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Radical Scavenging Activity of Peptide Fraction from *Curcuma pseudomontana* L. A Wild Variety Of Turmeric

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Abstract

The present investigation focusses on antioxidant activity of peptide fraction P4 from a wild variety of turmeric, *Curcuma pseudomontana* L. (*Zingiberaceae*). P4 was purified by standard protein purification protocol. 80% saturated ammonium sulphate protein precipitate exhibited 78±2.65% hydroxyl radical scavenging activity, was purified on Sephadex G-75 column that resulted in two peaks labelled as AS1 and AS2. AS2 showed 75±0.76% hydroxyl radical scavenging activity and AS1 exhibited 40±1.23% at 100µg/ml each compared to the reduced glutathione and butylated hydroxyltoluene. AS2 on preparative *rp*-HPLC eluted at different retention time into four fractions labelled as P1, P2, P3 and P4. The peptide fraction, P4 eluted at 39.73min showed 65±0.88% hydroxyl radical scavenging activity. Peak fractions 1, 2, and 3 exhibited 48±0.98%, 42±1.34% and 49±0.67% hydroxyl radical scavenging activities respectively. P4 efficiently quenched 2,2-diphenyl-1-picrylhydrazyl radicals by 68±0.94%. The ferric ion reducing ability of P4 was concentration dependent which showed 79±1.04% at 25µg/ml. P4 showed lipid peroxidation inhibition activity in a linoleic acid model system.

Keywords

Reduced glutathione (GSH), *Curcuma pseudomontana,* Peak fraction, Radical scavenging activity.

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1. INTRODUCTION

In multicellular organism the normal endogenous cellular metabolism releases reactive oxygen species that includes hydroxyl free radicals, superoxide radicals and reactive nitrogen species like nitric and nitrous oxide free radicals [1] that play deceptive role during cellular processes like apoptosis, cell division and are also involved in redox reactions at physiological cell concentration [2]. The cell's protective system is well equipped with endogenous enzymes such as thioredoxin, peroxidase, Catalase and super oxide dismutase, other natural compounds that can neutralize the effect of disproportionate amount of ROS/RNS, include peptide antioxidant glutathione, and natural antioxidants, carotenoids, flavonoids, vitamin derived compounds like tocopherols play a significant role in protecting the cell against these species. Adverse external factors like radiations, toxic chemicals released in the environment, inefficiently metabolised drugs and carcinogens present in cigarettes, xenobiotics [3] could deplete the concentration of these protective enzymes leading to an imbalance of superfluous ROS that in turn could cause lipid peroxidation and finally effecting the cellular macromolecules including DNA

damage and oxidative stress leading to non-communicable disease or declining the health condition of individuals suffering from chronic disease (atherosclerosis, neurodegenerative disease and aging) [4]**.** Chemically synthesised antioxidants such as butylated hydroxytoluene (BHT), butyl hydroxy anisole (BHA), and tert-Butylhydroquinone (TBHQ) are strong radical scavenging compounds and thus are utilized in food industry as supplement antioxidants. Continuous use of these chemicals has a deleterious harmful effect on human health. Hence, an alternative is to incorporate antioxidant rich food sources that could overcome the effect of ROS. Therefore, a large amount of scientific data exists to screen the antioxidant activities of secondary metabolites of plant, such as, carotenoids, polyphenols, and alkaloids all of which have radical scavenging activity. At present a lot of research is emphasised on generating peptide from protein hydrolysate that could serve as potent antioxidants in addition to health promoting activities because of they are harmless to the biological system, small, and easily utilizable [5]. From literature it has been proved that many antibacterial peptides also exhibit potent free radical scavenging abilities. Peptides isolated from hen egg white hydrolysates of Lysozyme enzyme have been reported to exhibit both antioxidant and antibacterial activity [6]. From literature it has been revealed that the structure (mass, type of amino acids and hydrophobicities) of the peptide directly influence the antioxidant property [7].

Innumerable research is being carried out in the last 25-30 years in the screening of turmeric with more than 400 publications revealing hundreds of novel bioactive components with potent biological activities. According to the data available approximately 20 molecules are antimicrobial 14 molecules as anticancer with a dozen of molecules exhibiting anti-tumour and anti-inflammatory activity with at least 10 molecules as antioxidant [8]. According to Ruby A. J et al 1995 and Sevam R et al 1995 [9,10] the major constituent of rhizome is the volatile oil, the major constituent present is the tumerone. The other coloured substances that are present are termed as curcuminoids. The most significant being curcumin (diferuloyl methane or 1, 7-bis (4-hydroxy-3-methoxyphenol)-1, 6-heptadiene-3, 5-Dione [11] and two of the related compounds, Curcumin desmethoxycurcumin, 5', -methoxy curcumin and dihydrocurcumin. The yellow colour of the root is due to the presence of these curcuminoids which are a group of polyphenols that exhibits excellent antioxidant properties [12].

The aim of the present investigation is to determine the radical scavenging activity of peptide isolated from the rhizome of plant *Curcuma pseudomontana*.

2. MATERIALS AND METHODS

2.1. Materials:

Wild variety of *Curcuma species, Curcuma Pseudomontana* was collected from Western Ghat region of India. Sephadex G-75, gels were procured from Sigma Aldrich Inc. Butylated hydroxy anisole (BHA), and *1,1*-diphenyl-*2*- picrylhydrazyl (DPPH), *2*-Deoxy-D-Ribose, were purchased from E-Merck India. All other chemicals used were of analytical grade.

2.2. Methodology:

Plant collection, authentication, and protein precipitation

Wild variety of *Curcuma species, Curcuma Pseudomontana* L. was collected from Western Ghat region of Southern India, during the month of February. The plant was authenticated by Dr. GVS Murthy, Director, Botanical Survey of India, Tamil Nadu Agricultural University campus, Coimbatore, Tamil Nadu, India. A voucher specimen was deposited in the laboratory for future reference (No.: BSIS/RC/5/23/2017/Tech./564). The sample was washed thoroughly with de-ionized water to remove extraneous and sand particles. The rhizome part of the sample was cut into small pieces, spread on thin paper and shade dried (temperature not exceeding 27°±2°C) separately. The shade dried sample was powdered and sieved through muslin cloth to remove fibers. The finely sieved powder was stored in -200˚C in airtight container until use for studies.

Fifty grams of the sample was taken and suspended in 1:1 ratio of extraction buffer (0.1M NaCl) for salting in of proteins and the mixture was stirred overnight at temperature not exceeding 80˚C. The suspension thus obtained was centrifuged at 12000g for 20min (to remove cellular debris) at 40˚C. The pellet was discarded, and the supernatant obtained was used for graded salting out of proteins (from 40% to 100% saturation). The samples were dialysed extensively with frequent changes of distilled water for 72 hrs to remove residual ammonium sulphate, centrifuged at 1500rpm for 10 min at 40˚C. Supernatant was collected and evaluated for hydroxyl radical scavenging activity. The salting out of proteins at 80% saturation showed maximum radical scavenging activity and thus was used for fractionation through Sephadex G-75. Approximately 10 mg of the protein was loaded on to the column pre-equilibrated with 0.1M phosphate buffer, pH 7.4 (Flow rate at 1.5ml/5min). The fractionation resulted in two peaks, AS1 and AS2. The second peak AS2

exhibited potent hydroxyl radical scavenging activity was lyophilized and further analysed using analytical and preparative rp-HPLC.

***2.3. rp*-HPLC of AS2 fraction:**

Reversed phase purification of AS2 was carried out using Agilent 1260 infinity quaternary LC system composed of G1311B/C quaternary pump/G1329B auto sampler G1330B thermostat and G4212B VWD. The HPLC system was equipped with eclipse C18 reverse phase column (4.6mmX150mm ID; 5mM particle). The analysis of the chromatographic data was carried out on open lab CDS Chem station software (A 01.05, updated in 2018). About 20µg of lyophilized AS2 was dissolved in deionized water and injected onto the column. The protein fraction was eluted with 0-90% water: acetonitrile gradient at a flow rate of 1ml/min for 70min. Protein elution was monitored at 280nm.

2.4. Preparative rp-HPLC:

Preparative rp-HPLC was carried out using Shimadzu LC20A model consisting of dual pump 2LC20AT, column oven CTO20A, fraction collector FRC10A and the detector SPD20A.The HPLC system was equipped with C18 column (Sepax Amethyst C18-H, 100 mm 3.00 mm). The analysis of the chromatographic data was carried out on LC Solutions software. About 20µg of lyophilized AS2 dissolved in deionized water was injected and eluted with 0-90% water: acetonitrile gradient at a flow rate of 5ml/min for 60min. The proteins eluted at different retention time (Peak P1 to P4) were collected through automated fraction collector and was monitored at 280nm. The protein samples were lyophilised using VD-800 Freeze drier. The protein concentration was estimated by Bradford method (Bradford, 1976). Peak4 (P4) showed maximum hydroxyl radical scavenging activity. Hence purity of peak P4 was checked again on analytical RP-HPLC with a retention time of 39.735 min.

2.5. Determination of hydroxyl radical scavenging activity by *2*-Deoxy D-ribose assay:

Hydroxyl radical scavenging activity of the 80% ammonium sulphate precipitated and fractionated on sephadex G-75 gel permeation chromatography were subjected to hydroxyl radical scavenging activity using, *2*-Deoxy D-ribose assay [13]. The reaction mixture containing $FeCl_3$ (100µM), ascorbate (100µM), EDTA (104µM), H_2O_2 (1mM), 2-deoxy-D-ribose (2.8mM) were mixed with 100µg each of the peak fractions (AS1 and AS2) in 20mM potassium phosphate buffer, pH7.4 and incubated for 1hr at 37°C. A similar assay was done with known antioxidant such as reduced glutathione was used as positive control. The reaction mixture was heated at 95^{0}C in boiling water bath for 15min following the addition of 1ml of TBA (0.5%). Finally, the reaction mixture was cooled on ice and optical density was measured at 535nm. The assay was carried out with appropriate blanks and controls. Antioxidant activity was expressed as percent inhibition of hydroxyl radical formation. The same protocol was followed for the peak fractions (P1-P4) obtained from preparative rp-HPLC.

Hydroxyl radical scavenging activity (%) = A_{blank}-A_{sample} /A_{blank} X 100

2.6. *1, 1*– diphenyl-*2*-picrylhydrazyl (DPPH) radical scavenging activity:

1, 1– diphenyl-*2*-picrylhydrazyl (DPPH) radical scavenging activity was assessed. According to the method of [14,15] with few modifications. The reaction mixture containing 10µg each of the peak fractions(Peak 1,2,3 and 4) was mixed in 1ml of freshly prepared 0.5mM ethanolic DPPH solution and 2.0ml of 0.1M acetate buffer, pH5.5. Similar assay was carried out with reduced glutathione as positive control. The resulting solutions were then left to stand at 37°C for 30min the reading was recorded at 517nm in Hitachi U-2900 spectrophotometer. Lower absorbance at 517nm represents higher DPPH scavenging activity. An appropriate Blank was also taken with the respective buffer. The % inhibition was calculated by comparing with that of control. Antioxidant activity was expressed as percent inhibition of DPPH radical formation.

DPPH=ABTS radical scavenging activity (%) = A_{blank}-A_{sample} / A_{blank} X 100

2.7. Test for ferric ion reducing power:

The reducing power was determined according to the method of [16].100µl of 4mM potassium ferricyanide solution was mixed with 200µl of 20mM phosphate buffer pH6.5 with 10µg each of the peak fractions (Peak 1,2,3 and 4). A similar assay was done with Ascorbic acid at 10µg concentration. The contents were incubated at 50°C for 20min. 200µl of 10%TCA was added to the reaction mixture and centrifuged at 5000rpm for 10min at room temperature. The resulting supernatant was taken and mixed with 100µl of 2mM ferric chloride solution and final volume was made up to 1ml with distilled water and then incubated at 37°C for 10min. The absorbance was recorded at 700nm. Absorbance increases with increase in reducing power. Distilled water and GSH were used respectively as the blank and positive control

2.8. Lipid Peroxidation Inhibition Assay:

The capacity of inhibiting linoleic acid peroxidation for P1, P2, P3 and P4 was evaluated according to the method of [17] with slight modifications. The mixture solution containing 10 µg of P1, P2, P3 and P4 lyophilized samples were made up to 1 ml using phosphate buffer pH 7.4. 1.0 ml of 2.5% (v/v) linoleic acid dissolved in anhydrous ethanol, and 1.0 ml of deionized water was taken in a crew capped conical flask, incubated in darkness at 40°C for 7 days. The linoleic acid oxidation inhibition of P1, P2, P3 and P4 was determined at 24h intervals by measuring the ferric thiocyanate values. An aliquot of 0.1 ml of the mixture of each peak fraction was mixed with 4.7 ml of 75% (v/v) ethanol, 0.1 ml of 30% ammonium thiocyanate and 0.1 ml of 20mM ferrous chloride dissolved in 3.5% (v/v) hydrochloric acid. The absorbance of thiocyanate was recorded at 500 nm after 3.0 min. Deionized water was used as the blank, and GSH was used as positive controls.

The inhibition of lipid peroxidation was calculated as **(1-A sample 532/ A blank532) × 100%.**

3. RESULTS AND DISCUSSION

Normal cellular metabolism releases Reactive Oxygen Species (ROS) that can be encountered by body's natural antioxidants such as GSH, superoxide dismutase (SOD), Catalase etc. that are in balance to prevent the accumulation of free radicals [18]. But under certain circumstances, the balance is affected, and the accumulation of these free radicals can have an adverse effect on the health potential of an individual, thus causing a chronic diseased state. Increase in free radicals can damage cellular structures such as membrane phospholipids and cellular macromolecules include proteins that may be enzymes, structural polysaccharides, and DNA. Synthetic antioxidants such as BHA (butylated hydroxyl anisole) and BHT (butylated hydroxyl toluene) used in food industry render side effects that are harmful in the long run [19]. Hence effective natural antioxidant peptides are a major area for exploration because of its advantage over chemical antioxidants such as low toxicity without any side effects, easily absorbable, high clearance rate, and low molecular weight with amino acid residues of most of the antioxidant peptides ranging from 2-20 are an added advantage. Antioxidant peptides may be released during in vivo digestion, in vitro enzymatic hydrolysis of meat products, cereals or during processing of food. Antioxidant peptides have been isolated and characterised by enzymatic hydrolysates from various sources such as walnut protein hydrolysates [20] sweet potato hydrolysates [21] foxtail millet [22] and Leek [23] *Curcuma* species exhibiting potent pharmacological activities, Turmerin, a 14KDa protein from *Curcuma longa* exhibits cytotoxic and myotoxic properties against NV-PLA2 [24] antioxidant activity exhibiting proteins (TAP) and lipid peroxidation were also isolated from aqueous extract of *Curcuma longa* [25]. The present investigation compiles the study of isolation, purification, and characterization of peptide P4 from the rhizome of *Curcuma pseudomontana* by a series of purification methods. The crude extract of 40%, 60%, 80% and 100% ammonium sulphate saturation were evaluated for hydroxyl radical scavenging activity. These are highly reactive species that can damage biological macromolecules. The 80% saturated crude extract showed significant scavenging activity, this precipitated sample was further dialysed and fractionated on sephadex G-75 that resulted in two peaks AS1 and AS2 (Fig 1) The peak AS2 exhibited 75 ±0.76% antioxidant activity comparable to the standard GSH and BHT that exhibited 83 ±0.67% and 78±1.054% radical scavenging activity at the same concentration of 100µg/ml compared to 40±1.23% activity of AS1 (Fig 4). Hence AS2 was further purified in RP-HPLC, eluted into four peaks P1-P4 (Fig 2) and each peak were evaluated for its hydroxyl radical scavenging activity (Fig 5). The peak P1, P2 and P3 did not exhibit significant antioxidant activity as compared to peak 4 fraction eluted at 39.73 min (Fig 3) exhibited 65±0.88% scavenging activity.

DPPH (2, 2-diphenyl-1-picrylhydrazyl) radicals scavenging activity of the eluted fractions (P1-P4) from the preparative rp- HPLC at the concentration of 10µg/ml was done. DPPH is very popular to determine the antioxidant capacity of natural antioxidants [26]. DPPH is a stable free organic radical, which accepts hydrogen from a potent donor and the absorbance is reduced at 517nm due to the colour change from deep purple to yellow. The hydrogen donating capacity of P1, P2, and P3 was 40±1.23% compared to 68±0.94% activity of P4. The antioxidant peptide GSH was used as positive control exhibiting 82±0.67% activity (Fig 6).

The reducing power is a measure of the ability of an antioxidant substance to donate electrons or hydrogen. The reducing capacity of the peak fractions (1-4) was dose dependent, there was a proportional increase in the reducing activity with increase in concentration from 5µg to 25µg concentration compared to the standard GSH (Fig 7). Lipid peroxidation involves a complex process of formation of primary oxidation products such as hydroperoxides and lipid radicals that are released in the presence of oxygen. It involves free radical mediated removal of electron from methylene

carbon of polyunsaturated fatty acids, the consequence is a membrane damage [27]. The assay is to evaluate the inhibitory activities of Peak fractions 1-4 by measuring lipid peroxidation *invitro* for 7 days and comparing with the standard antioxidant peptide and a synthetic antioxidant BHT. The result in the figure 8 shows that P4 exhibited maximum inhibition compared to P1, P2 and P3, but all the four fractions inhibited lipid peroxidation as compared to control. The maximum inhibitory activity of P4 could be attributed to radical scavenging ability of the P4 fraction that could be used in food packaging industry to inhibit the formation toxic substances that may result due to oxidation by contamination. The results of the present investigation suggest that the purified peptide P4 isolated from *Curcuma pseudomontana* exhibited excellent radical scavenging activities and lipid peroxidation inhibitory activities.

Fig 1: Elution profile of 80% saturated *Curcuma pseudomontana* proteins (6mg/ml) through Sephadex G-75. Proteins were eluted at 1.5ml/5min at 40°C, monitored at 280nm. The two peaks eluted obtained were designated as AS1 and AS2:

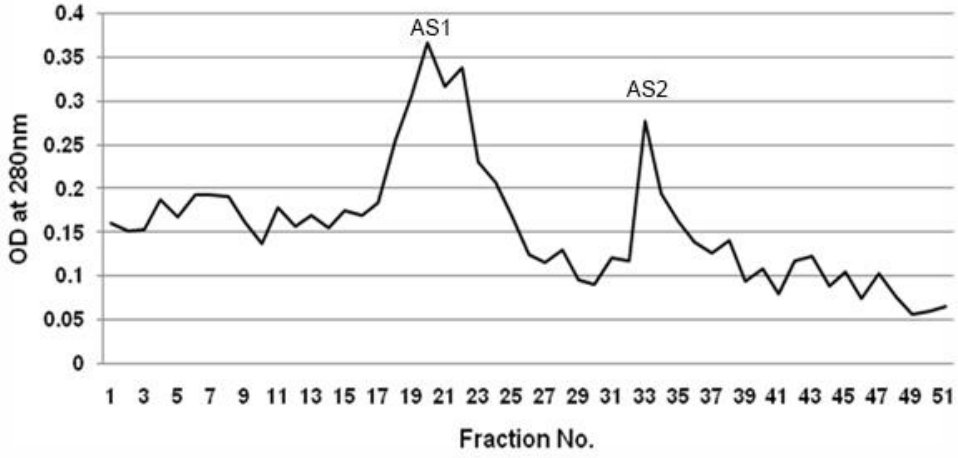


Fig 2: Reversed phase purification of AS2 was carried out using Agilent C18 reverse phase column (7.8mmX300mm), bead size 10µm and porosity 125Å) pre- equilibrated with 0.1% trifluoro acetic acid (TFA) in water. About 50µg of AS2, pre-incubated with 0.1% TFA was applied on to the column. Protein was eluted with 0-90% water: acetonitrile gradient at a flow rate of 1ml/min for 70min. Protein elution was monitored at 280nm:

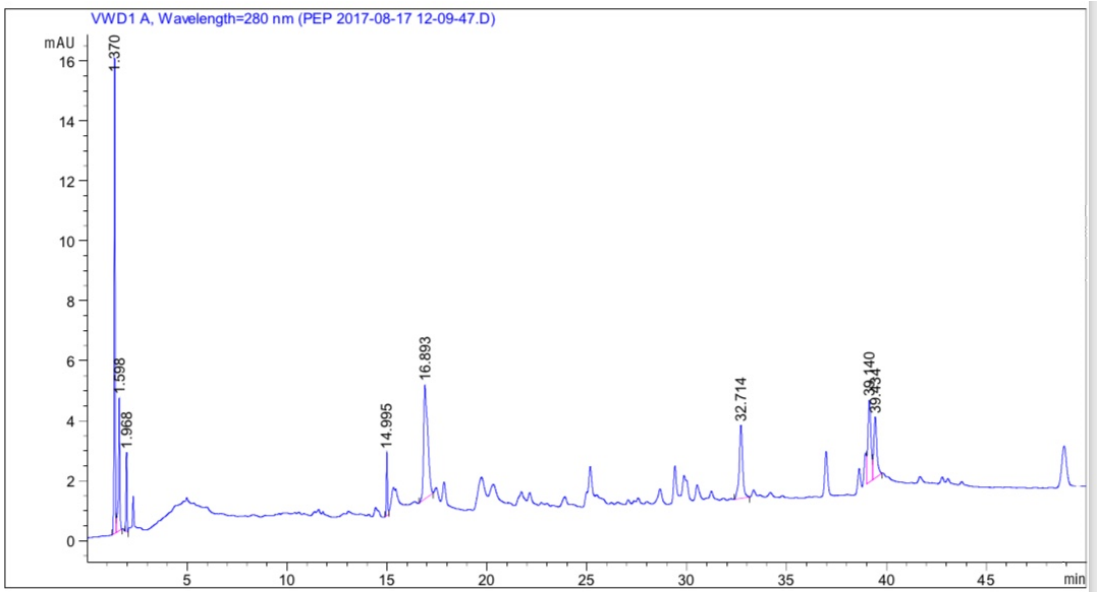


Fig 3: Reversed phase purification of peak 4 of concentration10µg was injected to eclipse C18 reverse phase column (4.6mmX150mm ID; 5mM particle). Protein was eluted with 0-90% water: acetonitrile gradient at a flow rate of 1ml/min for 50min and was monitored at 280nm. Peak eluted with the retention time of 39.735 minute was retained as P4:

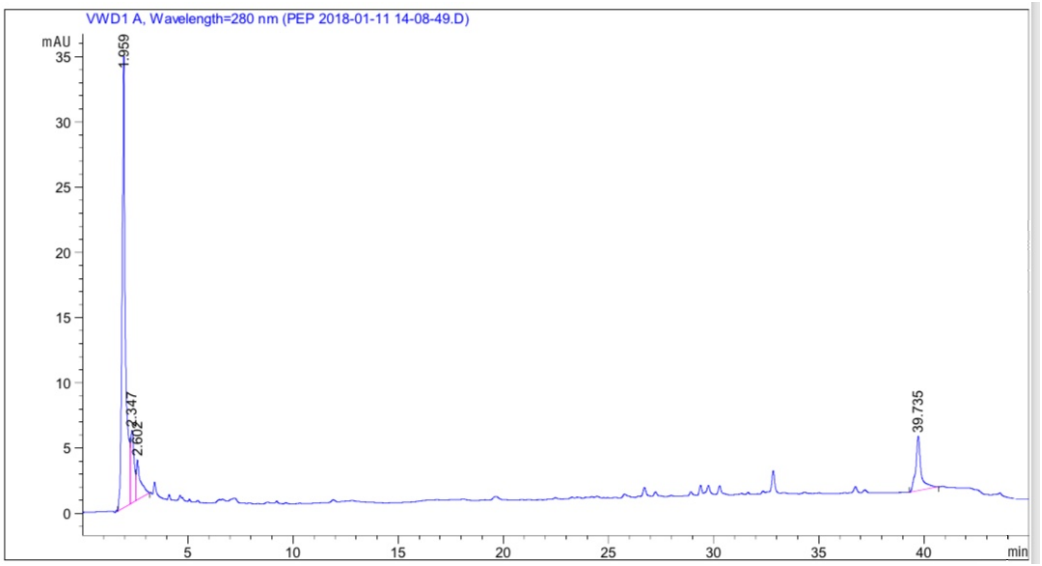


Fig 4: Hydroxyl radical scavenging activities of *Curcuma pseudomontana* 80% gel permeation fraction labelled as AS1 and AS2. GSH and BHT were used as positive control. All data are presented as the mean ± standard deviation (SD) of triplicate results:

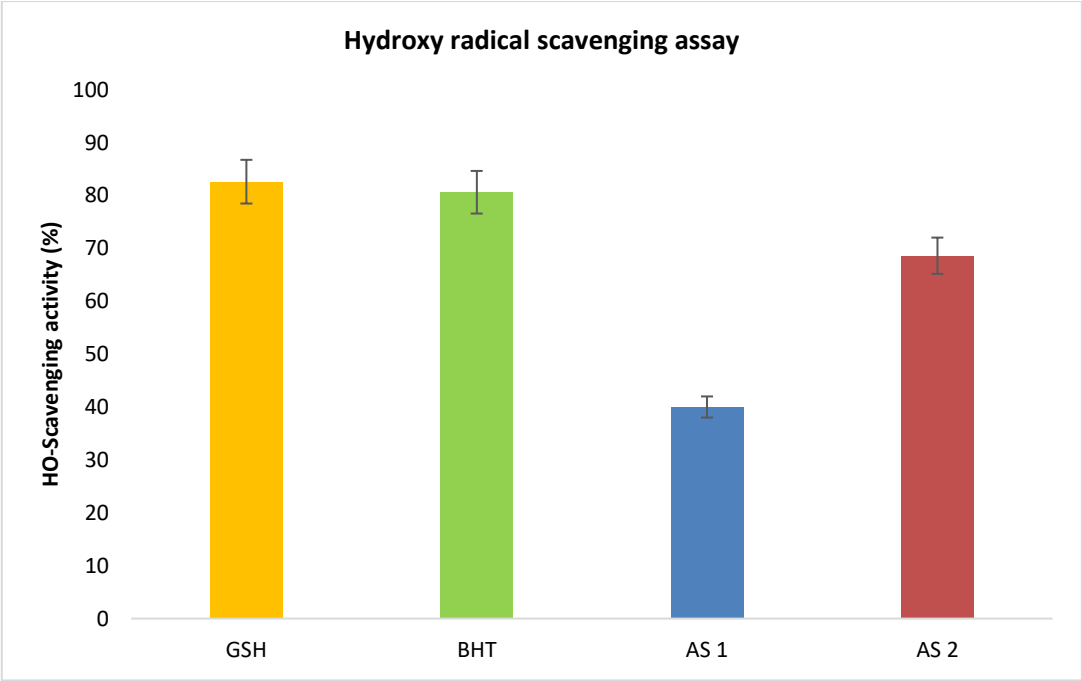


Fig 5: Hydroxyl radical scavenging activities P1-P4 fractions obtained from AS2 subjected to preparative rp-HPLC. GSH and BHT was used as positive control. All data are presented as the mean ± standard deviation (SD) of triplicate results:

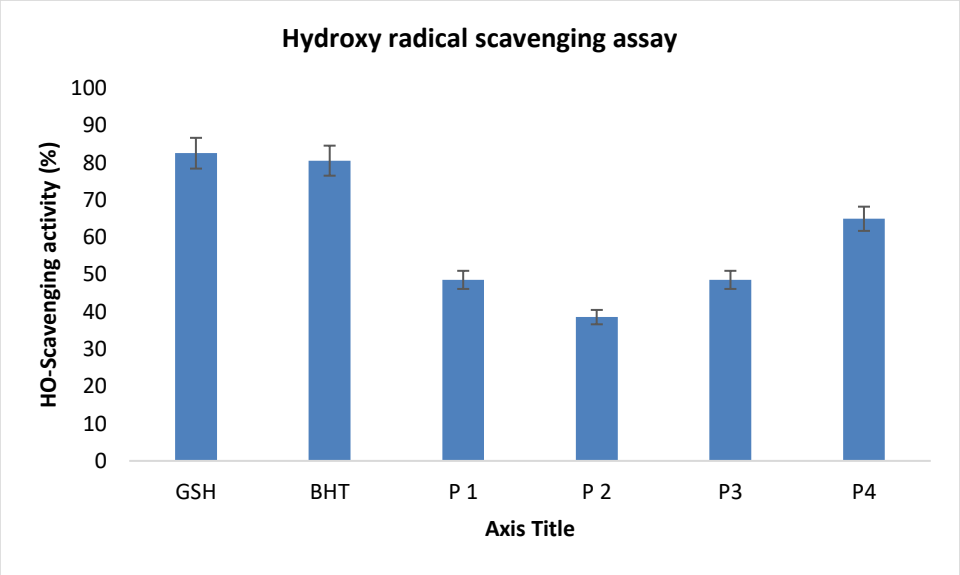


Fig 6: DPPH Assay: 2,2-diphenyl-1-picrylhydrazyl radicals scavenging activity of the eluted fractions (P1-P4) from the preparative rp- HPLC at the concentration of 10µg/ml. The antioxidant peptide GSH was used as positive control. All data are presented as the mean ± standard deviation (SD) of triplicate results:

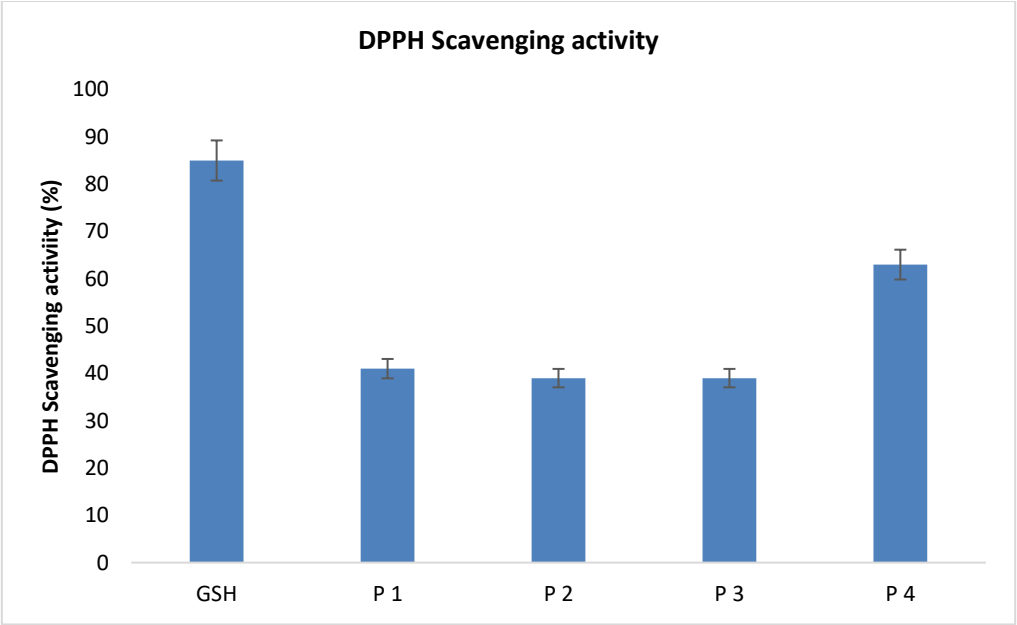


Fig 7: Reducing power of the eluted fractions (P1-P4) from the preparative rp- HPLC was dose dependent (5µg to 25µg) GSH was used as positive control. All data are presented as the mean ± standard deviation (SD) of triplicate results:

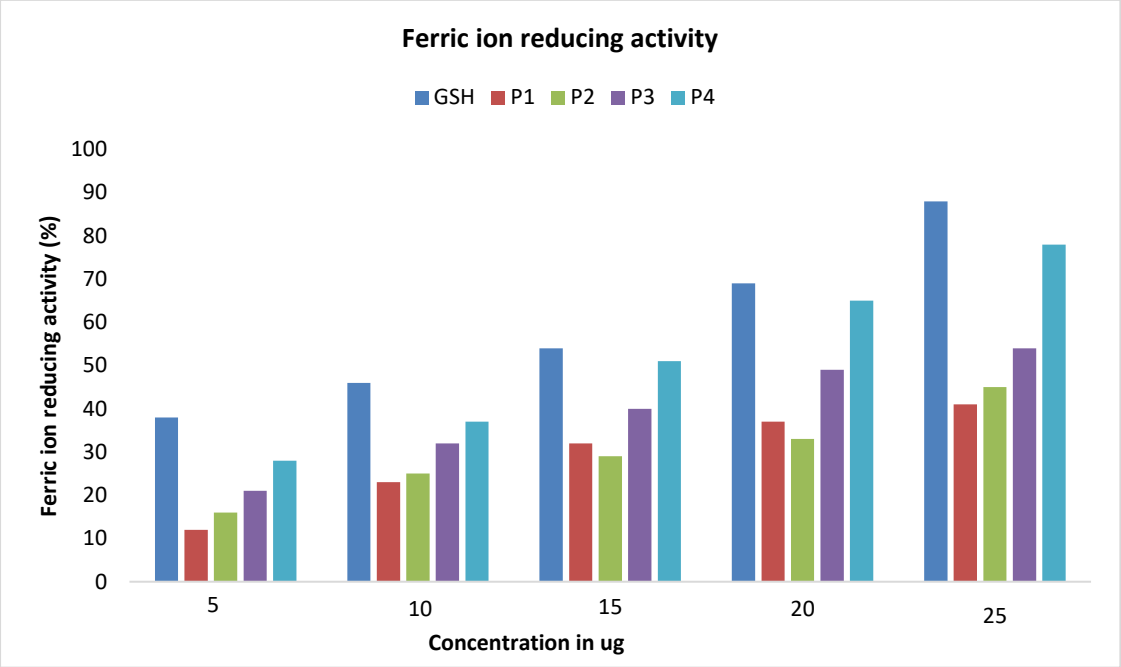
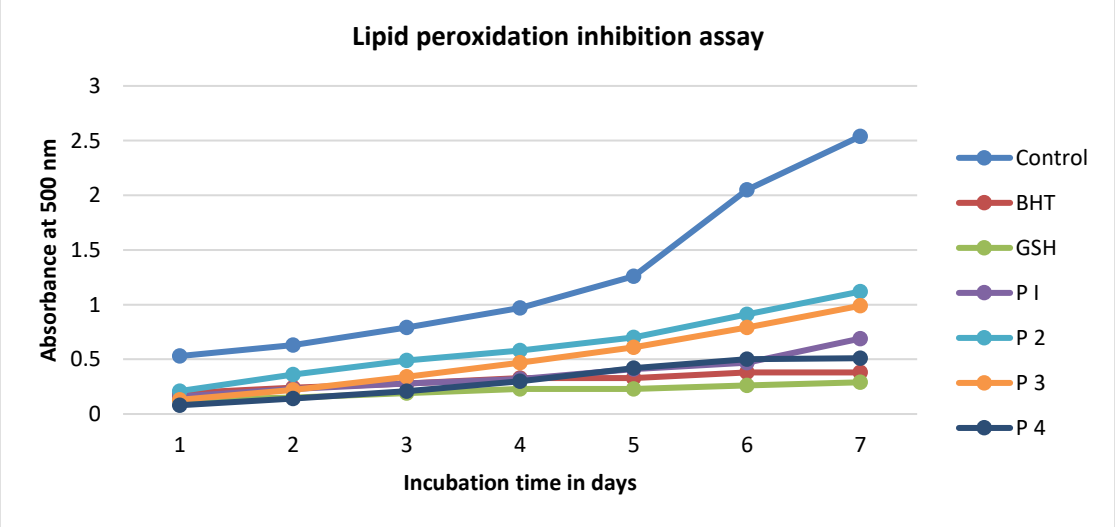


Fig 8: Lipid peroxidation inhibition assays of P1-P4. The degree of lipid oxidation was measured at 24 h intervals. The antioxidant peptide GSH and BHT was used as positive control. All the data are presented as the mean ± SD of triplicates:



4. CONCLUSION

In the present investigation, four peptide fractions were isolated and purified from *Curcuma pseudomontana* L. using standard protein purification techniques. All the four fractions exhibited good radical scavenging and lipid peroxidation activity. Based on the results of our studies natural antioxidant peptides could be an alternative to synthetic antioxidants used in food and packaging industry.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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