In Vitro Antioxidant Studies on the Benzyl Tetra Isoquinoline Alkaloid Berberine

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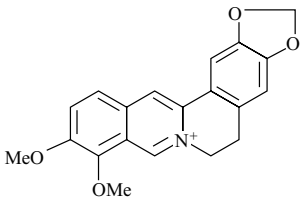
Berberine is a benzyl tetra isoquinoline alkaloid which is widely used as an antimicrobial and an antidiarrhoeal. As berberine containing plants are virtually used in all forms of traditional medicine, our study aimed to examine the antioxidant activity of berberine using 2,2-diphenyl 1-picrylhydrazyl (DPPH) radical scavenging, nitric oxide scavenging, lipid peroxidation, superoxide scavenging, iron chelating activity and 2,2-azino bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) radical scavenging methods. The IC_{50} values for all the models were calculated by regression analysis. In all the models tested, berberine showed its ability to scavenge the free radicals in a concentration dependent manner. The present study thereby justifies the therapeutic potential of berberine.

Key words berberine; antioxidant activity; superoxide; nitric oxide; lipid peroxidation

Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and our metabolism. They are continuously produced by the body's normal use of oxygen such as respiration and some cell mediated immune functions. Naturally, there is a dynamic balance between the amount of free radicals generated in the body and antioxidants to quench and/or scavenge them and protect the body against their deleterious effects. The origin of diseases of multifactorial nature is being understood now to be due to the vitiation in basic homeostatic balance phenomenon in the body. The cause of a majority of disease conditions like atherosclerosis, hypertension, ischaemic diseases, Alzhemier's disease, parkinsonism, cancer, diabetes mellitus and inflammatory conditions are being considered to be primarily due to the imbalance between prooxidant and antioxidant homeostasis. Antioxidant principles from natural resources possess multifacetedness in their multitude and magnitude of activities and provide enormous scope in correcting the imbalance. Hence, there is no doubt that phytochemicals deserve a proper position in the therapeutic armamentarium.

Berberine is an isoquinoline alkaloid present in a number of clinically important medicinal plants. Berberine containing plants are used medicinally in virtually all traditional medicinal systems, and have a history of usage in Ayurvedic and Chinese medicine dating back at least 3000 years. This alkaloid has demonstrated significant antimicrobial activity against bacteria,[1] fungi,[2] protozoa,[3] viruses,[4] helminthes[5] and chalmydia.[6] In addition, its action includes antagonism of the effects of cholera and *Escherichia coli* heat stable enterotoxin,[7] inhibition of intestinal ion secretion,[8] inhibition of smooth muscle contraction,[9] inhibition of ventricular tachyarrhythmias,[10] reduction in inflammation,[11] elevation of platelet count in patients with primary and secondary thrombocytopenia[12] and stimulation of bile secretion and bilirubin discharge.[13] Evidence also suggests that intravenous berberine administration can play a role in preventing the onset of ventricular tachyarrhythmia and sudden coronary death after myocardial ischemic damage.[14] Recently berberine has been reported to reduce the blood glucose level in diabetes.[15]



Berberine

The tetracyclic skeleton of berberine, a benzyl-tetra isoquinoline alkaloid derived from a benzyl tetra hydro isoquinoline system with the incorporation of an extra carbon atom, supplied from *S*-adenosyl methionine *via* an *N*-Methyl group. This extra skeletal carbon is known as a "berberine bridge." Formation of the berberine bridge is readily rationalized as an oxidative process in which the *N*-Methyl group is oxidized to an iminium ion, and cyclization to the aromatic ring occurs by the virtue of the phenolic group.[16] There is no data available for its antioxidant activity, though the alkaloid possesses a therapeutic potential of several kinds. Hence, the present study aims to investigate the *in vitro* antioxidant potential of berberine.

MATERIALS AND METHODS

Chemicals and Instruments All chemicals and solvents used in the study were of analytical grade. Berberine hydrochloride, DPPH and ABTS were obtained from Sigma Chemicals (St. Louis, Mo, U.S.A.). Sodium nitroprusside, ferrous sulfate, trichloroacetic acid, dimethyl sulfoxide, ethylenediaminetetraacetic acid (EDTA), sodium hydroxide, potassium chloride and sulphanilamide were obtained from Ranbaxy Fine Chemicals Ltd., India. UV spectrophotometer (Shimadzu 160 IPC), homogenizer, centrifuge (Remi, India) and pH meter (Elico Ltd., India) were the instruments used for the study.

Preparation of Berberine Stock Solution Berberine stock solution was prepared at the concentration of 1000 μg/ml in methanol. From the stock solution different concentrations *viz*. 2, 4, 8, 16, 32, 64, 128, 256 and 512 μg/ml were prepared in methanol and used for antioxidant studies.

Preparation of Ascorbic Acid Stock Solution Ascorbic acid used as a standard for the study. Ascorbic acid stock so-

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lution was prepared in the concentration of 1000 μg/ml in distilled water. It was prepared freshly and used immediately for the study to avoid the spontaneous decomposition of ascorbic acid in aqueous solution. From the stock solution different concentrations *viz.* 2, 4, 8, 16, 32, 64, 128, 256 and 512 μg/ml were prepared in water and used for antioxidant studies.

DPPH Radical Scavenging To 1 ml of various concentrations of berberine, 1 ml solution of DPPH 0.1 mM was added. An equal amount of methanol and DPPH served as control. After 20 min incubation in the dark, absorbance was recorded at 517 nm. The experiment was performed in triplicate. The percentage scavenging of berberine was calculated.[17]

ABTS Radical Scavenging ABTS 2 mM and potassium persulfate 70 mM were prepared in distilled water. 200 μl of potassium persulphate and 50 ml of ABTS were mixed and used after 2 h. This ABTS radical cation solution was used for the assay. To 500 μl of various concentrations of test compound, 300 μl of ABTS radical cation and 1.7 ml phosphate buffer pH 7.4 was added. For the control methanol was used, instead of the test compound. The absorbance was measured at 734 nm. The experiment was performed in triplicate.[18]

Nitric Oxide Radical Scavenging Sodium nitroprusside 5 mM was prepared in phosphate buffer pH 7.4. To 1 ml of various concentrations of test compound, sodium nitroprusside 0.3 ml was added. The test tubes were incubated at 25 °C for 5 h. After 5 h, 0.5 ml of Griess reagent was added. The absorbance of the chromophore formed during diazotization of nitrite with sulphanilamide and its subsequent coupling with *N*-(1) napthylethylenediamine was read at 546 nm. The experiment was performed in triplicate.[19]

Superoxide Scavenging Alkaline dimethyl sulfoxide (DMSO) was used as a superoxide generating system. To 0.5 ml of different concentrations of test compound, 1 ml of alkaline DMSO and 0.2 ml of nitro blue tetrazolium chloride (NBT) 20 mM in phosphate buffer pH 7.4 was added. The absorbance was measured at 560 nm. The experiment was performed in triplicate.[20]

Lipid Peroxidation Assay Stock Thiobarbituric Acid (TBA)–Trichloroacetic Acid (TCA)–HCl Reagent: 15% w/v tricholoroacetic acid, 0.375% w/v thiobarbituric acid was dissolved in 0.25 N hydrochloric acid. The solution was mildly heated to assist the dissolution of TBA.

Preparation of rat brain homogenate: Healthy Wistar albino rats of either sex weighing about 200—250 g were used for the study. The animals were housed in polypropylene cages, maintained under standard conditions (12 h light/12 h dark cycle; 25±3 °C; 35—60% humidity), and were fed with a standard rat pellet diet (Hindustan Lever Ltd., Mumbai, India) and water *ad libitum*. The study was approved by the Institutional Animal Ethical Committee, Kasturba Medical College, Manipal, India (IAEC/KMC/03/2003-04). After decapitation brain tissue was removed carefully and the tissue was homogenated with cold 0.15 M KCl to make 10% homogenate using a teflon homogenizer. The filtered homogenate was used as a source of polyunsaturated fatty acids for determining the extent of lipid peroxidation.

Method: 0.5 ml of brain homogenate was added to 1 ml of various concentrations of the test compound. The mixture was incubated for 30 min. Peroxidation was terminated by the addition of 2 ml TBA–TCA–HCl reagent. The solution was heated for 15 min in a boiling water bath and then cooled. The flocculent precipitate obtained after cooling was removed by centrifugation at 1000 rpm for 10 min. The absorbance of the supernatant was measured at 535 nm.[21]

Iron Chelating Activity The reaction mixture containing 1 ml 0.05% *o*-phenathroline in methanol, 2 ml ferric chloride 200 μM and 2 ml various concentrations of the test compound was incubated at ambient temperature for 10 min and the absorbance of the same was measured at 510 nm. The experiment was performed in triplicate.[22,23]

Statistical Analysis All results are expressed as mean ±S.E.M. Linear regression analysis was used to calculate the IC_{50} values.

RESULTS AND DISCUSSION

In the present investigation, we studied the antioxidant activity of the phytoconstituent berberine in some *in vitro* antioxidant models. In all the models tested, berberine showed its ability to scavenge the free radicals in a concentration dependent manner.

The free radical scavenging activity of berberine was studied by its ability to bleach the stable radical DPPH. This assay provides information on the reactivity of compounds with a stable free radical. Because of the odd electron, DPPH shows a strong absorption band at 517 nm in visible spectroscopy. As this electron becomes paired off in the presence of free radical scavenger, the absorption vanishes, and the resulting decolorisation is stochiometric with respect to the number of electrons taken up.[24] Maximum scavenging activity was found at a concentration of 256 μg/ml whilst the minimum scavenging activity was found at 2 μg/ml (Fig. 1). The IC_{50} value of berberine was found to be 42.7 μg/ml and that of ascorbic acid to be 62.4 μg/ml. The test compound was observed to scavenge free radicals in a concentration dependent manner between the concentrations of 2—256 μg/ml. From the DPPH assay results it may be postulated that berberine reduces the radical to the corresponding hydrazine on reacting with the hydrogen donors in berberine. The bleaching of DPPH represents the capacity of berberine to scavenge free radicals independent of enzymatic activity. The

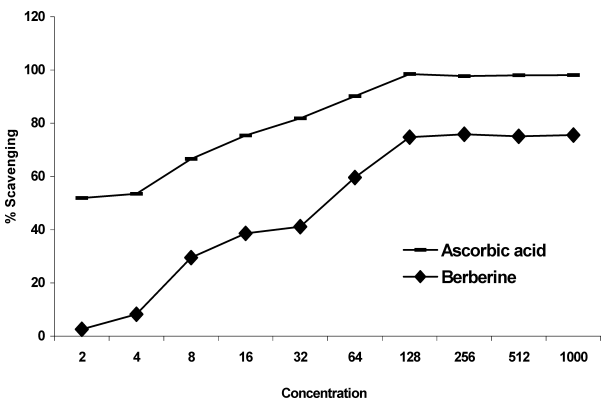

Fig. 1. DPPH Radical Scavenging

Antioxidant activity of different concentrations of berberine and ascorbic acid in DPPH radical scavenging. The results are expressed in terms of concentration *vs.* % scavenging. Each value represents mean±S.E.M.

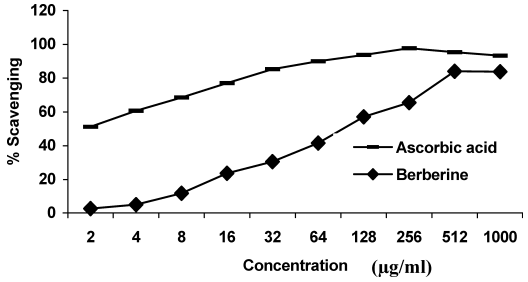

Fig. 2. ABTS Radical Scavenging

Antioxidant activity of different concentrations of berberine and ascorbic acid in ABTS radical scavenging. The results are expressed in terms of concentration *vs.* % scavenging. Each value represents mean±S.E.M.

present investigation shows that berberine is as effective as ascorbic acid in scavenging DPPH radicals.

ABTS is also a relatively stable free radical. The reduction of free radicals by the test compound using ABTS was measured at 734 nm. The free radicals were scavenged by berberine in a concentration dependant manner with the maximum scavenging activity observed at 512 μg/ml and the minimum scavenging activity found at 2 μg/ml (Fig. 2). IC_{50} was found to be 38.7 μg/ml for berberine and 74.6 μg/ml ascorbic acid. The ABTS chemistry which, involves the direct generation of ABTS radical mono cation with no involvement of any intermediary radical is a decolorization assay. Here, the radical cation is formed prior to addition of the antioxidant test system, rather than the generation of the radical taking place continually in the presence of antioxidant. This method, used for the screening of antioxidant activity, is applicable to both lipophilic and hydrophilic antioxidants.[25] The results imply that the activity of berberine may be by either inhibiting or scavenging the ABTS radicals.

Nitric oxide is a very unstable species under aerobic condition. It reacts with O_2 to produce its stable product nitrate and nitrite through intermediates NO_2, N_2O_4 and N_3O_4. It estimated by using Griess reagent. In the presence of a scavenging test compound, the amount of nitrous acid will decrease and can be measured at 546 nm. Berberine showed maximum scavenging activity at 256 μg/ml with an inhibition of 73.31% and minimum scavenging activity at 2 μg/ml with inhibition of 7.6%. The IC_{50} was found to be 46.5 μg/ml for berberine and 65.5 μg/ml for ascorbic acid (Fig. 3).

Nitric oxide (NO) is a potent pleiotropic mediator of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical, which plays many roles as an effector molecule in diverse biological systems including neuronal messenger, vasodilation, antimicrobial and antitumor activities. Studies in animal models have suggested the role for NO in the pathogenesis of inflammation and pain. Nitric oxide synthase inhibitor has been shown to have beneficial effects on some aspects of inflammation and tissue changes as seen in models of inflammatory bowel disease.[26] Berberine has also been found to protect the gastric mucosa and inhibit gastric ulcers,[27] but the pharmacological mechanisms for the protective effect of berberine are not clear. Accumulating evidence from both animal and human studies indicates that nitric oxide (NO) plays a key role in normal wound repair. The beneficial effects of NO on wound repair may be attributed to its

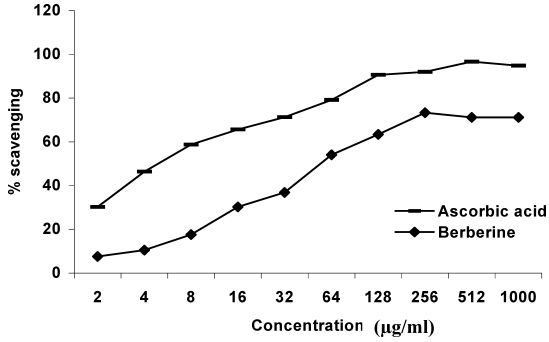

Fig. 3. Nitric Oxide Scavenging

Antioxidant activity of different concentrations of berberine and ascorbic acid in nitric oxide radical scavenging. The results are expressed in terms of concentration *vs.* % scavenging. Each value represents mean±S.E.M.

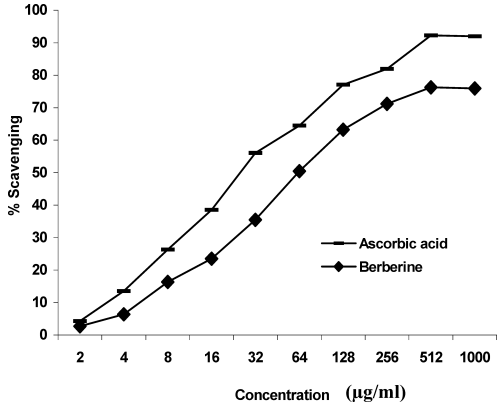

Fig. 4. Superoxide Radical Scavenging

Superoxide anion radical scavenging activity of different concentrations of berberine and ascorbic acid by NBT method. The results are expressed in terms of concentration *vs.* % scavenging. Each value represents mean±S.E.M.

functional influences on angiogenesis and inflammation.[28] It was reported that berberine reduced the production of NO and the expression of iNOS protein in a concentration-dependent manner in lipopolysaccharide (LPS)-stimulated macrophages.[29] Thus, the present study offers scientific support to earlier studies, which report the action of berberine in the reduction of inflammation, reduction of platelet aggregation and smooth muscle relaxation.[30—34]

Alkaline DMSO, used as a superoxide generating system reacts with NBT to give colored diformazan. In the presence of scavenger the reduction of NBT can be measured at 560 nm. Maximum scavenging activity was observed at 512 μg/ml with an inhibition of 76.26% (Fig. 4). The IC_{50} was found to be 35.9 μg/ml for berberine and 50.1 μg/ml for ascorbic acid. Superoxide is a highly reactive molecule that can react with many substrates, produced in various metabolic processes, including phagocytosis. It can cause oxidation or reduction of solutes depending on their reduction potential. Both aerobic and anaerobic organisms possess super oxide dismutase enzymes, which catalyze the breakdown of super oxide radical.[35] Superoxide scavenging exhibited in the alkaline DMSO method in our study indicates that berberine is a super oxide scavenger. It is well known that superoxide anions damage bio macromolecules directly or indirectly by forming H_2O_2, OH, peroxyl nitrite or singlet oxygen during pathophysiologic events such as ischemic reperfusion injury. The present findings corroborate with the earlier

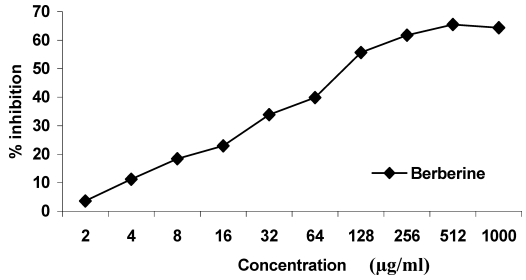

Fig. 5. Lipid Peroxidation

Antioxidant activity of different concentrations of berberine in lipid peroxidation assay. The results are expressed in terms of concentration *vs.* % inhibition. Each value represents mean±S.E.M.

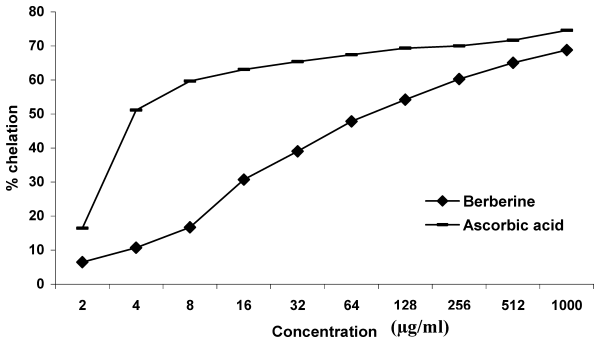

Fig. 6. Iron Chelating Activity

Reducing power of berberine and ascorbic acid by spectrophotometric detection of the Fe^{+3}–Fe^{2+} transformation. The results are expressed in terms of concentration *vs.* % chelation. Each value represents mean±S.E.M.

findings in animals and clinical trials in humans which suggest that intravenous administration of berberine may be effective in preventing the onset of ventricular tachyarrhythmias and sudden coronary death after myocardial ischemic damage.[36)]

Malondialdehyde formed from the breakdown of polyunsaturated fatty acids, serves as a convenient index for determining the extent of lipid peroxidation reaction. Malondialdehyde reacts with thiobarbituric acid to form TBARS to give red colour species, which is measured at 535 nm. Maximum scavenging activity was exhibited by berberine at 512 μg/ml and the minimum scavenging activity at 2 μg/ml (Fig. 5). The IC_{50} was fund to be 33.1 μg/ml. The hydroxyl radical is highly reactive and can damage biological molecules. Reaction with polyunsaturated fatty acid moieties of cell membrane phospholipids, lipid hydroperoxide produces[37)] lipid hydroperoxide can be decomposed to produce alkoxy and peroxyl radical, which eventually yield numerous carbonyl products such as malondialdehyde. The carbonyl products are responsible for DNA damage, generation of cancer and aging related disease. The decrease in the concentration of malondialdehyde level with increase in the concentration of berberine indicates the antioxidant role of berberine. The results suggest that the inhibition of lipid peroxidation by berberine may be due to its free radical scavenging properties.

Iron stimulates lipid peroxidation by the Fenton reaction and accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals which themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation.[38)] The observed results demonstrate the marked capacity of berberine for iron binding, suggesting that its action as a peroxidation protector may be related to its iron binding capacity. Maximum scavenging activity was exhibited at 1000 μg/ml with an inhibition of 68.76% (Fig. 6). The IC_{50} value was observed at 33.1 μg/ml for berberine and at 54.7 μg/ml for ascorbic acid. Metal chelating capacity is important since it reduces the concentration of the catalyzing transition metal in lipid peroxidation. It has been reported that chelating agents that form bonds with a metal are effective as secondary antioxidants because they reduce the redox potential thereby stimulating the oxidized form of the metal ion.[39)] The results of our study, suggest that berberine effectively chelates with metal ion.

In all the models tested, the antioxidant activity of berberine was studied in relation to that of ascorbic acid, a known standard potent antioxidant. Even though found to be less potent than ascorbic acid, our study has reported the ability of berberine to act as an antioxidant by several mechanisms *viz.* removal of oxygen, scavenging of reactive oxygen species and nitrogen species or their precursors, inhibition of reactive oxygen species and reactive nitrogen species, binding metal ions needed for catalysis of reactive oxygen generation and by the up-regulation of endogenous antioxidant defenses. The results of this study therefore justify the therapeutic potential of berberine and berberine containing plants in the traditional system of medicine, especially in diseases where the antioxidant property is an important component. The structure activity relationship, synergistic mode of action and its relative importance in different mechanisms will provide deeper insights in finding out better and safer therapeutics.

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