml) and AA (38.74µg/ml) respectively, for the 4-HPPP. The present investigation clearly indicated that the 4-HPPP possesses antioxidant properties and serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants.

KEYWORDS: Free radical scavenging, 4-HPPP, DPPH and superoxide scavenging.

INTRODUCTION

Majority of the diseases are mainly linked to oxidative stress due to free radicals. Free radicals are fundamental to any biochemical process and normal metabolism.[1] Antioxidant are substances that may protect cells from damage caused by unstable molecules known as free radicals. Antioxidants interact with and stabilize free radicals and may prevent some of the free radicals might otherwise cause. An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules.[2] Antioxidants play an important role in inhibiting and scavenging radicals, thus providing protection against infections and degenerative diseases. They can either directly scavenge or prevent generation of ROS.[3] In spite of comprehensive network of cellular defensive antioxidants, many ROS still escape this surveillance inflicting serious anomalies favouring such disease states.[4,5] Reactive oxygen species (ROS) is producing during normal physiologic events and they can easily initiate the peroxidation of membrane lipids, leading to the accumulation of lipid peroxides. Under pathological conditions, ROS is overproduced and results in oxidative stress. The imbalance between Reactive oxygen species and antioxidant defense mechanisms leads to oxidative modification in cellular membrane or intracellular Molecules.[6] Oxidative stress is initiated by free radicals, which seek stability through electron pairing with biological macromolecules such as proteins, lipids and DNA in healthy human cells and cause protein and DNA damage along with lipid peroxidation.[7] Oxidative stress plays a major part in the development of chronic and degenerative ailments such as cancer, autoimmune disorders, rheumatoid arthritis, aging, cardiovascular and neurodegenerative diseases.[8] Enzymes, particularly superoxide dismutase(SOD) as well as compounds like tocopherol, AA and glutathione play a vital role in protecting human cells from free radical mediated damage. Due to their potential, they are being used widely. One of the areas, which attracted a great treaty of attention in recent years, is antioxidant in the control degenerative disease in which oxidative dent has been implicated.[9,10] 4-HPPP a product from the oxidative splitting of stilbestrol, with an extremely low degree of estrogenicity. It has been found a valuable drug for checking the growth of lung metastases secondary to certain malignant tumors such as chorionepitheliomas or nephrobiastomas. The structure of 4-HPPP is depicted below Fig.1

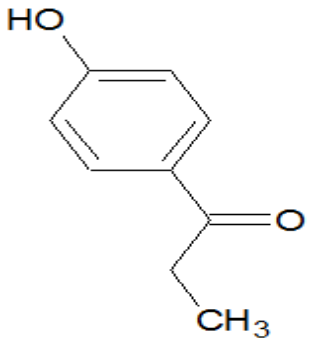

Fig. 1. 2D representation of 4-HPPP.

4-HPPP of greater therapeutic value and with even less estrogenic activity, several new fluorinecontaining aromatic hydroxy ketones were prepared for biological investigation.[11] The objectives of the present study were to investigate the *in vitro* free radicals scavenging activity of 4-HPPP.

MATERIALS AND METHODS

Chemicals

4-HPPP, DPPH and AA were purchased from Sigma- Aldrich (st Lousis, MO, USA). Sodium carbonate, sodium phosphate, potassium acetate, ethylene diamine tetra acetic acid (EDTA), methanol, ethyl acetate, chloroform, sulphuric acid, trichloroacetic acid (TCA), Ammoniumthiocyanate and hydrogen peroxide reagents were obtained from Qualigens (Mumbai). All other chemicals used were of high quality analytical grade.

DPPH radical scavenging activity

The antiradical efficiency was assessed using the DPPH method.[12] In this method commercially available methanol soluble stable free radical DPPH was used. In its radical form, DPPH has an absorption band at 515 nm, which disappears upon reduction by an antioxidant compound or a radical species. For the photometric assay, different volumes of the 4-HPPP were taken in different test tubes. 5.0 mL of 0.1mM methanolic solution of DPPH was added to these tubes and shaken vigorously. The tubes were allowed to stand for 20 min at 27ºC. AA was used as control. Changes in the absorbance of the samples were monitored at 517 nm. The percentage of DPPH decolorization of the samples was calculated using the following formula:

(%) decolorization = $[(ABS_{control} – ABS_{sample}) /ABS_{control}] \times 100$.

IC_{50} Value (mg/ml) where the inhibitory concentration at which DPPH radicals was scavenged by 50%. AA was used for comparison.

ABTS $^{+}$ Assay

The total antioxidant activity of the samples was measured by [2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)] $ABTS^{+}$ radical cation decolorization assay according to the method.[13] ABTS+ was produced by reacting 7mM ABTS+ aqueous solution with 2.4mM potassium persulfate in the dark for 12-16 hours at room temperature. The radical was stable in this form for more than two days when stored in the dark at room temperature. Then, 2ml of diluted ABTS + solution was added to the sample varying concentrations of 4-HPPP. After 30 minutes of incubation at room temperature, the absorbance was recorded at 734nm and compared with standard Ascorbic acid. Percentage of inhibition was calculated.

$$\% \text{ Scavenging} = \frac{\text{Control OD - Test OD}}{\text{Control OD}} \times 100$$

Ferric Reducing Antioxidant Potential (FRAP) Assay

Ferric reducing ability was evaluated using different concentrations of 4-HPPP. The FRAP reagent contained 10mM of TPTZ solution in 40mm. HCl, 20m M $FeCl_3$, $6HO_2$ and acetate buffer (300mM, pH 3.6) (1:1:10, v/v/v). A 100µL 50% aqueous ethyl acetate of the test compounds was added to 3mL of the FRAP reagent and the absorbance was measured at 593nm after incubation at room temperature for 6min, using the AA reagent as a blank.[14]

Nitric oxide scavenging activity

Nitric oxide scavenging activity was determined according to the method.[15] Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacted with oxygen to produce nitrite ions, which can be estimated using the Griess reagent. Scavengers of nitric-oxide act against oxygen, leading to reduced production of nitrite ions. In brief, 3.0 mL of 10 mM sodium nitroprusside in phosphate buffered saline was mixed with different concentrations of the 4-HPPP compound and incubated at 25°C for 150 min. 0.5 mL of the incubated solution was removed and diluted with 0.5 mL of Griess reagent (1% sulphanilamide, 2% orthophosphoric acid and 0.1% N-1-naphthylethylenediamine dihydrochloride). The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with N-1-naphthylethylenediamine

dihydrochloride was measured at 546 nm and percentage of scavenging activity was measured with reference to standards.

Superoxide anion scavenging activity assay

The superoxide anion radicals are produced in 2 ml of phosphate buffer (100 mM, pH 7.4) with 78 M -nicotinamide adenine dinucleotide (NADH), 50 M nitroblue tetrazoliumchloride (NBT) and test samples at different concentrations. The reaction mixture is kept for incubation at room temperature for 5 min. It is then added with 10 M of 5-methylphenazinium methosulphate (PMS) to initiate the reaction and incubated for 5 min at room temperature. The color reaction between superoxide anion radical and NBT is read at 560 nm. Percent inhibition was calculated against a control.[16] The scavenging activity is calculated as follows,

% Scavenging activity = [(Absc– Abss)/Absc] × 100.

Hydroxyl radical scavenging activity

The scavenging activity for the sample extracts on hydroxyl radical was measured according to the method.[17] 20µg concentration of the 4-HPPP was added with 1.0 mL of iron – EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5mL of EDTA solution (0.018%) and 1.0 mL of DMSO (0.85% v/v in 0.1 M phosphate buffer, pH 7.4) sequentially. The reaction was initiated by adding 0.5 mL of AA (0.22%) and incubated at 80-90ºC for 15 min in a water bath. The reaction was terminated by the addition of 1.0 mL of ice – cold TCA (17.5% w/v). Then, 3.0 mL of Nash reagent (75.0 g of ammonium acetate, 3.0 mL of glacial acetic acid, and 2.0 mL of acetyl acetone were mixed and raised to 1L with distilled water) was added and left at laboratory temperature for 15 min. The reaction mixture without sample was used as control. The intensity of the colour formed was measured spectrophotometrically at 412 nm against reagent blank. The percentage of hydroxyl radical scavenging activity (HRSA) was calculated using the following formula:

HRSA (%) = [A control– A sample / A control] × 100.

Scavenging Activity against Hydrogen Peroxide

The scavenging capacity of 4-HPPP on hydrogen peroxide was determined according to the method.[18] Test tubes were prepared with 2.0 ml of various extracts (5-50µg/mL) and a solution of H_2O_2 (1.2 ml, 40 mM) in phosphate buffer (pH 7.4). A blank solution was prepared in the same way but without H_2O_2. After incubation of the mixture during 10 min, the absorbance

was recorded at 230 nm. AA was used as reference standard. The scavenging activity was calculated using the following

% scavenging activity = [(Ac – At)/ Ac] 100.

Where,

Ac absorbance of the control.

Reducing Ability Assay

The reducing power of 4-HPPP was evaluated according to the metho.[19] Different amounts of the 4-HPPP (5-40µg/ml) were suspended in distilled water and mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% $K_3Fe(CN)_6$. The mixture was incubated at 50°C for 20 min; 2.5 ml of 10% TCA was added to the mixture and centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%) and the absorbance was measured at 700 nm. Increase in absorbance of the reaction mixture indicated the ability of reducing power. AA was used as standard.

Metal Chelating Activity

In this assay, 1 m1 of ferric chloride (2 mM; diluted 20 times) is mixed with different dilutions of the 4-HPPP (1 ml). The reaction is initiated by the addition of 1 ml of ferrozine (5 mM; diluted 20 times). The absorbance is measured at 562 nm after 10 minutes.[20] The positive controls that can be used in this assay are EDTA, citric acid. The ability of the sample to chelate ferrous ions canfrom the following equation:

Chelating Effect (%) = (A0 – A1/A0) × 100.

where; A0 is the absorbance of control, A1 is the absorbance in the presence of sample.

Phosphomolybdenum Complex Method

In the phosphomolybdenum complex method, the reduction of Mo (VI) to Mo (V) is detected at 695 nm by spectrophotometer due to the formation of green phosphate Mo (V) compounds at acidic pH.[21] For the total antioxidant capacity assay, 0.1 ml of 4-HPPP is mixed with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in eppendorff tube. The tubes are then caped and incubated at 95ºC for 90 minutes in a thermal block. After incubation, the reaction mixture is cooled to room temperature and the absorbance is measured at 695 nm against reagent blank. AA may be used as the standard antioxidant.

Statistical Analysis

All the assays were carried out in triplicate. Experimental results are expressed as mean ± standard deviation. The results were analyzed using one-way analysis of variance and the group means were compared using Duncan's multiple range tests using SPSS version 16.

RESULT AND DISCUSSION

DPPH radical scavenging capacity

DPPH is a stable free radical. When antioxidant reacts with this stable radical, the electron becomes paired off and bleaching of the colour stochiometrically depends on the number of electron taken up. From our findings, it can be postulated that 4-HPPP reduces the radical moderately to the corresponding hydrazine when it reacts with the hydrogen donors in the antioxidant principles.[22] 4-HPPP exhibited a significant dose dependent inhibition of DPPH activity. The activity was compared with AA (standard). The results were depicted in Fig. 2, the IC_{50} value of AA and 4-HPPP was 37.74µg/ml and 35.62µg/ml respectively.

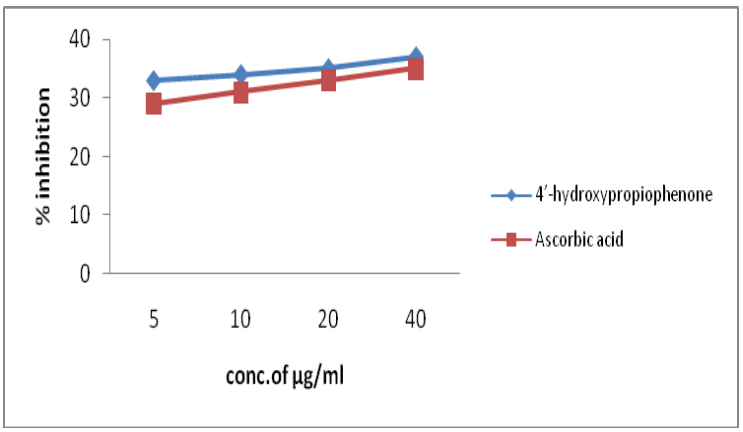

Fig. 2. DPPH radical scavenging activity of 4'-hydroxypropiophenone Each value is expressed as mean ± standard deviation (n=3).

ABTS Assay

$ABTS^{+}$ assay is a decolorizing assay, which involves the direct generation of ABTS radical into mono cation, which has a long wavelength absorption spectrum without the involvement of any intermediary radical. The antioxidant activity of the 4-HPPP by this assay implies that the action may be either inhibiting or scavenging radicals since both inhibition and scavenging properties of antioxidant towards this radical have been reported in earlier studies.[23] The inhibition capacity of radical ABTS and the AA values, expressed in 4-HPPP, for the different concentration were presented in Fig. 3, The IC50 showed that 4-HPPP present the highest AA behaviour, with values of 62.31µg/ml and 57.74µg/ml respectively.

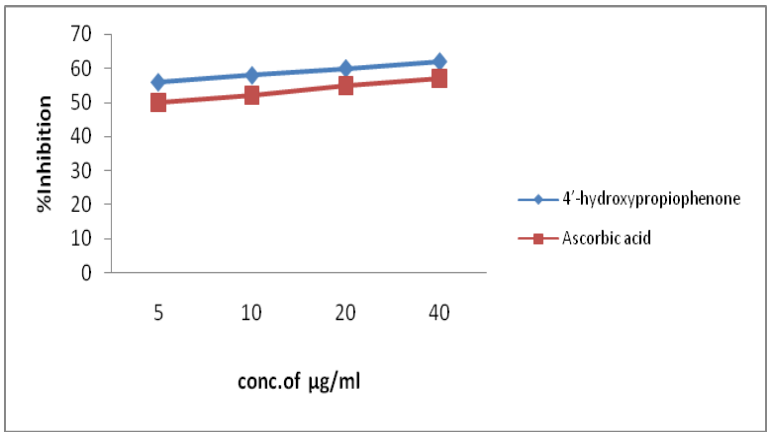

Fig. 3, ABTS radical scavenging activity of 4'-hydroxypropiophenone Each value is expressed as mean ± standard deviation (n=3).

FRAP Assay

Antioxidants can be explained as reductants, and inactivators of oxidants.[24] Some previous studies have also reported that the reducing power may serve as a significant indicator of potential antioxidant activity. Antioxidant activity is the ability to be related to reducing power. In this study, we used a FRAP assay because it is quick and simple to perform, and the reaction is reproducible and linearly related to the molar concentration of the antioxidant. FRAP assay was used by several authors for the assessment of antioxidant activity of various food products samples.[25] Suggested most of the secondary metabolities and redox- active compounds that will be picked up by the FRAP assay Fig. 4, revealed the reductive capability of 4-HPPP. The reducing power of the 4-HPPP increases with the increasing concentration was 42.79µg/ml and IC_{50} value of AA was 39.74µg/ml.

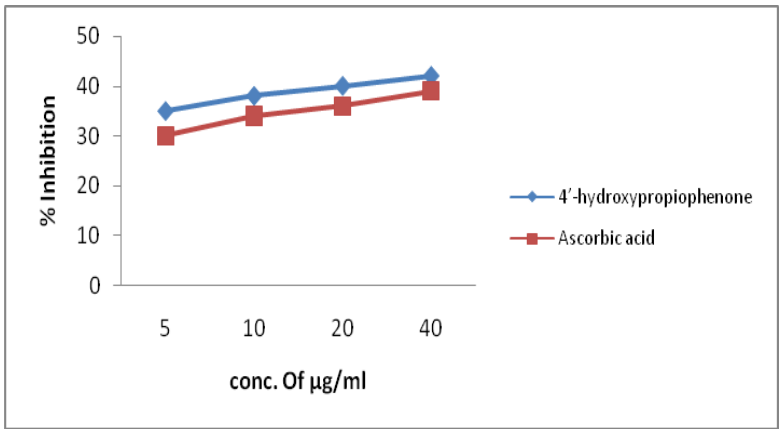

Fig. 4, FRAP radical scavenging activity of 4'-hydroxypropiophenone Each value is expressed as mean ± standard deviation (n=3).

Nitric Oxide Assay

Nitric oxide is a free radical produced in mammalian cells, involved in the regulation of various physiological process including neurotransmission, vascular homeostasis, antimicrobial and antitumor activities. However, excess production of NO is associated with several diseases. It would be interesting to develop potent and selective inhibitors of NO for potential therapeutic use.[26] No generated from sodium nitroprusside in aqueous solution at physiological pH reacts with oxygen to form nitrite ion. 4-HPPP inhibited nitrite formation in a concentration dependent manner (5-40µg/ml). This may be due to the presence of antioxidant principles in the 4-HPPP, which compete with oxygen to react with nitric oxide. The scavenging of nitric oxide by 4-HPPP was increased in a dose –dependent manner as illustrated in Fig. 5, The IC_{50} value of AA and 4-HPPP was 49.74µg/ml and 45.28µg/ml respectively.

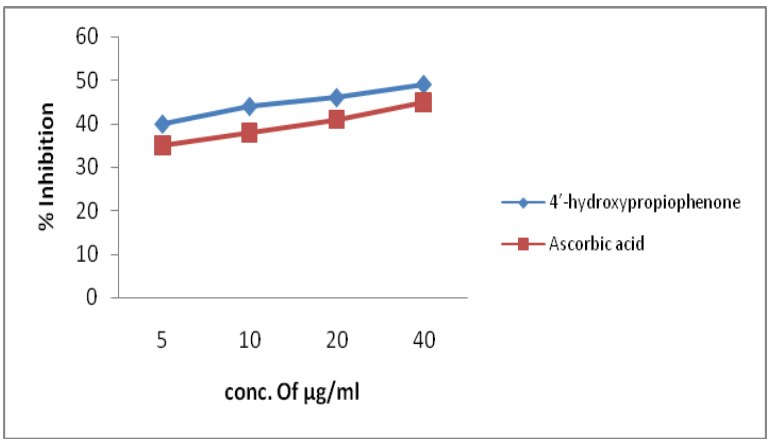

Fig. 5, Nitric oxide radical scavenging activity of 4'-hydroxypropiophenone Each value is expressed as mean ± standard deviation (n=3).

Superoxide Anion Assay

Superoxide is a highly reactive molecule that can react with many substrates produced in various metabolic process including phagocytosis. It can cause the oxidation or reduction of solutes depending on their reduction potential. Both aerobic and anaerobic organisms possess superoxide dismutase enzyme which catalyzes the breakdown of superoxide radical.[27] The superoxide anion derived from dissolved oxygen by phenazine methosulphate/nadh coupling reaction reduces nitro blue tetrazolium. The decrease in 4-HPPP of absorbance at 560nm indicated the consumption of superoxide anion in the reaction mixture. As mentioned in Fig. 6, 4-HPPP as well as AA showed the scavenging activity: IC_{50} value, 54.48µg/ml and 50.74µg/ml respectively.

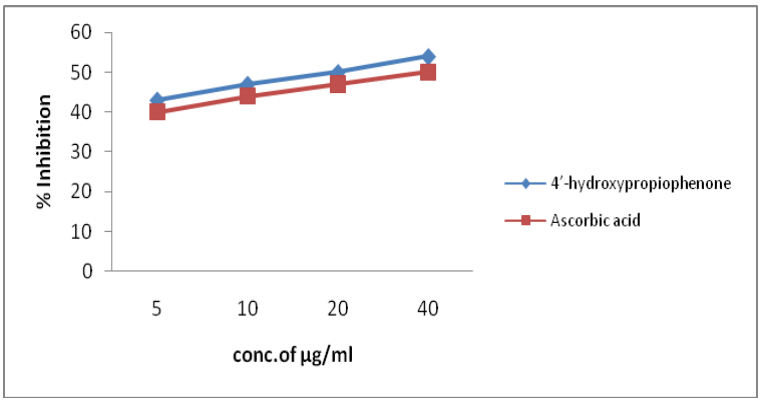

Fig. 6, Superoxide anion radical scavenging activity of 4'-hydroxypropiophenone Each value is expressed as mean ± standard deviation (n=3).

Hydroxyl Radical Assay

Hydroxyl radical itself is not very reactive, but sometimes it is toxic to cells because it may give to hydroxyl radical. Therefore, removing hydrogen peroxide is very important for antioxidant defence in a cell system.[28] Hydroxyl radical scavenging capacity of 4-HPPP is directly related to its antioxidant activity. This method involved *in vitro* generation of hydroxyl radicals using fe $^{3+}$/ascorbate/EDTA/H_2O_2 system using fenton reaction. The oxygen-derived hydroxyl radicals along with the added transition metal ion (Fe^{2+}) cause the degradation of deoxyribose into malondialdehyde, which produces a pink chromogen with thiobarbituric acid. To attack the substrate deoxyribose hydroxyl radicals were generated by reaction of ferric EDTA together with H_2O_2 and Ascorbic acid. When the 4-HPPP was incubated with the above reaction mixture, it could prevent the damage against sugar. The result for hydroxyl scavenging assay are depicted in Fig. 7, it is clear from the result that 4-HPPP have shown a concentration dependent radical scavenging activity. The concentration for 50% inhibition was found to be 36.57µg/ml and 31.74µg/ml for the 4-HPPP and AA respectively.

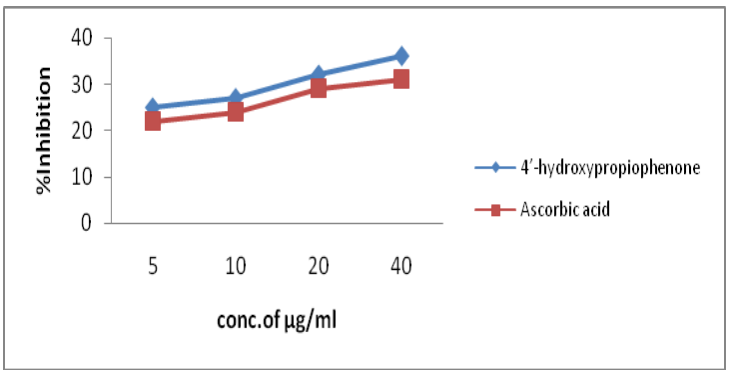

Fig. 7, Hydroxyl radical scavenging activity of 4'-hydroxypropiophenone Each value is expressed as mean ± standard deviation (n=3).

Hydrogen Peroxide Assay

Hydrogen peroxide (H_2O_2) is a weak oxidizing agent that inhibits the oxidation of essential thiol (-SH) group directly by few enzymes. Many of its toxic effects are because H_2O_2 has the ability to rapidly cross the cell membrane and once inside the cell, it can probably react with Fe^{2+} and possible Cu^{2+} ions to form hydroxyl radicals.[29] From the result, 4-HPPP was capable of scavenging H_2O_2 in a concentration dependant manner. The free radical scavenging activity of 4-HPPP was evaluated by hydrogen peroxide (H_2O_2) scavenging method. In Fig. 8, 4-HPPP showed the concentration dependent activity and the H_2O_2 scavenging effect. The result showed that the IC_{50} values of AA and 4-HPPP was 74.02µg/ml and 68.74µg/ml respectively.

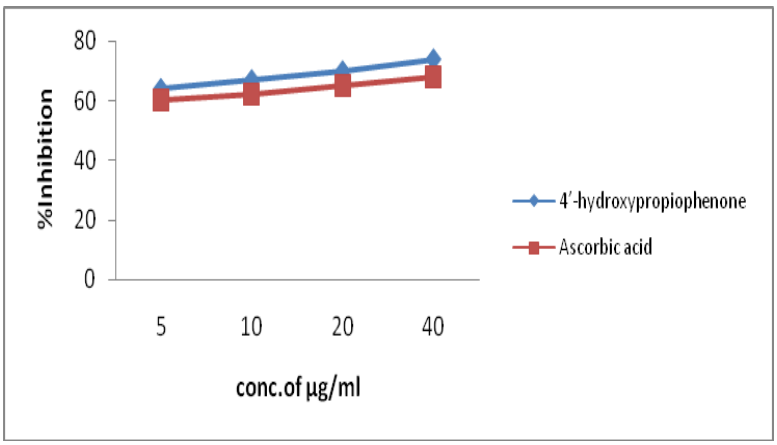

Fig. 8, Hydrogen peroxide radical scavenging activity of 4'-hydroxypropiophenone Each value is expressed as mean ± standard deviation (n=3).

Reducing Power Assay

Reducing the power of the 4-HPPP was evaluated by the transformation of Fe^{3+} to Fe^{2+} through electron transfer ability, which serves as a significant indicator of its antioxidant activity. The reducing power of the 4-HPPP are generally associated with the presence of reductants, which have been shown to exert antioxidant activity by donating a hydrogen atom by breaking the free radical chain. reductants are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. the presence of antioxidant substances in the compound samples causes the reduction of Fe^{2+} can be monitored by measuring the formation of perl's Prussian blue at 700nm.[30] Fig. 9, elucidated the reduced capabilities of 4-HPPP compared to Ascorbic acid. 4-HPPP could reduce the most Fe^{3+} ions, which had a lesser reductive activity than the standard of Ascorbic acid. The IC_{50} values of 4-HPPP and AA was 39.09µg/ml and 34.74µg/ml respectively.

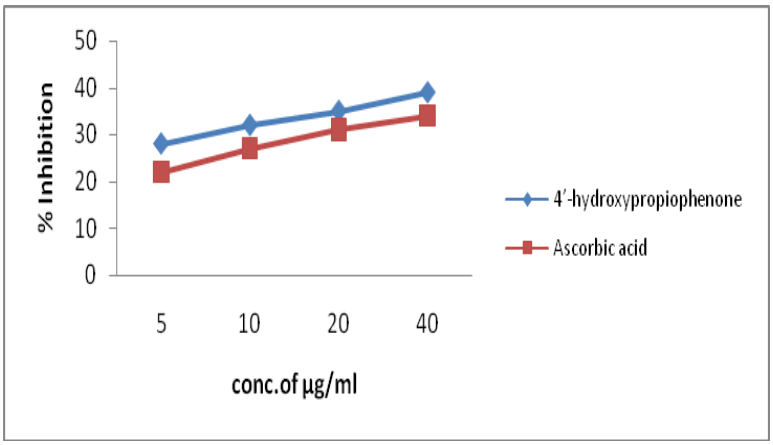

Fig. 9, Reducing ability radical scavenging activity of 4'-hydroxypropiophenone Each value is expressed as mean ± standard deviation (n=3).

Metal Chelating Assay

Iron is an essential mineral for normal physiology, but an excess of it, may result in cellular injury. If they undergo fenton reaction, these reduced metals may form reactive hydroxyl radicals and thereby contribute to oxidative stress.[31] An important mechanism of antioxidant activity is the ability to chelate/deactivate transition metals, which posess the ability to catalyze hydroperoxide decomposition and Fenton typereactions. Therefore, it is considered important to screen the iron by the 4-HPPP was estimated by the method. Ferrozine can quantitively from complex formation is disrupted with the result that red color of the complex is decreased Fig. 10, depicated the iron chelating activity of 4-HPPP. Thus result showed that the IC_{50} values of AA and 4-HPPP was, 55.74µg/ml and 37.90µg/ml respectively.

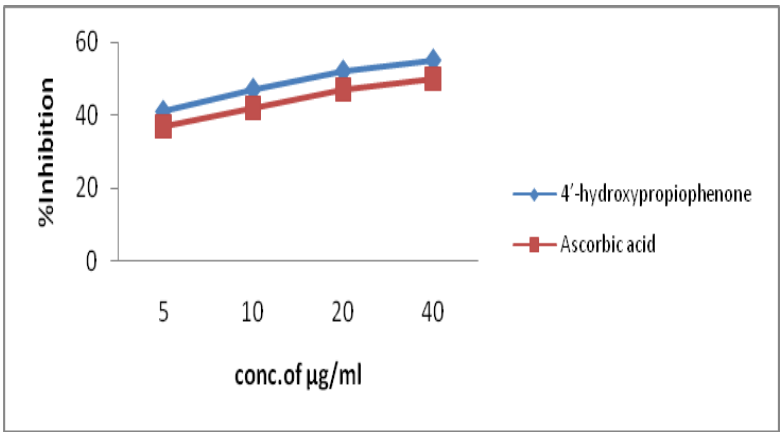

Fig. 10, Metal chelating radical scavenging activity of 4'-hydroxypropiophenone Each value is expressed as mean ± standard deviation (n=3).

Phosphomolybdenum Assay

This assay has been routinely used to evaluated the antioxidant capacity of 4'-hydroxypropiophenone. Various concentration of 4-HPPP used to determine their antioxidant capacity by the formation of green phosphomolybdenum complexes. The formation of the complex was measured by the intensity of absorbance in 4-HPPP at a concentration of 5-40 µg/ml at 95^0C. The phosphomolybdenum method is based on the reduction on M_0(VI) to M_0(V) by the antioxidant compounds and the formation of green phosphate/ M_0(V) complex with the maximal absorption at 695nm Fig. 11, illustrated the phosphomolybdenum assay of 4-HPPP. This revealed that increased phosphomolybdenum reduction of 4-HPPP to the quantity of the sample. The IC50 values of 4-HPPP was 43.35µg/ml and 38.74µg/ml as a standard Ascorbic acid. This may be explained by fact that the transfer of electron/hydrogen from antioxidants depends on the structure of the antioxidants.[32]

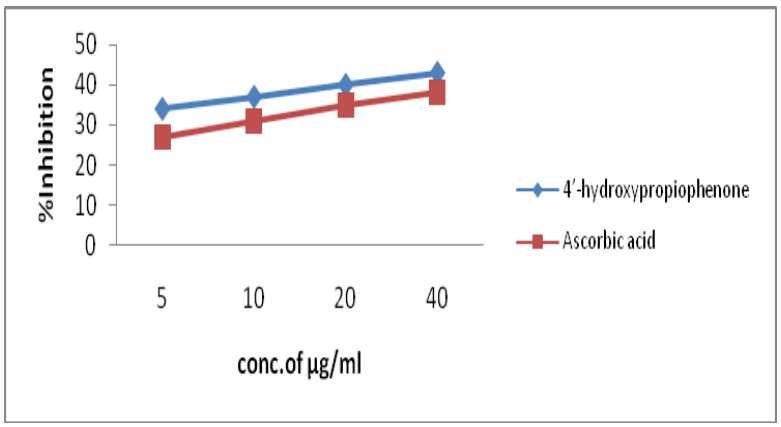

Fig. 11, Phosphomolybdenum scavenging assay of 4'-hydroxypropiophenone Each value is expressed as mean ± standard deviation (n=3).

CONCLUSION

The results of the present work indicated that the 4-HPPP was a fairly active scavenging assay system. The present findings seem promising to facilitate further experiments on the identification and characterization specific of compounds which are responsible for the relatively high antioxidant activities. Importantly, this research may contribute to a rational basis for the application of 4-HPPP in possible therapy of diseases associated with oxidative stress.

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