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ANTIOXIDANT PROPERTIES OF FUNGAL METABOLITE NIGERLOXIN *IN VITRO*

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We have recently reported the beneficial influence of the fungal metabolite nigerloxin, a new aldose reductase inhibitor and a lipoxygenase inhibitor on oxidative stress in streptozotocin induced diabetic rats. In the present study we have investigated the antioxidant potential of nigerloxin *in vitro* as compared to one of the well known natural antioxidant, curcumin. The fungal metabolite nigerloxin was found to be an effective antioxidant in different *in vitro* assays including the phosphomolybdenum, 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{\bullet +}$) and ferric reducing antioxidant power (FRAP) methods. The antioxidant potency of nigerloxin may be attributed to its electron donating nature. The ferric reducing potency of nigerloxin as demonstrated by FRAP assay method was even found to be superior to that of the natural antioxidant curcumin.

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The role of free radicals (molecular species carring one or more unpaired electrons in their molecular orbitals) in the pathogenisis of many diseases is well documented [1]. In biological systems cellular metabolism is the most important source of production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS and RNS include radicals such as superoxide $\left(O_2^{\bullet -}\right)$, hydroxyl ($OH^{\bullet}$), hydroperoxyl $\left(HO_2^{\bullet}\right)$, alkoxyl ($RO^{\bullet}$), peroxyl ($ROO^{\bullet}$), nitric oxide ($NO^{\bullet}$), nitrogen dioxide $\left(NO_2^{\bullet}\right)$ and lipid peroxyl ($LOO^{\bullet}$) [2]. The deleterious influence of free radicals on biological system is termed oxidative and nitrosative stresses [3–5]. These stresses may be due to overproduction of ROS/RNS or deficiency of enzymatic and non-enzymatic antioxidants in biological system. Some of other non radicals like hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3), peroxynitrite ($ONOO^-$), nitrous acid (HNO_2), dinitrogentrioxide (N_2O_3), lipid peroxide (LOOH) are also termed as oxidants and they are capable of leading to free radical reactions in living organisms.

The toxic influence of oxidative stress in many pathological conditions including cardiovascular disease, cancer, neurological disorders, diabetes, ischemia/reperfusion, other diseases and ageing has been well studied [6–9]. Compounds capable of donating electrons or hydrogen atom are able to protect from deleterious influence of free radicals. Antioxidants have exhibited beneficial influence in many pathological conditions [10]. There are several novel approaches in the study of free radicals/antioxidants for the improvement of human health.

A new aldose reductase inhibitor molecule of [2-amido-3-hydroxy-6-methoxy-5-methyl-4-(prop-1'-enyl) benzoic acid] (nigerloxin) obtained from solid-state fermentation of *Aspergillus niger* CFR-W-105 has been reported to exhibit inhibitory activity on the partially purified aldose reductase and lipoxygenase *in vitro* [11]. Animal study have been recently carried out to verify if this fungal metabolite exhibits aldose reductase inhibitory activity *in vivo* and to examine the influence of nigerloxin on modulation of the renal and retinal lesions [12] associated with diabetes in experimental rats, by virtue of its aldose reductase inhibitory potential. The aim of the study was to assess antioxidant potential of nigerloxin *in vitro* as compared to one of the major natural antioxidant, curcumin.

MATERIALS AND METHODS

Chemicals. Nigerloxin was produced through solid state fermentation of *A. niger* CFR-W-105 (Central Food Technological Research Institute, Mysore, India) according to the procedure given by Rao et al. [13]. 2,2-Diphenyl-1-picrylhydrazyl (DPPH); 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS); 2,4,6-tris-(2-pyridyl)-S-triazine (TPTZ); dimethyl sulfoxide (DMSO) and curcumin were obtained from Sigma-Aldrich (USA). All other chemicals and solvents used were of analytical grade obtained from Sisco Research Laboratories (India).

Antioxidant capacity of nigerloxin assessed by phosphomolybdenum method. Antioxidant potential of nigerloxin was evaluated by the protocol given by Prieto et al. [14] and compared with that of curcumin. Brief-

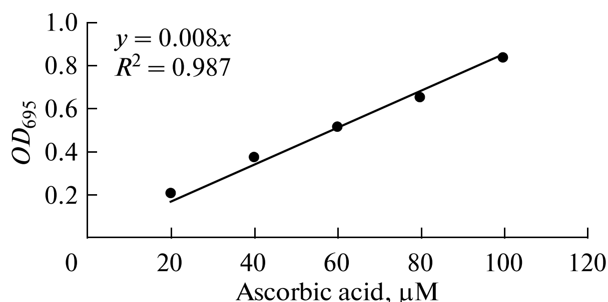

Fig. 1. The linear regression curve of ascorbic acid.

ly, nigerloxin (2–10 μg) or curcumin (2–10 μg) taken in different concentrations in 10 μL of DMSO was added to 1.0 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Blank solution was constituted by adding 10 μL of DMSO in place of sample solution with 1 mL of reagent. The tubes were capped and incubated in a boiling water bath for 90 min. After the samples had cooled to room temperature, the OD_{695} was measured against blank. Ascorbic was used as a standard and antioxidant capacities of nigerloxin and curcumin were expressed as ascorbic acid equivalents (μmol/mg of sample).

Free radical scavenging activity of nigerloxin assessed by DPPH assay. The DPPH radical scavenging potential of nigerloxin and curcumin were determined according to the method of Hatano et al. [15]. Briefly, nigerloxin (5–50 μg) or curcumin (2–10 μg) taken in different concentrations in 10 μL of DMSO, was mixed with 1 mL of 0.2 mM DPPH in methanol. The mixture was vigorously shaken and incubated at 28°C for 30 min. The absorbance was measured at 517 nm. The free radical quenching ability of nigerloxin and curcumin were determined as percentage decrease in the absorbance with respect to control having appropriate concentration of solvent. The effective concentration (EC_{50}) value was determined as the concentration of the sample required to reduce 50% of the absorbance with respect to control. Inhibition of free radical DPPH was calculated as follows:

$$\text{Inhibition of DPPH radical } \% = (OD_{\text{control}} - OD_{\text{sample}}) \times 100/OD_{\text{control}}.$$

ABTS radical cation decolorization assay. The ABTS radical scavenging activity was determined according to the protocol given by Re et al. [16]. ABTS radical was generated by incubating a mixture of 2 mM ABTS and 2.45 mM potassium persulfate in water for 16 h in the dark at room temperature. The ABTS radical solution was diluted with ethanol to produce absorbance of around 0.7 at 734 nm. Nigerloxin (5–50 μg) or curcumin (2–10 μg) taken in different concentrations in 10 μL of DMSO was mixed with 1 mL of pre-diluted ABTS radical solution. The mixture was vigorously shaken and incubated at room temperature for 30 min and OD_{734} was recorded. The percentage of radical scavenging was calculated for each concentration relative to a blank containing appropriate amount of solvent using the following equation and EC_{50} value was expressed in μmol.

$$\text{Inhibition of ABTS radical } \% = (OD_{\text{control}} - OD_{\text{sample}}) \times 100/OD_{\text{control}}.$$

Ferric reducing antioxidant power (FRAP) assay. The ferric reducing potential of nigerloxin and curcumin were estimated by the procedure given by Benzie and Strain [17]. The reagent was constituted by adding 2.5 mL of a 10 mM TPTZ solution in 40 mM HCl, 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$ and 25 mL of 300 mM acetate buffer, pH 3.6. Reagent was freshly prepared and warmed at 37°C before used. From one to five μg of nigerloxin or curcumin in several concentrations was prepared in 10 μL of DMSO and added to 1 mL of FRAP reagent. Reaction mixture was mixed thoroughly and incubated at 37°C for 30 min and OD_{593} was recorded. Ferrous sulphate was used as standard and FRAP value was expressed as mM of ferrous sulphate equivalents/mg sample.

RESULTS

A dose-dependent antioxidant influence of nigerloxin and curcumin on phosphomolybdenum complex is shown in Fig. 1 and 2. The regression curve of ascorbic acid was constructed by plotting absorbance against concentration and antioxidant potency of nigerloxin and curcumin were calculated using regression equation. The antioxidant potential of nigerloxin and curcumin by this method was found to be 3791 and 4684 μM of ascorbic acid equivalents/mg, respectively. DPPH radical scavenging potential of nigerloxin and curcumin are presented in Fig. 3. Nigerloxin scavenged DPPH radical in a dose-dependent manner. Nigerloxin and curcumin exhibited EC_{50} value at the concentration of 68.56 and 22.57 μM against DPPH radicals, respectively.

The dose-dependent inhibition of ABTS radical by nigerloxin and curcumin is shown in Fig. 4. Nigerloxin and curcumin potentially scavenged this radical and the EC_{50} value was found to be 8.97 μM and 6.75 μM, respectively. The ferric ion reducing potential of nigerloxin and curcumin is presented in Fig. 5 and 6. The linear regression curve, absorbance against concentration of ferrous sulphate was constructed and regression equation was used to calculate the reducing strength of nigerloxin and curcumin. Nigerloxin and curcumin reduced ferric complex in a dose depended manner. The FRAP value of nigerloxin and curcumin was found 14.44 and 8.20 mM ferrous sulphate equivalents/mg, respectively.

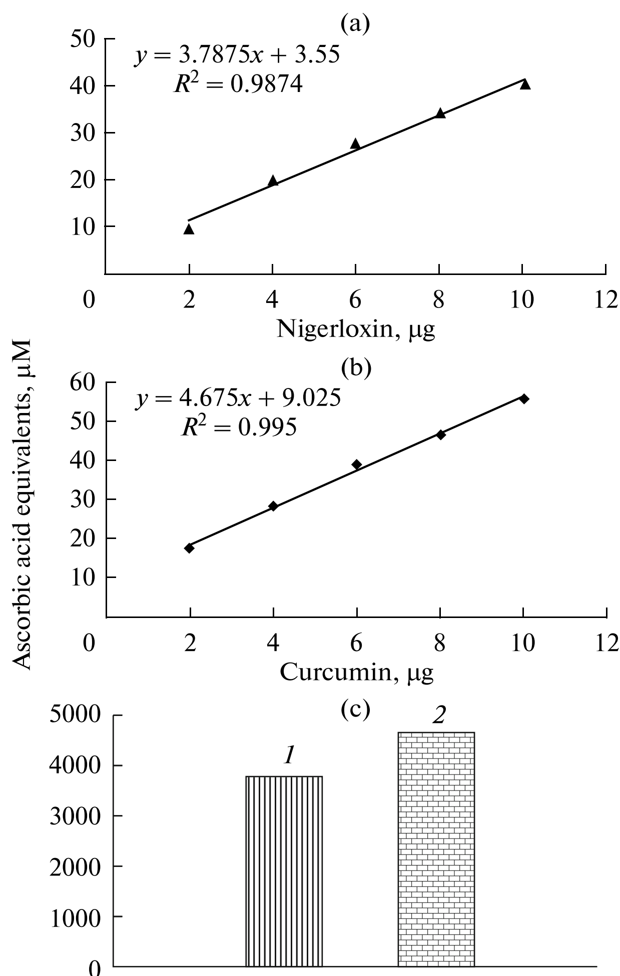

Fig. 2. Antioxidant capacities of nigerloxin (a) and curcumin (b) and comparison of them (c) for nigerloxin (*1*) and curcumin (*2*) assessed by phosphomolybdenum method.

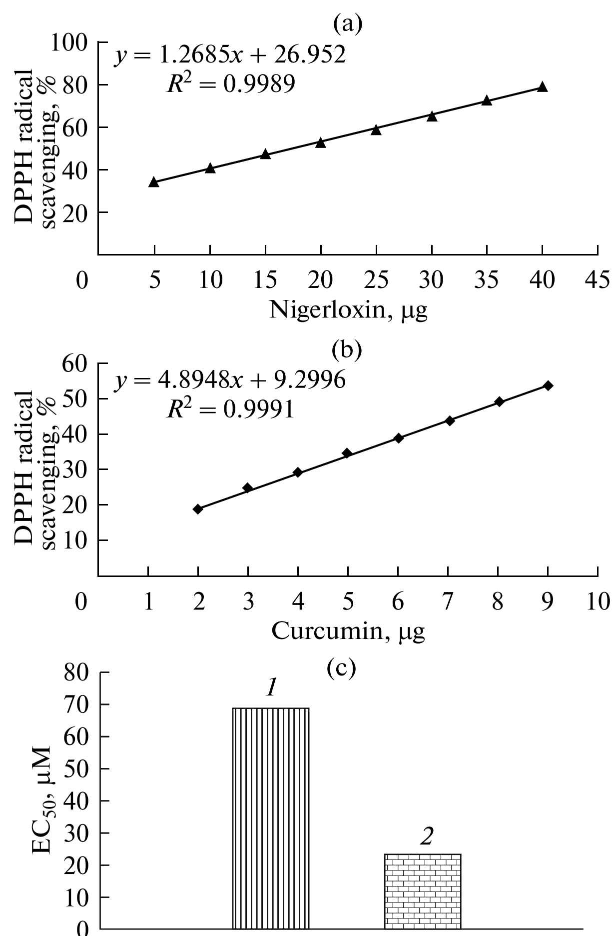

Fig. 3. DPPH radical scavenging potential of nigerloxin (a) and curcumin (b) and comparison of their EC_{50} values (c) for nigerloxin (*1*) and curcumin (*2*) against DPPH radical.

DISCUSSION

The beneficial influence of fungal metabolite nigerloxin on the diabetes induced oxidative stress has been recently studied in experimental rats in the context of oxidative stress playing a key role in the progression of diabetes and its complications [18]. Administration of nigerloxin for 30 days at a daily dose of 100 mg/kg of body weight to diabetic rats significantly decreased the plasma and liver lipid peroxides and elevated the nonenzymatic antioxidants – ascorbic acid, reduced glutathione, and total thiols, and the activities of antioxidant enzymes in blood and liver.

This prompted us to examine the antioxidant potential of nigerloxin *in vitro* to assess it in comparison to the well known natural antioxidant biomolecule, curcumin. Curcumin is a natural antioxidant biomolecule present in turmeric (*Curcuma longa*). Several assay methods have been frequently used to estimate antioxidant potentials. In the present study mainly four assay methods were employed to accomplish an electron donating and hydrogen atom donating strength of nigerloxin *in vitro*. The methods included ABTS radical cation and DPPH radical scavenging assays, ferric ion reducing activity and total antioxidant activity evaluated by phosphomolybdenum method. The total antioxidant capacity of nigerloxin was established by the formation of phosphomolybdenum complex. Antioxidant capable to reduce Mo(VI) to Mo(V) results in an increase of OD_{695}. Nigerloxin and curcumin exhibited different degrees of antioxidant capacities, viz., 3791 and 4684 µM/mg of ascorbic acid equivalents, respectively.

DPPH and ABTS radical scavenging assays are most common spectrophotometric methods to assess the antioxidant potential of chemical moieties. Advantages of these methods include easy performing, high sensitivity and rapidity. DPPH is commercially available long lived organic nitrogen radical and freely soluble in organic solvents. It produces stable purple

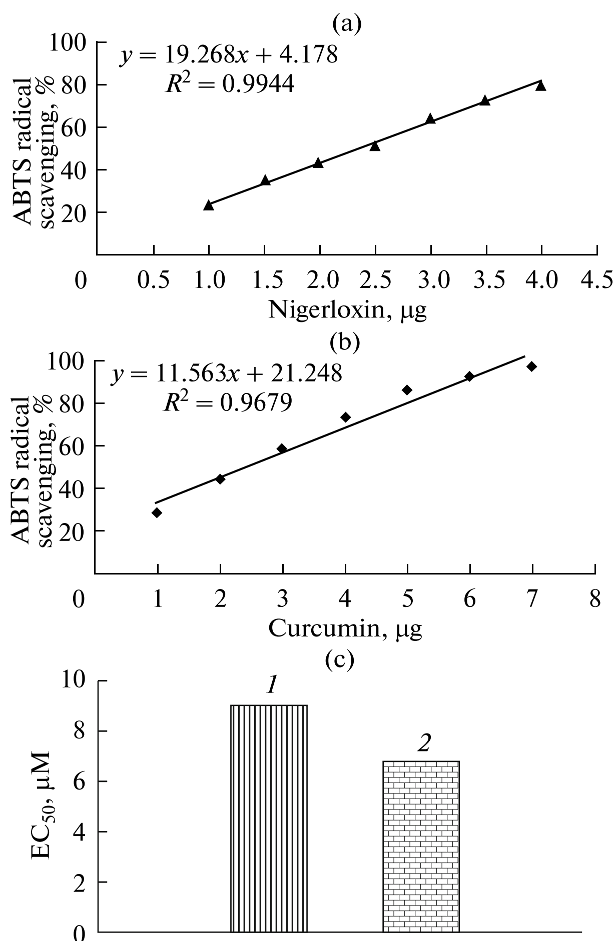

Fig. 4. ABTS radical scavenging potential of nigerloxin (a) and curcumin (b) and comparison of their EC_{50} values (c) for nigerloxin (*1*) and curcumin (*2*) against ABTS radical.

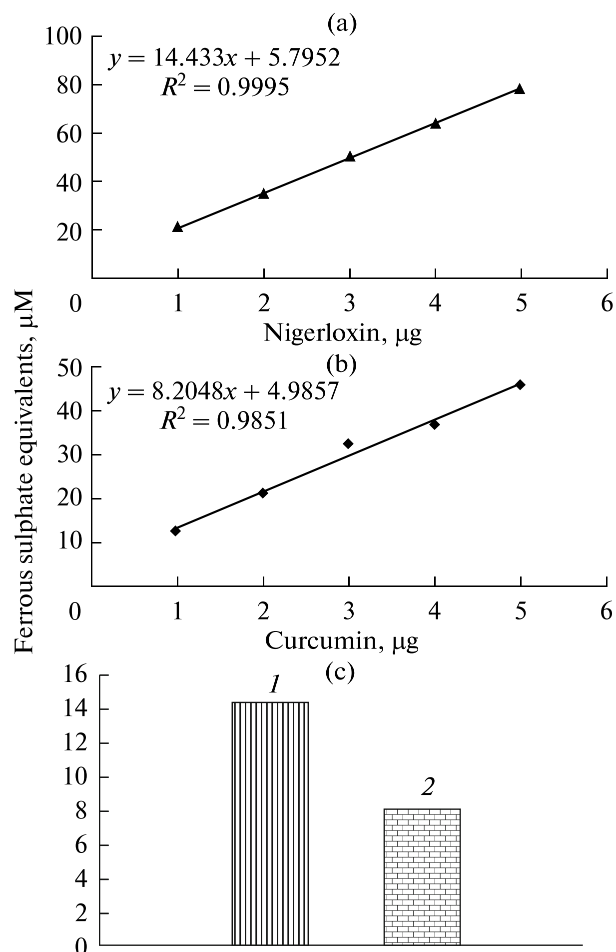

Fig. 6. Ferric reducing antioxidant potential of nigerloxin (a) or curcumin (b) and comparison of their FRAP values (c) for nigerloxin (*1*) and curcumin (*2*).

colored radical, having an absorption maximum at 515 nm. The DPPH assay is believed to involve hydrogen atom transfer reaction. On the other hand, Foti and coworkers [19] suggested that electron transfer reaction is the major principle involved in this assay. Therefore, the antioxidant strength of the compound depends upon its hydrogen or electron donating nature. The antioxidant potential of nigerloxin was measured by the bleaching of the purple colored methanol solution of the stable DPPH radical and compared with that of the most extensively studied natural antioxidant, curcumin. Nigerloxin exhibited EC_{50} of 68.56 μM against this radical. The potency of DPPH radical scavenging activity of curcumin was around three times less and had an EC_{50} value of 22.57 μM.

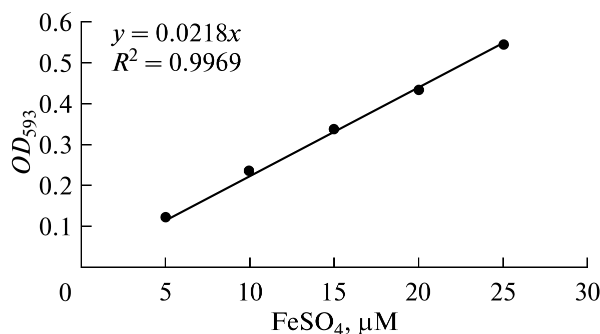

Fig. 5. The linear regression curve of ferrous sulphate.

ABTS also forms a relatively stable free radical, which decolorizes in its non-radical form [20]. ABTS and potassium persulfate in aqueous media produce blue-green $ABTS^{\bullet+}$ chromophore. $ABTS^{\bullet-}$, the oxidant, is generated by potassium persulfate oxidation of $ABTS^{2-}$ and the radical cation is measured spectrophotometrically. This is a direct generation of a stable form of radical to create a blue-green $ABTS^{\bullet+}$ chro-

mophore prior to the reaction with antioxidants [21]. Bleaching of the preformed solution of the blue-green radical cation $ABTS^{\bullet +}$ was used here to assess the antioxidant capacity of nigerloxin and compared with curcumin. Nigerloxin and curcumin exhibited dose depended decolorization of this radical. The EC_{50} value of nigerloxin against ABTS radical cation was found to be 8.97 μM as compared to curcumin having 6.75 μM.

The electron donating potential of a bioactive compound reflects its reducing power [22]. The ferric reducing potency of nigerloxin was demonstrated by FRAP assay method. This assay takes advantage of electron-transfer reactions, wherein the ferric salt, viz., $Fe(III)(TPTZ)_2Cl_3$ is used as an oxidant [17]. In the presence of electron donating chemicals, ferric-tripyridyltriazine complex is reduced to blue colored ferrous-tripyridyltriazine complex. Measurement of the intensity of this color at 593 nm [17, 23] is one of the major assay methods used to demonstrate electron donating property of antioxidants. Our results revealed that nigerloxin has a tendency to donate electron. The ferric reducing potency of nigerloxin was in fact higher than that of curcumin.

Thus, in this study, the fungal metabolite nigerloxin was found to be an effective antioxidant in different *in vitro* assays including the phosphomolybdenum, $DPPH^{\bullet}$, $ABTS^{\bullet +}$ and FRAP methods. The antioxidant potency of nigerloxin may be attributed to its electron donating nature. The ferric reducing potency of nigerloxin as demonstrated by FRAP assay method was even found to be superior to that of the natural antioxidant curcumin.

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