Original Article

In vitro antioxidant potential and deoxyribonucleic acid protecting activity of CNB-001, a novel pyrazole derivative of curcumin

Abstract

Background: Free radicals are underpinned to initiate cascade of toxic events leading to oxidative stress and resultant cell death in many neurodegenerative disorders. Now-a-days antioxidants have become mandatory in the treatment of various diseases apart from the drug's modes of action. CNB-001, a novel hybrid molecule synthesized by combining curcumin and cyclohexyl bisphenol A is known to possess various biological activities, but the antioxidative property of the compound has not yet been elucidated. **Aim:** The present study is aimed to analyze various free radicals scavenging by employing *in vitro* antioxidant assays and to evaluate the deoxyribonucleic acid (DNA) protecting the ability of CNB-001 against hydroxyl radicals. **Materials and methods:** The *in vitro* antioxidant potential of CNB-001 was evaluated by analyzing its ability to scavenge DPPH, ABTS, nitric oxide, superoxide, hydrogen peroxide, superoxide anion, hydroxyl, hydrogen peroxide radicals and reducing power using spectroscopic method. The DNA protecting activity of CNB-001 was also evaluated on pUC19 plasmid DNA subjected to hydroxyl radicals using standard agarose gel electrophoresis. **Results:** From the assays, it was observed that CNB-001 scavenged free radicals effectively in a dose dependent manner. CNB-001 scavenged 2,2-diphenyl-1-picrylhydrazyl (IC50 = 44.99 μg/ml), 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (IC50 = 17.99 μg/ml), nitric oxide (IC50 = 1.36 μg/ml), superoxide radical (IC50 = 77.17 μg/ml), hydrogen peroxide (IC50 = 492.7 μg/ml), superoxide (IC50 = 36.92 μg/ml) and hydroxyl (IC50 = 456.5 μg/ml) radicals effectively and the reducing power was found to be 11.53 μg/ml. CNB-001 showed considerable protecting activity against plasmid DNA (pUC19) strand scission by •OH at dose dependent manner. **Conclusion:** Results from these assays concluded that CNB-001 has a good antioxidant potential by reducing reactive oxygen and reactive nitrogen radicals and it showed significant protecting activity against DNA scission by hydroxyl radicals. Hence, CNB-001 can be further developed as potential drug for free radical induced neurodegenerative disorders.

Key words:
2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid), 2,2-diphenyl-1-picrylhydrazyl, antioxidant, -001, deoxyribonucleic acid protecting, free radicals

Introduction

Atoms or molecules having a retiring unpaired electron in the outer shell are called as free radicals. The tendency of free radicals is that they react chemically with other molecules to capture electron in order to pair the radicals unpaired electron.[1] Free radicals in biological systems were first investigated by Denham Harmon in 1956. His first proposal was the role of free radicals in ageing.[2] Later, based on the importance of free radicals in human health, various researches have been carried out. Superoxide dismutase, an important antioxidant enzyme first discovered in the year 1969 by McCord and Fridovich gave strong evidence on free radicals in living system[3] Mitochondrial oxidative phosphorylation, reactive oxygen species (ROS) like superoxide radicals (O2•—), hydrogen peroxide (H_2O_2),

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hydroxyl radical (•OH), peroxynitrite (ONOO—) and reactive nitrogen species (RNS) are free radicals that are byproducts of various metabolism occurring within the body systems[4,5] possessing beneficial as well as deleterious effects. Beneficial effects include energy production, phagocytosis, intracellular signaling and cell growth regulation.[6] Though the beneficial effects of ROS occurs at lower concentration, the body cells are prone to the destructive effect by free radicals, which is termed as oxidative stress and nitrosative effect causing various biological damage.[7] Naturally, biological systems have the capability to counteract free radical production with the help of a mechanism called "redox regulation". During this mechanism, redox homeostasis is maintained[8] with the help of free radical detoxifying antioxidant enzymes namely superoxide dismutase, glutathione peroxidase, catalase, glutathione-S-transferase and also by the support of non-enzymatic antioxidants like ascorbic acid, α-tocopherol, glutathione, carotenoids and flavanoids. When there is a disturbance in prooxidant/antioxidant reactions, these free radicals cause disorientation of the cell membrane, Protein damage, attack lipids in cell membranes, causes deoxyribonucleic acid (DNA) damage by means of single strand breaks and double strand breaks leading to cancer,[9,10] cardiovascular diseases, neurodisorders[11] and reduces immunity. Mitochondrium is the primary source of ATP production involving complex I-IV. During energy transduction, small amount of electrons react with oxygen to form oxygen free radical "superoxide" and this free radical generation and impairment of normal metabolism leads to decreased ATP synthesis and ultimately cell death. Free radicals initiated mitochondrial dysfunction is proposed to be a major mechanism underlying many neurodegenerative disorders.[12] These toxic effects are balanced by antioxidants by donating electrons to free radicals, making them harmless molecules and preventing radical-chained reaction.[13] Hence antioxidants play an important role in maintaining harmony between the body and mind. Sometimes free radical scavenging activity of the system are alone not sufficient to fight free radicals and hence the need in search of drugs with antioxidant activity are of prime importance.[14]

CNB)-001[4-((1E)-2-(5-(4-hydroxy-3-methoxystyryl-)-1-phenyl-1H-pyrazoyl-3-yl)vinyl)-2-methoxy-phenol)] [Figure 1] is a hybrid molecule synthesized by combining cyclohexyl bisphenol A (CBA), a molecule with neurotrophic activity and curcumin, a spice with potent neuroprotective activity.[15] This compound has been studied for enhancement of memory,[16] anti-inflammatory activity[17] and its protection against ischemic stroke[18] but the antioxidant potential of the compound has not been proved *in vitro*. The focus of the current study is to analyze various free radical scavenging ability of CNB-001 by employing 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), H_2O_2 scavenging activity, hydroxyl radical scavenging activity,

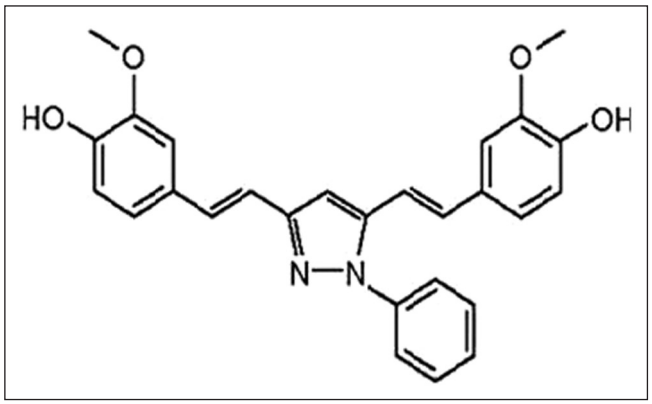

Figure 1: Structure of CNB-001

nitric oxide scavenging activity, reducing power, superoxide radical scavenging activity and superoxide scavenging activity. Since free radicals cause DNA nicks, we also analyzed the DNA protecting activity of CNB-001 against $FeSO_4$ and H_2O_2 induced DNA damage on plasmid DNA (pUC19).

Materials and Methods

DPPH radical scavenging activity

DPPH radical scavenging activity was determined according to the method of Blois.[19,20] Briefly, 1 ml of 0.1 mM DPPH radical solution was mixed with 3 ml of different concentrations of CNB-001 (20, 40, 60, 80 and 100 μg/ml) dissolved in methanol. The mixture was then thoroughly vortexed and left in dark for 30 min at 40°C. For the baseline control, 3 ml of methanol was used. The absorbance was measured at 517 nm.

Percentage of inhibition – ([A0–A1]/A0) × 100 where A0 was the absorbance of the control (blank, without compound) and A1 was the absorbance in the presence of the compound.

96-Multiwell plate assay

Aliquots of 0.5 ml of 0.04 mM DPPH solution in methanol was added into 96-multiwell plate. 0.5 ml (20, 40, 60, 80 and 100 μg/ml concentration) of CNB-001 was added into each well except that of the control. The concentrations of samples were increasing sequentially as shown in Figure 1. The plate was incubated for 10 min in room temperature and photographs were taken. Ascorbic acid was used as a standard.[21]

Radical cation 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^{•+}$) scavenging activity

Radical cation scavenging capacity of CNB-001 was observed against ABTSd + generated by chemical methods.[22,23] In brief, $ABTS^{•+}$ was prepared by oxidizing a 5 mM aqueous solution of ABTS diammonium salt with manganese dioxide at room temperature for 30 min. The reaction mixture contained 1.0 ml of $ABTS^{•+}$with an absorbance of 0.7 at 734 nm, 100 μl of CNB-

001 with various concentrations and 1.0 ml of 5 mM phosphate buffered saline (PBS) buffer (pH 7.4). The absorbance was read after 1 min at 734 nm and the % inhibition was calculated. The experiment was performed in triplicate. Percentage of inhibition – ([A0–A1]/A0) × 100, where A0 was the absorbance of the control (blank, without compound) and A1 was the absorbance in the presence of the compound.

Determination of reducing power

The reducing power of CNB-001 was evaluated by the method of Oyaizu.[24,25] In brief, various concentrations of CNB-001 (0.2 ml) were mixed with 1.0 ml of 200 mM sodium phosphate buffer (pH 6.6) and 1.0 ml of 1% potassium ferricyanide. The mixture was incubated for 30 min at 50°C. Later 1.0 ml of 10% trichloroacetic acid (w/v) was added and the mixture was centrifuged at 4000 rpm for 10 min. The supernatant (2 ml) was mixed with 2 ml of deionized water and 0.4 ml of 0.1% of ferric chloride was added. The absorbance was measured spectrophotometrically at 700 nm. Control was prepared using distilled water instead of CNB-001. Higher absorbance of the reaction mixture indicated greater reductive potential. The experiment was carried out in triplicate.

Percentage inhibition was calculated using, percentage of inhibition – ([A0–A1]/A0) × 100 where A0 was the absorbance of the control (blank, without compound) and A1 was the absorbance of the mixture in presence of CNB-001.

Nitric oxide radical scavenging activity

Nitric oxide radical scavenging activity was measured spectrophotometrically.[26,27] Sodium nitroprusside (5 mM, 1.5 ml) in PBS was mixed in different concentration of CNB-001 and incubated at 25°C for 30 min. Control without test compound but with an equal amount of methanol was taken. 30 min after incubation, 1.5 ml of the incubated solution was diluted with 1. 5 ml of greiss reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride in 2% H_3PO_4). The absorbance of the chromophore formed during diazotization of nitrite with sulphanilimide and subsequent coupling napthyl ethylene diamine was measured at 546 nm. Percentage radical scavenging activity of the sample was calculated as follows:

Percentage of inhibition – ([A0–A1]/A0) × 100 where A0 was the absorbance of the control (blank, without compound) and A1 was the absorbance in the presence of the compound.

Superoxide (O_2) radical scavenging activity

The assay was based on the capacity of the sample to inhibit blue formazan formation by scavenging the superoxide radicals generated using riboflavin-light-nitroblue tetrazolium (NBT) system.[28] Briefly, 3 ml reaction mixture contained 50 mM phosphate buffer (pH 7.6), 20 μg riboflavin, 12 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mg NBT/3 ml and various concentrations (20-100 μg/ml) of CNB-001. Reaction was started by illuminating the reaction mixture for 90 s. Immediately after illumination the absorbance was measured at 590 nm. The entire reaction assembly was enclosed in a box lined with aluminum foil. Identical tubes with the reaction mixture except CNB-001 were kept in dark and served as blank. The percentage inhibition of superoxide anion generation was calculated as: Percentage of inhibition – ([A0–A1]/A0) × 100 where A0 was the absorbance of the control (blank, without compound) and A1 was the absorbance in the presence of the compound.

Superoxide anion scavenging activity assay

Superoxide anion scavenging activity of CNB-001 was validated.[29] Briefly, the reaction mixture contains 1 ml of nitro blue tetrazolium solution (156 μM in 100 mM phosphate buffer, pH 7.4), 1 ml nicotine amide adenine dinucleotide solution (468 μM in 100 mM phosphate buffer, pH 7.4) and 0.1 ml of different concentrations of CNB-001. The reaction was initiated by adding 100 μl of phenazine methosulfate solution (60 μM) in 100 mM phosphate buffer, (pH 7.4) to the mixture. The reaction mixture was incubated at room temperature for 5 min and the absorbance was measured at 560 nm. The superoxide anion scavenging activity was calculated according to the following equation. Percentage of inhibition – ([A0–A1]/A0) × 100 where A0 is the absorbance of the control (blank, without compound) and A1 is the absorbance in presence of compound.

H_2O_2 radical scavenging activity

This activity was determined according to a method described elsewhere[30] with minor modifications. Various concentrations (20, 40, 60, 80 and 100 μg/ml) of CNB-001 and aliquot of 50 mM H_2O_2 were mixed (1:1 v/v) and incubated for 30 min at room temperature. After incubation, 10 μl methanol and 0.9 ml FOX reagent (prepared in advance by mixing 9 volumes of 4.4 mM BHT in HPLC grade methanol with 1 volume of 1 mM xylenol orange and 2.56 mM ammonium ferrous sulfate in 0.25 M H_2SO_4) was added to 90 μl of the incubated mixture. The reaction mixture was vortexed and incubated at room temperature for 30 min. The absorbance of the ferric-xylenol orange complex was measured at 560 nm. All tests were carried out in triplicate and ascorbic acid was used as the standard.

Percentage of inhibition – ([A0–A1]/A0) × 100 where A0 was the absorbance of the control (blank, without compound) and A1 was the absorbance in the presence of the compound.

Hydroxyl radical (•OH) scavenging activity

Hydroxyl radicals generated by the Fenton reaction were measured as per the method of Chung *et al*.[31] Fenton reaction mixture constituted of 0.2 ml $FeSO_4 \cdot 7H_2O$ (10 mM), 0.2 ml EDTA (10 mM) and 0.2 ml 2-deoxyribose

(10 mM) mixed with 1.2 ml phosphate buffer (0.1 M, pH 7.4). CNB-001 at various concentrations (20, 40, 60, 80 and 100 μg/ml) was added to Fenton reaction mixture followed by the addition of 0.2 ml H_2O_2 (10 mM) and incubated at 37°C for 4 h. Later, 1 ml TCA (2.8%) and 1 ml TBA (1%) were added to the reaction mixture and placed in a boiling water bath for 10 min. The resultant mixture was brought to room temperature and centrifuged at 395 ×*g* for 5 min and absorbance was obtained at 532 nm. Hydroxyl radical scavenging activity of the compound is calculated using Percentage of inhibition – ([A0–A1]/A0) × 100 where A0 was the absorbance of the control (blank, without compound) and A1 was the absorbance in the presence of the compound.

IC50

IC50 value was determined for all the assays from the plotted graph of scavenging activity versus the concentration of CNB-001 and Ascorbic acid using GraphPad Prism 5 software, GraphPad Software, Inc., La Jolla, USA. IC50 is defined as the amount of antioxidant necessary to decrease the initial radical concentration by 50%. Triplicate measurements were carried out and their activity was calculated by the percentage of radical scavenged. The IC50 values obtained are shown in Table 1.

DNA protecting activity

This assay was performed to analyze the ability of CNB-001 to protect DNA against hydroxyl radicals (•OH).[32,33] In brief, damage solution was prepared by adding (1 mM •OH) was prepared by adding 1.6 μl of 30% H_2O_2 into 50 ml of 1 mM $FeSO_4$. Total reaction solution consists of 5 μl plasmid DNA (pUC19), 5 μl of CNB-001 (20, 40, 60, 80 μg/ml), 5 μl of damage solution and irradiated in ultraviolet (UV) by placing on UV transilluminator (300 nm). Curcumin (20, 80 μg/ml) was used for comparison. The reaction solution was incubated in dark for 1 h. Later, 3 μl of gel loading buffer (30 mM EDTA, 36% [v/v] glycerol, 0.05% [w/v] bromophenol blue) was added and the reaction products were electrophoresized in 1% agarose gel for 1 h under 50 V condition. The gel was then stained with 0.05% (w/v) ethidium bromide and analyzed using gel documentation system (Alpha imager mini, Alpha Innotech, USA).

Results

DPPH radical scavenging activity

CNB-001 has an antiradical activity by inhibiting DPPH radical with an IC50 value of 44.99 μg/ml compared with that of reference standard, ascorbic acid of 90.81 μg/ml. The inhibiting potential of CNB-001 is shown in Figure 2.

96-Multiwell plate assay

The color reaction shows the ability of the compound to reduce DPPH radical. Pink color (high intensity) indicated the presence of DPPH radical and yellow color (low intensity) indicates the scavenging capacity of compound in a dose dependent manner [Figure 3]. Lower the intensity, higher the DPPH radical scavenging activity.

ABTS radical scavenging activity

The antioxidant activity of CNB-001 was calculated by decolorization of ABTS radical. Radical scavenging activity of CNB-001 is expressed by percentage inhibition. IC50 value was found to be 17.99 μg/ml. These values are superior to standard antioxidant compound ascorbic acid (45 μg/ml). The percentage of inhibition corresponding to the concentration is shown in Figure 4.

Reducing power

To investigate the reducing potential of CNB-001, the reduction of Fe^{3+} to Fe^{2+} was studied in the presence of CNB-001. Observed results showed that CNB-001 and ascorbic acid have a dose dependent quenching ability [Figure 5]. Similarly the IC50 values were closer which was found to be 11.53 and 9.55 μg/ml respectively.

Nitric oxide scavenging activity

The concentration of CNB-001 is directly proportional to the nitric oxide scavenging property [Figure 6]. The ability of CNB-001 to quench 50% of nitric oxide was found to be 1.36 μg/ml and ascorbic acid was found to be 8.13 μg/ml. Hence the test compound has higher nitric oxide scavenging activity at a very low dose.

Superoxide radical scavenging activity

The ability of the compound to scavenge superoxide radical generated from dissolved oxygen by phenazine methosulfate-NADH coupling can be measured by their capability to reduce NBT. Superoxide anion is one of the important radical generated in living systems. Hence the ability of test compound to scavenge superoxide anion were studied. The results indicate that CNB-001 has superoxide scavenging activity at a very low dose and as the dose increases, the scavenging activity

Table 1: IC50 values of CNB-001 and ascorbic acid. Values are represented as mean ± SD

IC50 μg/ml	DPPH	ABTS	TRAP	Hydroxyl radical	Superoxide radical	Superoxide scavenging	Nitric oxide	Reducing power	Hydrogen peroxide
CNB-001	44.99±1.28	17.99±1.35	128.4±1.38	456.5±3.20	77.17±1.05	36.92±0.75	1.36±0.04	11.53±0.29	492.7±1.14
Ascorbic acid	90.81±0.58	45±0.23	64.53±0.49	4.088±0.04	739.5±2.54	1301±0.78	8.13±0.13	9.55±0.28	450±2.25

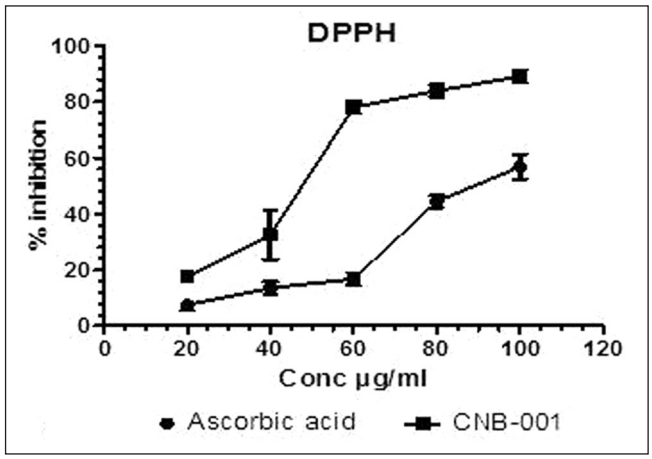

Figure 2: 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity of CNB-001 at different concentration. Data are presented as mean ± SD of each of three replicates ($n = 3$)

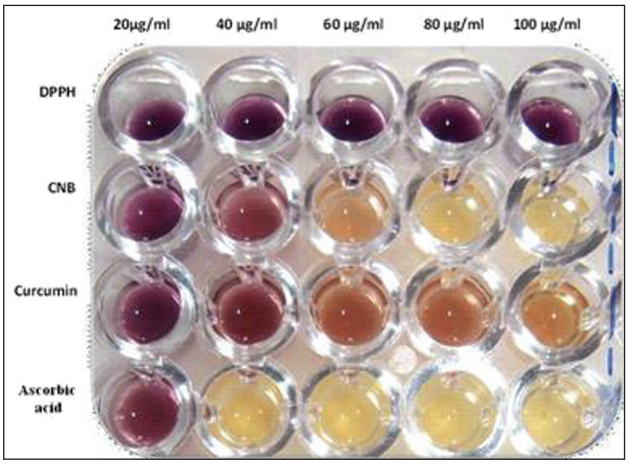

Figure 3: Part of 96 multi-well plate showing the gradually change in color from deep purple to pink to yellow for comparative study of the antioxidant activities of compounds at different concentrations after addition of 0.4 mM 2,2-diphenyl-1-picrylhydrazyl solution in methanol

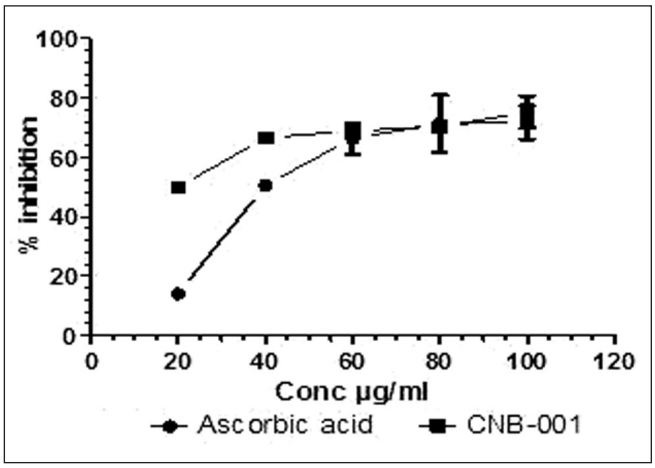

Figure 4: 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity of CNB-001 at different concentration. Data are presented as mean ± SD of each of three replicates ($n = 3$)

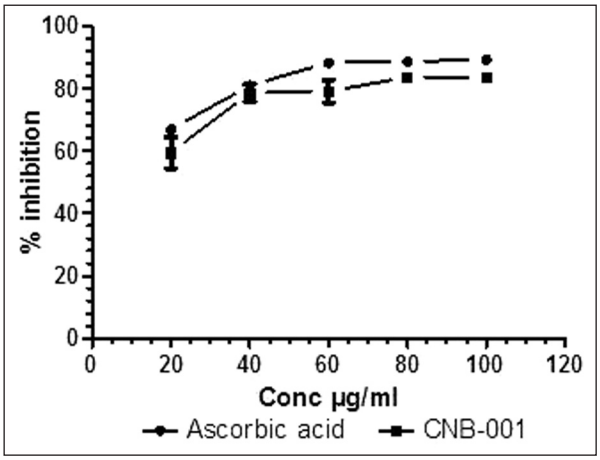

Figure 5: Reducing power of CNB-001 at different concentration. Data are presented as mean ± SD of each of three replicates ($n = 3$)

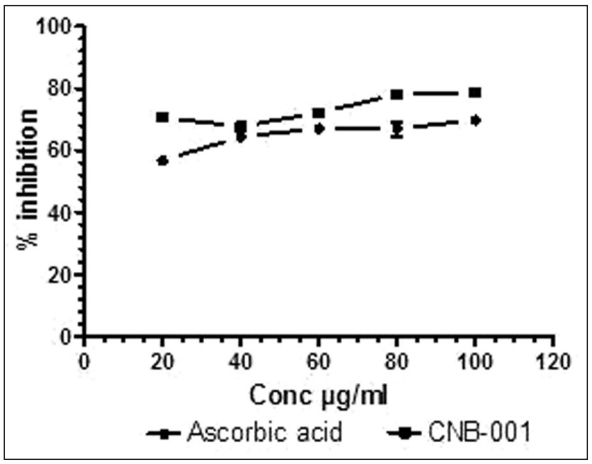

Figure 6: Nitric oxide radical scavenging activity of CNB-001 at different concentration. Data are presented as mean ± SD of each of three replicates ($n = 3$)

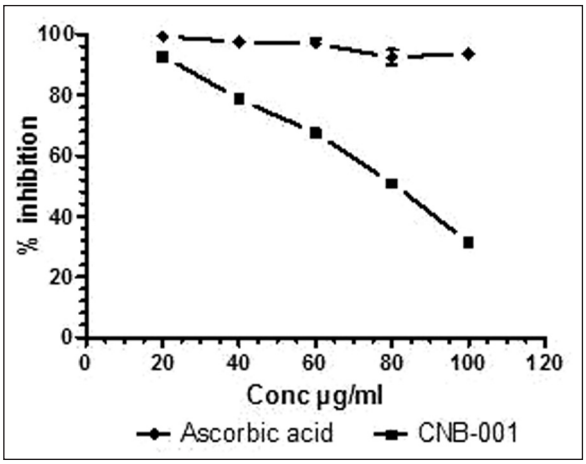

Figure 7: Superoxide radical scavenging activity of CNB-001 at different concentration. Data are presented as mean ± SD of each of three replicates ($n = 3$)

decreases [Figure 7]. The IC50 values of CNB-001 and ascorbic acid were found to be 77.17 and 739.5 µg/ml respectively.

Superoxide anion scavenging activity

Similar to superoxide radical scavenging activity, CNB-001 has higher superoxide anion quenching effect at a low dose when compared to standard [Figure 8]. IC50 values of CNB-001 and ascorbic acid were found to be 36.92 and 1301 µg/ml respectively.

H_2O_2 scavenging activity

H_2O_2 scavenging activity was assayed by FOX reagent method. The scavenging effect of CNB-001 on H_2O_2 was in a concentration dependent manner [Figure 9]. CNB-001 and reference standard both had inhibitory percentage of 22 at a concentration of 20 µg/ml. The IC50 value of CNB-001 and ascorbic acid were 492.7 and 450 µg/ml respectively.

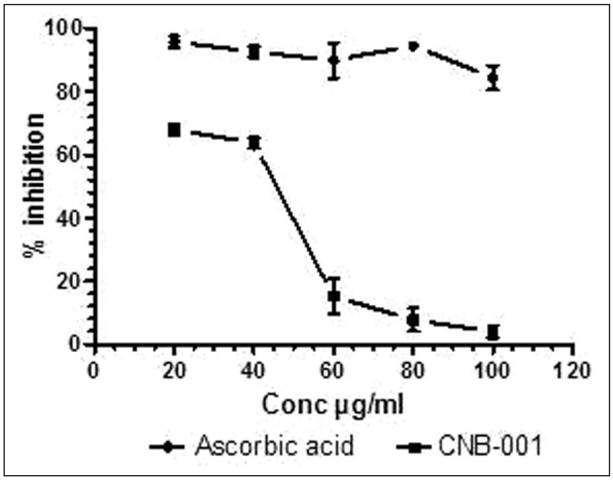

Figure 8: Superoxide anion scavenging activity of CNB-001 at different concentration. Data are presented as mean ± SD of each of three replicates ($n = 3$)

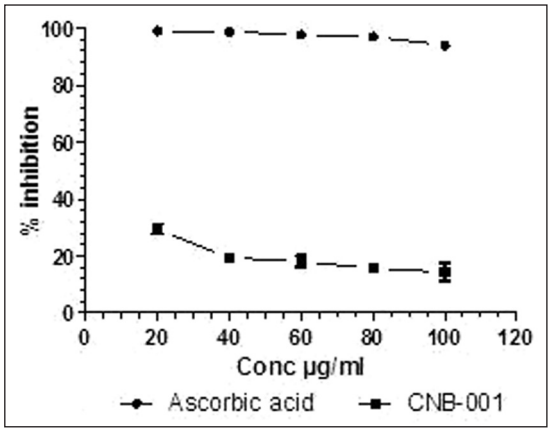

Figure 10: Hydroxyl radical scavenging activity of CNB-001 at different concentration. Data are presented as mean ± SD of each of three replicates ($n = 3$)

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity of the test compound was comparatively lesser than ascorbic acid [Figure 10]. Similarly IC50 value of CNB-001 was found to be 456.5 µg/ml and ascorbic acid was found to be 4.088 µg/ml. Hence ascorbic acid has superior hydroxyl radical scavenging activity.

DNA protecting activity

CNB-001 was found to protect plasmid DNA (pUC19) from hydroxyl radicals in a dose dependent manner [Figure 11]. Plasmid DNA has three forms when run of gel electrophoresis namely supercoiled circular DNA, Open circular form and linear DNA. UV radiation and hydroxyl radicals cleaved supercoiled form into open and linear DNA (Lane B, C, D) as shown in Figure 11, CNB-001and curcumin rescued the plasmid DNA from H_2O_2 and UV induced DNA scission by scavenging hydroxyl radicals in a dose-dependent manner.

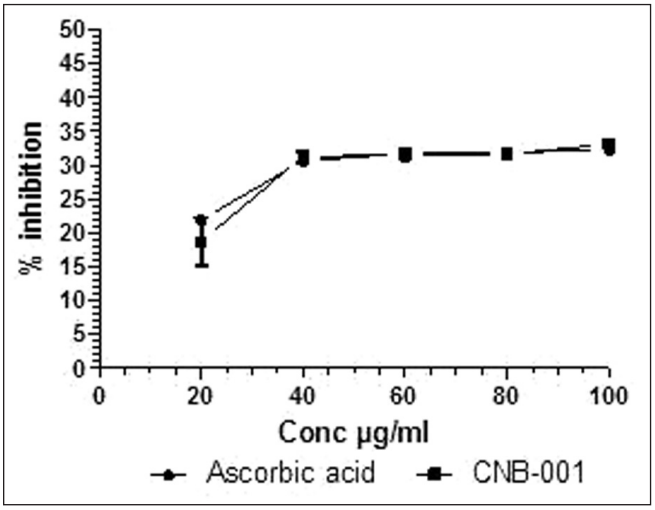

Figure 9: Hydrogen peroxide radical scavenging activity of CNB-001 at different concentration. Data are presented as mean ± SD of each of three replicates ($n = 3$)

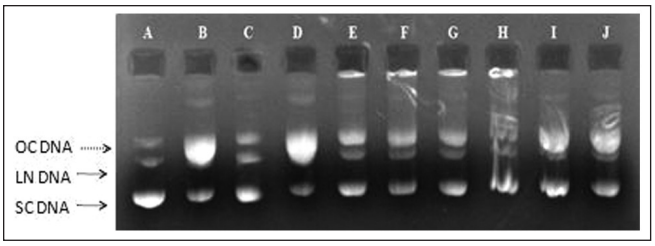

Figure 11: Electrophoretic pattern of plasmid (pUC19) deoxyribonucleic acid (DNA) in presence of CNB-001 and curcumin. (a) water; (b) damage solution; (c) ultraviolet (UV) treated; (d) damage solution + UV; (e-h) damage solution + UV + CNB 20, 40, 60, 80 µg/ml respectively; (i and j) damage solution + curcumin 20 and 80 µg/ml respectively. OC DNA: Open circular DNA; LN DNA: Linear DNA; SC DNA: Supercoiled DNA

Discussion

Free radicals are the main culprits in the initiation of various diseases.[34] Hence any drug of interest is nowadays analyzed for its antioxidant property.[14] CNB-001, a pyrazole derivative is synthesized from curcumin, a neuroprotective compound[35] and CBA, a neurotrophic molecule. Though, both the parental compounds have excellent biological properties, its activities are limited. Curcumin, a potent antioxidant fails to inhibit excitotoxicity in cortical neurons and has poor bioavailability.[15] Recent cell culture work reported by Liu *et al*. showed that CNB-001 was far more superior than curcumin and CBA as evinced by trophic factor withdrawal, excitotoxicity, glucose starvation and amyloid toxicity assays. CNB-001 also showed neurotrophic factor like activity many folds higher than CBA. Neurotrophic factors plays an important role in maintaining and regulating brains microenvironment during CNS injury. Moreover, both the parental compounds have higher EC50 value compared to CNB-001. Hence CNB-001 was synthesized to enhance the effectiveness of curcumin and CBA. Insilico analysis done in our lab (data not shown) also showed that CNB-001 is non-toxic, has good ADMET properties and protected SK-N-SH cells *in vitro* against rotenone toxicity.[36] Therefore, this compound is tested for antioxidant and DNA protecting activity, which stands as a baseline property for a drug. It is believed that the present work would enhance and highlight the potentiality of CNB-001 for further drug targeted studies. Stereo selectivity of radicals affects the interaction between the compounds which in turn affects the scavenging activity of the compound.[37] A study by Wang *et al*. concluded that compound which has good ability to scavenge ABTS + did not scavenge DPPH radical.[38] Contradictory to this result, CNB-001 was found to scavenge different radicals in different systems.

DPPH is a stable free radical which accepts electron or hydrogen radical from other molecules to become a stable diamagnetic molecule. This assay is based on electron transfer reaction. Hence the antioxidant or radical scavenger turns from violet to yellow by the presence of hydrogen or electron donation.[39] This is one of the most common antioxidant assays to analyze the free radical scavenging activity of the test compound. In our study the EC50 of test compound was found to be 44.99 μg/ml whereas standard ascorbic acid was found to be 90.81 μg/ml. This scavenging ability was in turn proved using 96 multi well plate assay. Free radicals were of pink color (high intensity) and when CNB-001 was added, the radicals were quenched in a dose dependent manner and the color changes to yellow (low intensity). This assay was supported by the work done by Mon *et al*.[21]

ABTS is an improved version of Trolox Equivalent antioxidant capacity assay.[40] $ABTS^{\bullet+}$ is a blue chromophore produced by the reaction between ABTS and potassium persulfate. This assay is used to understand the oxygen radical scavenging capacity. Our experiment revealed that CNB-001 has an excellent radical scavenging capacity.

Ferric reducing power is an electron transfer based assay where Ferric salt, Fe (III)(TPTZ)$2Cl_3$ is used as an oxidant. It shows the ability of antioxidants to reduce Fe^{3+} to Fe^{2+}. The principle behind this assay is depicted below

Oxidant + electron from antioxidant → reduced oxidant + oxidized antioxidant.

The reducing capacity of antioxidant is directly proportional to the color change. CNB-001 has a good redox potentiality than ascorbic acid. Antioxidant capacity of CNB-001 involves the reduction of ferricyanide complex to ferrous form, which is indicated by the formation of Pearl's Prussian blue color at 700 nm.[41] The reducing power of CNB 001 was observed to be in a dose-dependent manner, which is measured in terms of inhibiting potential. CNB-001 was found to be highly potent in reducing Fe^{3+} than the standard antioxidant, ascorbic acid.

Nitric oxide radical has different roles in a biological system.[42] Peroxynitrite (ONOO—) is one of the important RNS causing oxidative damage in biological systems. Biological system has a self-defense mechanism to convert harmful ROS to harmless species. Radicals like O_2•— and H_2O_2 can be converted to non-radicals by SOD and Catalase respectively. But few radicals like ROO•, HO•, $1O_2$ and ONOO— cannot be scavenged by enzymatic defense mechanism and hence the body purely depends upon non enzymatic antioxidants and supplements. CNB-001 has a good RNS scavenging activity than ascorbic acid.

Superoxide radical scavenging and superoxide anion scavenging assays are based upon singlet oxygen scavenging capacity. Superoxide anion indirectly initiates lipid oxidation[43] and reduces certain iron complexes such as cytochrome.[44] Immune cells, during Inflammation process generate superoxide radicals where NADPH oxidase initiates vascular complications.[8] Narumoto *et al*. proved that CNB-001 showed superior anti-inflammatory property *in vivo* compared to curcumin by suppressing the expression of vital inflammatory proteins interleukin-6 and tumor necrosis factor-alpha possibly by scavenging superoxide radicals and inhibiting NF-κB and p38MAPK activation.[17] This result is supported by the superoxide radical scavenging property in the present study which showed potent superoxide radical scavenging activity of CNB-001 even ate a very low dose of 20 μg/ml.

Lipids present in cell membrane are prone to peroxidation by hydroxyl radicals resulting in lipid hydroperoxide free radicals.[34] Hydroxyl radicals have a short life time and

can hydroxylate biomolecules very rapidly. The conversion of H_2O_2 to H_2O and O_2 is done by Catalase and when this defense mechanism fails, antioxidants play a vital role in scavenging hydroxyl radicals. These radicals are known to be highly mutagenic and carcinogenic. H_2O_2 readily crosses cell membrane and reacts with Fe^{2+} and Cu^{2+} ions which results in many toxic effects.[45] The scavenging capacity of CNB-001 and ascorbic acid were higher at a lower concentration and decreases slightly as the concentration increases. This might be because the interference substance(s) won't be able to donate protons at critical higher concentration. The other reason for prooxidant effect may be the formation of phenoxy radicals which participates in radical chain propogation as corroborated by others.[46,47]

Hydroxyl radicals has the capacity to interfere with the DNA by degrading deoxyribose using Fe^{2+} salt as a catalytic complex which results in mutation and carcinogenesis.[48,49] DNA damage was performed by exposing DNA to damage solution (1.6 µl of 30% H_2O_2 and 1 mM $FeSO_4$) for radical nicking and UV for photodegradation (Lane B-D). Treatment with CNB-001 at different concentrations (20, 40, 60, 80 µg/ml) (Lane E-H) and curcumin (20, 80 µg/ml) (Lane I and J) showed DNA protecting activity by reducing hydroxyl radical and inhibiting DNA strand scission. DNA protection was observed to be in a dose dependent manner. CNB-001, a pyrazole derivative of curcumin lacks labile dicarbonyl group of curcumin with 1H-Pyrazole as a basic nucleus. The presence of substituted styryl groups at 3,5-positions of pyrazole has electron donating/releasing groups like -OH,-OCH3 which might be responsible to its free radical scavenging property. Moreover, CNB-001 has two methoxyphenol group separated by β-diketone bridge which is accountable for iron chelation. These structural modifications accounts for its various biological properties and can be effectively developed as a novel drug for therapeutic intervention.

Conclusion

Etiological factors of many diseases reveal that free radicals are basic culprit in initiation of various toxic events and resultant cell death. To combat the disease progression, drugs with antioxidative property will be an effective way of treatment. The results from this study show that CNB-001 has an excellent antioxidative property by scavenging ROS and RNS. The protective role of CNB-001 in various *in vivo* experiments may be catalyzed by the antioxidative property of the compound and can be a superior target for therapeutic potential.

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