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Antioxidant and free radical scavenging activity of curcumin determined by using different *in vitro* and *ex vivo* models

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Antioxidant potential and free radical scavenging abilities of curcumin were evaluated using *in vitro* models such as, reducing potential, 1,1-diphenyl-2-picryl-hydrazil (DPPH), superoxide (O_2^-), hydrogen peroxide (H_2O_2) and nitric oxide (NO) radical scavenging. Curcumin recorded dose-dependent effect and higher reducing potential (OD_{max} = 0.266). Curcumin could efficiently scavenged DPPH (IC_{50} = 1.08 ± 0.06 µg/ml), H_2O_2 (IC_{50} = 10.08 ± 2.01 µg/ml), NO (IC_{50} = 37.50 ± 1.54 µg/ml), ferric reducing antioxidant power assay (FRAP) (1240 ± 18.54 µM Fe (II)/g) and superoxide anion (IC_{50} = 29.63 ± 2.07 µg/ml) radicals in a dose-dependent manner. *Ex vivo* models like erythrocyte lipid peroxidation and erythrocyte haemolysis were also studied. Curcumin could efficiently scavenged the peroxy radicals which can induce haemolysis in erythrocytes (IC_{50} = 282.7 ± 4.82 µg/ml) and inhibit the erythrocyte membrane lipid peroxidation (IC_{50} = 12.02 ± 1.03 µg/ml). The presence of antioxidants was confirmed by comparing with natural antioxidant ascorbic acid. The total phenolic content and total antioxidant activity in curcumin were determined as gallic acid and ascorbic acid equivalents, respectively. Results obtained herein can be attributed to the presence of polyphenols (448.4 ± 8.3 mg GAE/g gallic acid equivalent-polyphenols) in curcumin. These observations suggest the antioxidant and free radical scavenging potentials of curcumin.

Key words: Curcumin, free radical scavenging activity, *in vitro*, *ex vivo.*

INTRODUCTION

The oxygen consumption inherent in cell growth leads to the generation of a series of reactive oxygen species (ROS). These ROS are molecules such as superoxide anion radicals (O_2^-) and hydroxyl radicals (OH). However, non free radical species such as hydrogen peroxide (H_2O_2) and singled oxygen ($1O_2$) are formed *in vivo* also. Both oxygen species play a positive role in energy production, phagocytosis and regulation of cell growth, intercellular signalling, and synthesis of biologically important compounds. However, ROS may also be very damaging, they can attack the lipids of cell membranes and DNA. The oxidation induced by ROS can result in

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cell membrane disintegration, membrane protein damage and DNA mutation, which can further initiate or propagate the development of many diseases (Valento et al., 2002; Gulcin et al., 2003c).

ROS are continuously produced during normal physiologic events and are removed by antioxidant defence mechanisms (Buyukokuroulu et al., 2001; Chang et al., 2001; Gulcin et al., 2002a, b). It is well known that ROS are closely involved in various human diseases such as Alzheimer's disease, aging, cancer, inflammation, rheumatoid arthritis and atherosclerosis (Singh, 1989; Freeman, 1984). It is commonly recognized that antioxidants can neutralize potentially harmful reactive free radicals in body cells before they cause lipid and protein oxidation and may reduce potential mutation and therefore, help prevent cancer or heart diseases (Vaidyaratnam and Variers, 1994).

Currently, synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tert-butylhydroquinone (TBHQ) are widely used in the food industry. However, restriction on the synthetic antioxidants is being imposed because of their toxicity to liver and carcinogenicity (Grice, 1986; Wichi, 1988). Therefore, the development and utilization of more effective antioxidants of natural origins are desired. Of various kinds of natural antioxidants, phenolic compounds have received much attention (Espin et al., 2000; Luo et al., 2002). Therefore, the phenolic constituents of plants are of interest as potential chemo preventive agents and plants may be an attractive alternative to currently available commercial antioxidants because they are biodegradable to non-toxic products (Lim et al., 2002; Kayano et al., 2002).

Curcumin (Diferuloylmethane) is a naturally occurring yellow pigment isolated from ground rhizomes of the plant *Curcuma longa L*. (Zingiberaceae). As a powder, called turmeric, it has been in continuous use as a colouring and flavouring spice in foods as well as in folk medicine in the management of various inflammatory disorders and wound healing (Araujo and Leon, 2001). Although molecular mechanisms of action of curcumin are not fully understood, in several animal models it has been demonstrated to exert potent anti-inflammatory and anti-tumour and hypolipidemic properties (Aggarwal, 2003; Duvoix et al., 2005). In view of these, we aimed to evaluate the antioxidant activity of pure form of curcumin in comparison with ascorbic acid through different *in vitro* and *ex vivo* test models.

MATERIALS AND METHODS

Chemicals

Curcumin was a gift from Laila Pharmaceuticals Pvt. Ltd. (with 99% purity synthesized in Laila Impex R&D Centre). The standard L-Ascorbic acid was obtained from Hi-Media lab. Ltd., Mumbai, India. 1,1-diphenyl-2-picryl hydrazyl (DPPH), nitro blue tetrazolium (NADH), phenazine metho sulphate, 2,2'-Azobis(2-methypropionamidine) dihydro chloride (AAPH), 2,4,6-tripyridyl-s-triazine were purchased from Sigma Chemical Co, St. Louis, MO, USA. The solvents used were of analytical grade.

Total phenolic content

Total phenolic content was determined using Folin-Ciocalteu (FC) reagent according to the method of Singleton et al. (1999). Briefly, ethanolic solution of the curcumin in the concentration of 1 mg/ml was used in the analysis. The reaction mixture was prepared by mixing 0.5 ml of ethanolic solution of curcumin (20 to 100 µg/ml), 2.5 ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml 7.5% $NaHCO_3$. Blank was concomitantly prepared containing 0.5 ml methanol, 2.5 ml 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml of 7.5% of Na_2CO_3. The samples were thereafter incubated in a thermostat at 45°C for 45 min. The absorbance was determined using spectrophotometer (Shimadzu UV-1800) at λ_{max} = 765 nm. The same procedure was repeated for the standard solution of gallic acid. Based on the measured absorbance, the concentration of phenolics was read (mg/ml) from the calibration line; then the content of phenolics in extract was expressed in terms of gallic acid equivalent (mg of GA/g of extract).

DPPH free radical scavenging assay

The scavenging activity for DPPH free radicals was measured according to the procedure described by Blios (1958). Curcumin at various concentrations (1 to 5 µg/ml) was added separately to each 5 ml of 0.1 mm methanolic solution of DPPH and allowed to stand for 30 min at room temperature. After incubation, the absorbance of each solution was determined at 517 nm using spectrophotometer (Shimadzu UV-1800). Ascorbic acid was used as standard.

Superoxide scavenging assay

Superoxide scavenging potential of curcumin was determined using the method described by Nishimiki et al. (1972). To 1 ml of nitroblue tetrazolium (NBT) solution (156 µM NBT in 100 mM phosphate buffer, pH 7.4), 1 ml NADH solution (468 µM in 100 mM phosphate buffer, pH 7.4) and 1 ml sample solution of curcumin (10 to 50 µg/ml) in ethanol was mixed. The reaction started by adding 100 µl of phenazine methosulphate (PMS) solution (60 µM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25°C for 5 min and the absorbance at 560 nm was measured against blank sample. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity.

Nitric oxide radical scavenging assay

The method of Garrat (1964) was used to determine the nitric oxide radical scavenging activity of curcumin. A volume of 2 ml of 10 mM sodium nitroprusside prepared in phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of curcumin at various concentrations ranging from 10 to 50 µg/ml and ascorbic acid at various concentrations ranging from 25 to 200 µg/ml. The mixture was

incubated at 25°C. After 150 min, 0.5 ml of incubation solution was withdrawn and mixed with 0.5 ml of Griess reagent [1.0 ml sulfanilic acid reagent (0.33% prepared in 20% glacial acetic acid at room temperature for 5 min with 1 ml of naphthylethylene diamine dihydrochloride (0.1% w/v)]. The mixture was incubated at room temperature for 30 min, followed by the measurement of absorbance at 540 nm using spectrophotometer (Shimadzu UV-1800).

Reducing power assay

Reducing power capacity of the curcumin was determined using the method described by Oyaizu (1986). 1 ml of curcumin (40 to 200 µg/ml) was mixed with 2.5 ml of 0.2 M phosphate buffer, pH 6.6 and 2.5 ml of 1% aqueous potassium hexacyanoferrate [$K_3Fe(CN)_6$] solution. After 30 min of incubation at 50°C, 2.5 ml of 10% trichloro-acetic acid was added and the mixture was centrifuged for 10 min. Finally, 2.5 ml of the upper layer was mixed with 2.5 ml of water and 0.5 ml of 0.1% aqueous $FeCl_3$ and the absorbance was recorded at 700 nm using spectrophotometer (Shimadzu UV-1800).

Total anti oxidant capacity

The total antioxidant activity of the extract was evaluated by the phosphomolybdenum method according to the procedure described by Prieto et al. (1999). An aliquot (0.1 ml) of the sample at different concentrations (20 to 100 µg/ml) was mixed with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The mixture was covered and incubated at 95°C for 90 min. After the mixture was cooled, the absorbance was measured at 695 nm against blank. A typical blank solution contained 1 ml of reagent solution and the appropriate volume of the same solvent used for dissolving the sample, and it was incubated under the same conditions. The total antioxidant activity was expressed as the absorbance value at 695 nm using spectrometer (Shimadzu UV-1800) against blank. Ascorbic acid was used as reference standard. Based on the measured absorbance, the total antioxidant activity was read (mg/ml) from the calibration line; the antioxidant activity is expressed as the number of equivalents of ascorbic acid (AscAE).

Hydrogen peroxide scavenging assay

Hydrogen peroxide scavenging potential of curcumin was determined using the method described by Jayaprakasha et al. (2004). A solution of hydrogen peroxide (20 mM) was prepared in phosphate buffer saline (PBS, pH 7.4). Different concentrations of curcumin (4 to 20 µg/ml) in ethanol (1 ml) were added to 2 ml of hydrogen peroxide solution in PBS. After 10 min, the absorbance was measured at 230 nm using spectrophotometer (Shimadzu UV-1800) against a blank solution that contained hydrogen peroxide solution without curcumin.

Ferric reducing antioxidant power assay

The determination of the ferric reducing activity is a modified method of Benzie and Strain (1996). The stock solutions included 300 mM acetate buffer (3.1 g $C_2H_3NaO_2 \cdot 3H_2O$ and 16 ml $C_2H_4O_2$), pH 3.6, 10 mM 2,4,6-tripyridyl-*s*-triazine (TPTZ) solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The fresh working solution was prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ, and 2.5 ml $FeCl_3 \cdot 6H_2O$. The temperature of the solution was raised to 37°C before use. Curcumin (200 µl) were allowed to react with 2800 µl of the FRAP solution for 30 min in the dark condition. Readings of the coloured product (ferrous tripyridyltriazine complex) were taken at 593 nm. The standard curve was linear between 100 and 500 µM $FeSO_4$. Results are expressed in µM Fe(II)/g dry mass and compared with ascorbic acid.

AAPH induced hemolysis in erythrocytes

Haemolysis of erythrocytes mediated by 2,2′-azobis-2-amidinopropane dihydrochloride (AAPH) was determined using a modification of a method described by Miki et al. (1987). Blood samples were obtained from healthy volunteers in heparinised tubes and centrifuged (1,500 rpm, 10 min). After removing the supernatant, the pellet was washed three times with PBS. During the last washing, the erythrocytes were centrifuged (1,500 rpm, 10 min). A 10% suspension of erythrocytes was prepared by adding phosphate buffered saline (PBS, pH 7.4). Test samples (0.2 ml) at different concentrations and 0.2 ml of 100 mM AAPH were added in succession to 0.2 ml of erythrocyte suspension. The reaction mixtures, curcumin and ascorbic acid in various concentrations ranging from100 to 500 µg/ml were incubated at 37°C for 3 h with gentle shaking. After incubation, the reaction mixture was diluted 20 times with PBS and centrifuged at 1000 × *g* for 10 min. The absorbance of the supernatant at 540 nm was read. Similarly, another tube of the reaction mixture was diluted with distilled water to yield complete haemolysis and the absorbance of the supernatant after centrifugation was measured at 540 nm using spectrophotometer (Shimadzu UV-1800).

Inhibition of erythrocyte lipid peroxidation

Lipid peroxidation was estimated calorimetrically by thiobarbituric acid reactive substances (TBARS) using the modified method of Niehius and Samuelsson (1968). In order to induce lipid per oxidation, 200 µl of erythrocyte suspension and 100 µl of 100 µM hydrogen peroxide was added into a test tube containing curcumin and ascorbic acid in various concentrations ranging from 20 to 100 µg/ml. The contents were incubated at 37°C for 1 h. The reaction was stopped by the addition of 2 ml of thiobarbituric acid (TBA) stock reagent (0.375% TBA, 15% TCA, 0.2 HCl). The solution was incubated in water bath for 1 h. After cooling, the solution was centrifuged at 3,000 rpm for 5 min. The absorbance of the supernatant was measured at 532 nm using spectrophotometer (Shimadzu UV-1800).

Statistical analysis

Data were expressed as mean ± standard deviation (SD) for three parallel measurements using Graph Pad Prism version 6.0 for windows, Graph Pad Software, San Diego, California, USA. Statistical analysis was done by student's *t* test and $p < 0.05$ considered as significant. The 50% inhibitory concentration (IC_{50}) was calculated from the dose response curve (Graph Pad Prism Version 6.0) obtained by plotting percentage inhibition versus concentrations.

Inhibition (%) = Absorbance of control – absorbance of test sample/ absorbance of control × 100.

RESULTS AND DISCUSSION

Total phenolic content

The determination of total phenolics was based on the absorbance value of curcumin solution (0.1 mg/ml) that react with Folin-Ciocalteu reagent, followed by comparing with the standard solution of gallic acid equivalents. The standard curve of gallic acid (Figure 1) was done by using gallic acid concentration ranging from 20 to 100 µg/ml. The following equation expressed the absorbance of gallic acid standard solution as a function of concentration:

$Y = 0.4819x + 0.1659$, $R^2 = 0.9968$

Where x is the absorbance and Y is the gallic acid equivalent (mg/g). Curcumin exhibited the greater phenolic content as mg gallic acid equivalent/g weight (mg GAE/g) for a value of 448.4 ± 8.3 mg GAE/g.

Phenolic compounds are hydroxylated derivatives of benzoic and cinnamic acids that are responsible for the antioxidant activity. In general, the antiradical and antioxidant activities of plant extracts are associated to the phenolic content (Khattak et al., 2008). The reason that curcumin elicited the higher total phenolic content may be due to the fact that it contains two phenolic groups. It is clear that the number of phenolic groups present in the structure of an antioxidant molecule is not always the only factor to determine its antioxidant activity. Positions of the phenolic groups, presence of other functional groups in the molecules such as double bonds and conjugation to phenolic and ketone groups also play important roles in antioxidant activities and have been reported by another study that studied the activity relationship of antioxidant activity of flavonoids and phenolic acid (Rice-Evans et al., 1996). The two phenolic groups of curcumin are attached to different unadjacent benzene rings that give the two OHs the mobility to work freely without hindrance. Additionally, polarity and hydrophobicity of antioxidants plays an important factor in the antioxidant activity especially in the bio membrane systems (Wu et al., 2007).

DPPH free radical scavenging assay

Free radical scavenging activity of curcumin and ascorbic acids were tested using the DPPH method and the results are shown in Figure 2. In this study, the free radical scavenging ability of each sample was evaluated through recording the change of absorbance produced by the reduction of $DPPH^{\cdot}$. The percentage scavenging activity of each sample against DPPH. ranged from 55.60 to 71.64% and 57.35 to 79.27% for curcumin, ascorbic acid, respectively. The IC50 was found to be 1.08 ± 0.06 µg/ml for curcumin and for standard ascorbic acid, it was found to be 1.34 ± 0.02 µg/ml. The results obtained indicated that higher percentage of DPPH. scavenging activity may be attributed to the high reducing power and higher total phenolic contents present in curcumin. DPPH is a stable radical showing maximum absorbance at 515 nm. In DPPH assay, the anti oxidants were able to reduce DPPH to yellow coloured diphenyl picrylhydrazone (Frankel and Meyer, 2000). The method based on the reduction DPPH in alcoholic solution in the presence of hydrogen donating anti oxidant due to formation of the non radical form DPPH-H in the reaction. DPPH is usually used as a reagent to evaluate free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Oyaizu, 1986).

Superoxide anion scavenging activity

The percentage superoxide scavenging activities of the ascorbic acid and curcumin on superoxide radicals are shown in Figure 3. The percentage superoxide scavenging activities of curcumin and ascorbic acid were ranging from 36.69 to 52.79% and 40.0 to 54.05%, respectively. The IC_{50} was found to be 29.63 ± 2.07 µg/ml for curcumin and for standard ascorbic acid, it was found to be 34.56 ± 2.11 µg/ml. Superoxide has been observed to directly initiate lipid peroxidation. It has also been reported that antioxidant properties of some flavonoids are effective mainly through scavenging of superoxide anion radical (Yen and Duh, 1994). Superoxide anions derived from dissolved oxygen by the phenazine methosulfate (PMS)-Nicotinamide adenine dinucleotide (NADH) system will reduce NBT in this system. In this method, superoxide anion reduces the yellow dye (NBT^{2+}) to produce the blue formazan, which is measured spectrophotometrically. Antioxidants are able to inhibit the blue NBT formation (Parejo et al., 2002). The decrease of absorbance in presence of antioxidants indicates consumption of superoxide anion in the reaction mixture.

Nitric oxide radiacl scavenging assay

Incubation of solutions of sodium nitroprusside in phosphate buffer saline at 25°C for 150 min resulted in the generation of NO. The curcumin effectively reduced the generation of NO. The IC_{50} was found to be 37.50 ± 1.54 µg/ml for curcumin and 190.46 ± 3.87 µg/ml for ascorbic acid (Table 1). NO radical plays multiple roles in a variety of biological processes which includes: as an effector molecule, neuronal messenger, vasodilator, antimicrobial agent, etc (Hagerman et al., 1998). It has

Table 1. IC_{50} values of *in vitro* and ex-vivo antioxidant activities of curcumin.

Sample	DPPH (µg/ml)	Nitric oxide (µg/ml)	H_2O_2 (µg/ml)	Superoxide Anion (µg/ml)	AAPH induced Haemolysis (µg/ml)	Lipid peroxidation (µg/ml)
Curcumin	1.08±0.06	37.50±1.54	10.08±2.01	29.63±2.07	282.7±4.82	12.02±1.03
Ascorbic acid	1.34±0.02	190.46±3.87	41.50±1.64	34.56±2.11	313.4±5.46	14.82±1.91

Data are presented as Mean± SD.

Table 2. Total phenolic content, Total anti oxidant capacity and Ferric reducing activities of curcumin.

Sample	Total phenolic content (mg GAE/g)	Total anti oxidant capacity (mg AscAE/g)	FRAP assay (µM Fe (II)/g)
Curcumin	448.4±8.3	319.80±2.54	1240±18.54
Ascorbic acid	-	-	1615±22.64

Data are presented as Mean ± SD.

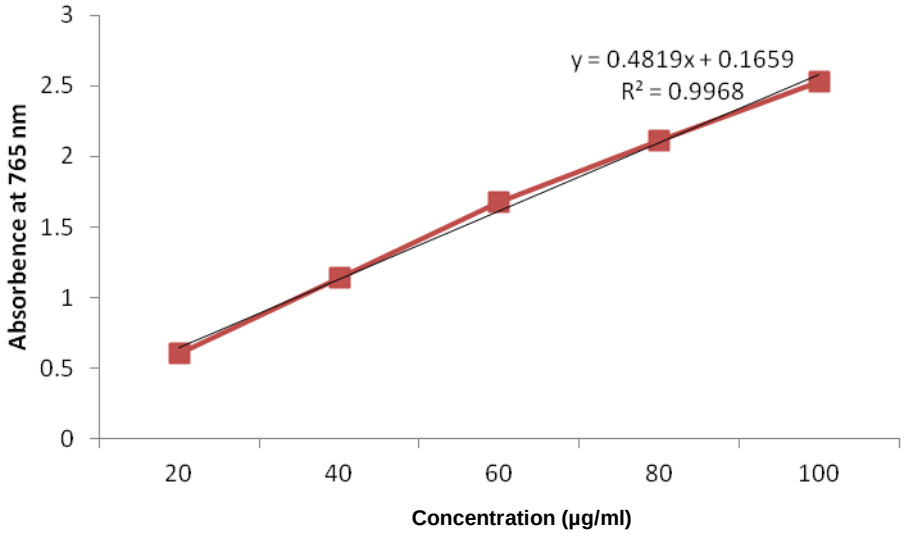

Figure 1. Total phenolic content of curcumin expressed in gallic acid equivalents (mg GAE/g).

been reported to react with $^{\cdot}O_2$ radical to form peroxynitrite radicals ($ONOO^{\cdot}$) that cause toxicity to bio molecules such as proteins, lipids and nucleic acids (Yermilov et al., 1995). During the process of inflamemation, cells of the immune system generate superoxide radicals in which NADPH oxidase plays an important role in induction of vascular complications (Droge, 2002). $^{\cdot}O_2$ further decomposes into singlet oxygen and HO. that result in massive mitochondrial damage. Curcumin significantly inhibits generation of $NO^{\cdot}$ and $HO^{\cdot}$ radicals in a dose-dependent manner. These observations further highlight the importance of curcumin in preventing physiological deleteriation caused by $NO^{\cdot}$ and $^{\cdot}O2$ radicals.

Reducing power assay

The reducing power of the curcumin increased and correlated well with the increasing concentration. However, the reducing power of ascorbic acid was relatively more pronounced than that of curcumin. A high absorbance

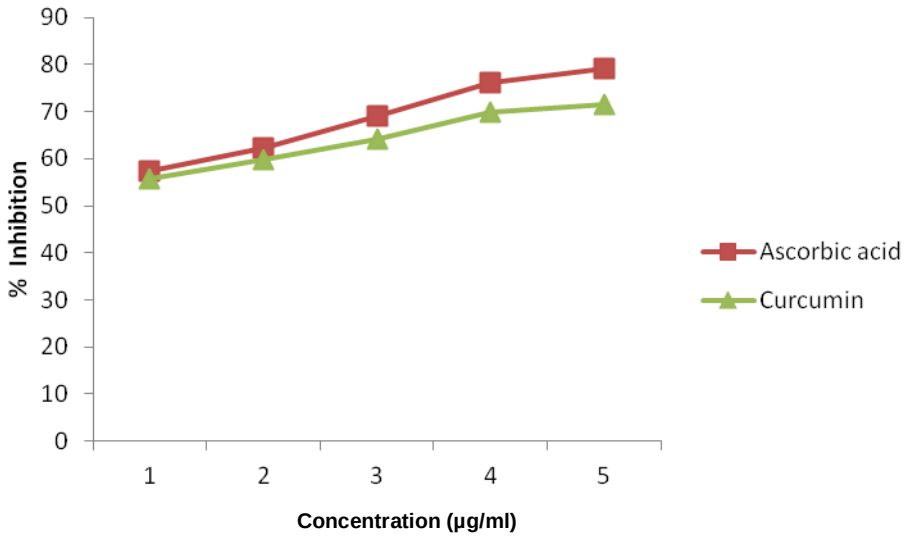

Figure 2. DPPH radical scavenging activity of curcumin and ascorbic acid. Data are presented as the percentage of DPPH radical scavenging, Mean± SD.

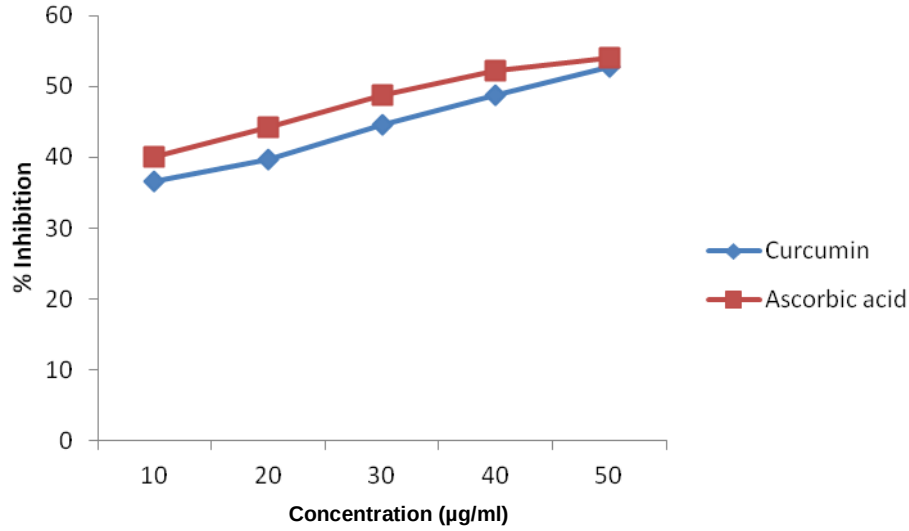

Figure 3. Superoxide anion scavenging activity of curcumin and ascorbic acid. Data are presented as the percentage of superoxide anion radical scavenging, Mean± SD.

value of the sample indicates its strong antioxidant activity. The reducing power of curcumin and ascorbic acid ranged from 0.059 to 0.266 and 0.082 to 0.346 Abs for 40 to 200 µg/ml, respectively (Figure 4). The antioxidant activity of phenolic compounds is mainly due to their redox properties which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides (Osawa, 1994). For the measurements of the reductive ability, it has been found that the Fe^{3+} to Fe^{2+} transformation occurred in the presence of curcumin samples which were postulated previously by Oyaizu (1986). Tanaka et al. (1988) have observed a direct correlation between antioxidant activities and reducing power of certain plant extracts. The reducing properties are generally associated with the presence of reductones (Duh et al., 1999) which have been shown to exert antioxidant action by breaking the free radical chain by

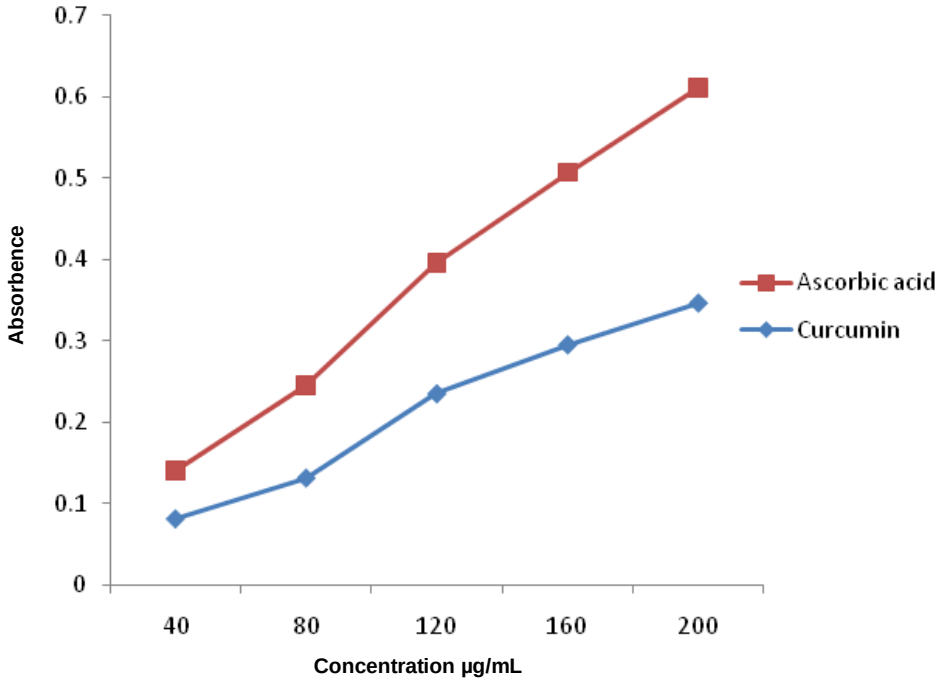

Figure 4. Reducing power activity of curcumin and ascorbic acid. Data are presented as Mean± SD.

donating a hydrogen atom (Gordon, 1990). Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. In this assay, depending on the reducing power of antioxidant compounds, the yellow colour of the test solution changes into various shades of green and blue. Therefore, by measuring the formation of Perl's Prussian blue at 700 nm, we can monitor the Fe^{2+} concentration. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity.

Total antioxidant capacity

The antioxidant activity of the curcumin was evaluated by the phosphomolybdenum method, followed by comparing with the standard solution of ascorbic acid equivalents. The standard curve of ascorbic acid was done by using ascorbic acid concentration ranging from 20 to 100 µg/ml. The following equation expressed the absorbance of ascorbic acid standard solution as a function of concentration:

$Y = 0.5636x + 0.0478$, $R^2 = 0.998$

Where x is the absorbance and Y is the ascorbic acid equivalent (mg/g). Curcumin showed an increase in antioxidant capacity with an increase in dose. Total antioxidant capacity of curcumin was found to be 31.98 ± 2.54 µg ascorbic acid equivalents at 100 µg/ml curcumin concentration (Table 2). It determines the total antioxidant capacity. The phosphomolybdenum method is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and the subsequent formation of green phosphate Mo (V) complex with a maximum absorption at 695 nm. The extract reduced molybdenum VI to a green coloured phosphomolybdenum V complex. This good antioxidant activity might be attributed to the presence of high amounts of polyphenols in these extracts (Prieto et al., 1999).

Hydrogen peroxide scavenging assay

The ability of curcumin to scavenge hydrogen peroxide is shown in Table 1 and compared with that of ascorbic acid as reference compound. Hydrogen peroxide scavenging activities of curcumin and ascorbic acid are 18.20 to 29.13% and 30.9 to 77.15%, respectively at the same concentration. The IC_{50} was found to be 10.08 ± 2.01 µg/ml for curcumin and for standard ascorbic acid, it was found to be 41.50 ± 1.64 µg/ml (Figure 5). Biological systems can produce hydrogen peroxide. Hydrogen peroxide can be formed *in vivo* by several oxidizing

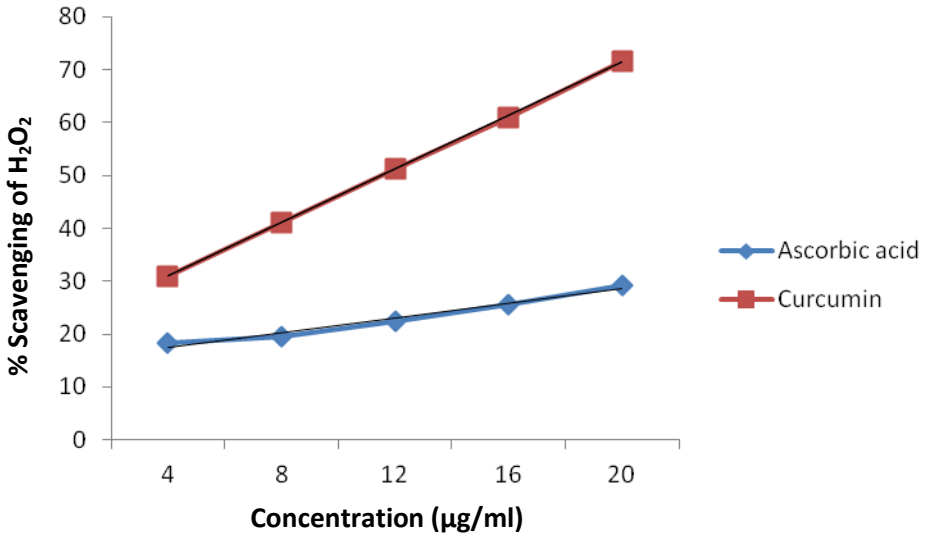

Figure 5. Hydrogen peroxide scavenging activity of curcumin and ascorbic acid. Data are presented as the percentage of H_2O_2 radical scavenging, Mean ± SD.

enzymes such as superoxide dismutase. It can cross membranes and may slowly oxidize a number of compounds. It is used in the respiratory burst of activated phagocytes (MacDonald-Wicks et al., 2006). Curcumin has effective hydrogen peroxide scavenging activity. It is known that H_2O_2 is toxic and induces cell death *in vitro* (Aoshima et al., 2004). Hydrogen peroxide can attack many cellular energy-producing systems. For instance, it deactivates the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (Hyslop et al., 1988).

Ferric reducing antioxidant power assay

Ferric reducing antioxidant power is widely used in evaluating antioxidant activity of plant polyphenols. The standard curve of $FeSO_4$ was done by using $FeSO_4$ concentration ranging from 100 to 500 µM. The following equation expressed the absorbance of FeSO4 standard solution as a function of concentration:

$Y = 0.1063x – 0.0393$, $R^2 = 0.9999$

Where x is the absorbance and Y is the $FeSO_4$ equivalent µM Fe(II)/g. Curcumin showed an increase in ferric reducing power with an increase in dose. Total ferric reducing power of curcumin was found to be 1240 ± 18.54 µM Fe(II)/g and for ascorbic acid 1615 ± 22.64 at 100 µg/ml concentration (Table 2). Principally, FRAP assay treats the antioxidants in the sample as reductant in a redox linked colorimetric reaction. FRAP assay measures the reducing potential of antioxidant to react on ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex and produce blue colour of ferrous form which can be detected at absorbance 593 nm. Antioxidant compounds which act as reducing agent exert their effect by donating hydrogen atom to ferric complex and thus break the radical chain reaction (Benzie and Strain, 1996).

AAPH induced hemolysis in erythrocytes

Scavenging of peroxyl radicals by different concentrations of curcumin and ascorbic acid were evaluated by its inhibitory effect on RBC haemolysis induced by peroxyl radicals. Percent of haemolysis decreased in a concentration dependent manner at concentrations ranging from 100 to 500 µg/ml (Figure 6). The IC_{50} was found to be 282.7 ± 4.82 µg/ml for curcumin and for standard ascorbic acid, it was found to be 313.4 ± 5.46 µg/ml. The peroxyl radicals was generated by AAPH on addition to erythrocyte suspension and its subsequent scavenging action produced by graded concentrations of curcumin and standard ascorbic acid. An increase in inhibition was noticed at all concentrations of curcumin and ascorbic acid. The azo compound generates free radicals by its unimolecular thermal decomposition. The rate of generation of peroxyl radicals can be easily controlled and measured by adjusting the concentration of AAPH. Therefore, the haemolysis induced by AAPH clearly demonstrates the oxidative erythrocytes membrane damage by peroxyl radical attack from the outside of the membrane. AAPH as an azo compound can decompose to form carbon-centered radicals that

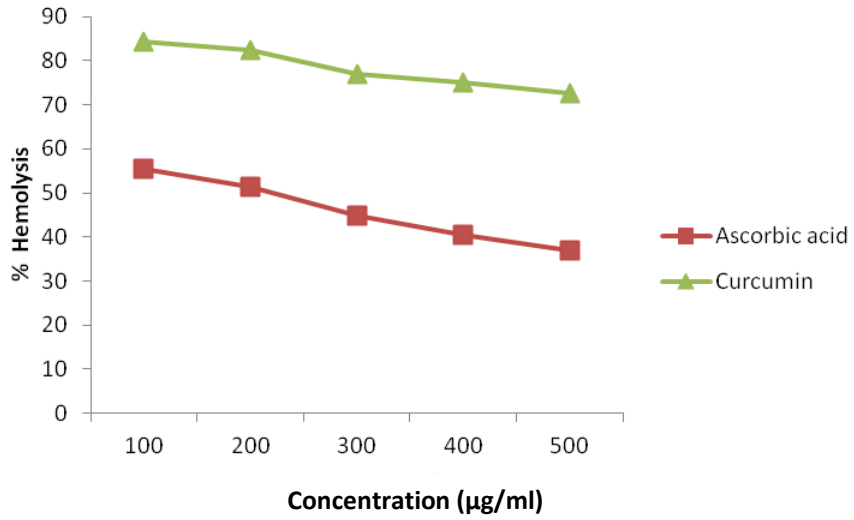

Figure 6. AAPH induced haemolysis activity of curcumin and ascorbic acid. Data are presented as the percentage of haemolysis, Mean ± SD.

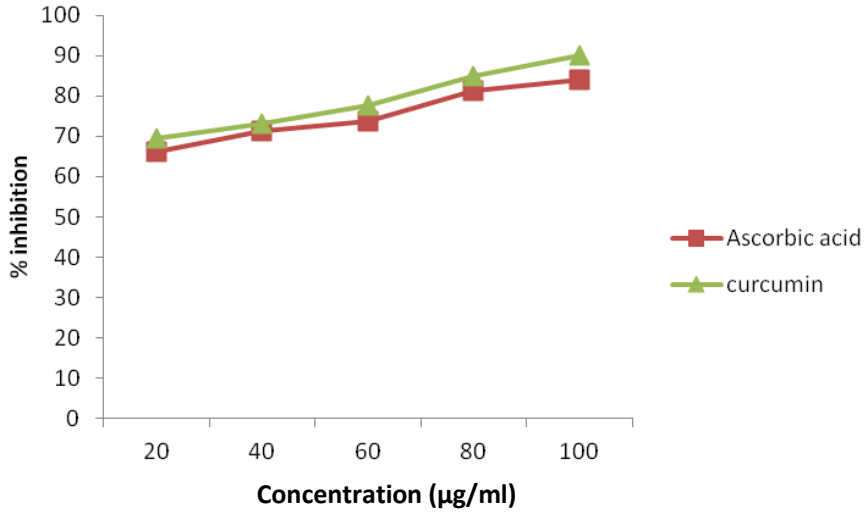

Figure 7. Inhibition of erythrocyte membrane lipid peroxidation activity of curcumin and ascorbic acid. Data are presented as the percentage of lipid peroxidation inhibition, Mean± SD.

can react with O_2 to yield peroxyl radicals. The erythrocyte membrane contains abundant polyunsaturated fatty acids which are very susceptible to free radical induced peroxidation. Since the generation rate of free radical from the decomposition of AAPH at physiological temperature can be easily controlled, this water-soluble azo compound can be used as free radical resource to attack the erythrocyte membrane to induce haemolysis. Hence, the AAPH-induced haemolysis provides a good approach to research the free-radical-induced membrane damage (Zou et al., 2001).

Inhibition of erythrocyte lipid peroxidation

The percentage inhibition of erythrocyte membrane lipid peroxidation activities of the ascorbic acid and curcumin are shown in Figure 7. The percentage inhibition activities of curcumin and ascorbic acid ranged from 69.37 to 89.91% and 66.25 to 83.92%, respectively. The IC_{50} was

found to be 12.02 ± 1.03 µg/ml for curcumin and for standard ascorbic acid, it was found to be 14.82 ± 1.91 µg/ml. Malondialdehyde (MDA) is the by-product of peroxidation of phospholipids and generally regarded as a marker for oxidative stress, rendering its determination in biological samples rather interesting (Rodriguez, 2006). The MDA levels of H_2O_2-induced hemoglobin oxidation treated with different concentrations of the curcumin decreased as compared to H_2O_2-induced haemoglobin (Li et al., 2009).

Conclusion

Based on the various *in vitro* and *ex vivo* assays, it can be concluded that the curcumin possesses strong antioxidant activity as evidenced by the free radical scavenging property, reducing power property *ex vivo* assays like AAPH induced haemolysis in erythrocytes and inhibition of erythrocyte lipid per oxidation, can be a very effective antioxidant and can protect biological systems against the oxidative stress that is found to be an important pathophysiological event in a variety of diseases including aging, cancer, diabetes, cardio-vascular disorders and rheumatoid arthritis. This may be due to the presence of phenolic components in the curcumin. Overall, curcumin is a source of natural antioxidant that can be important in disease prevention and health preservation.

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