International Journal of Sciences & Applied Research

www.ijsar.in

ANTIOXIDANT POTENTIALS OF COMBINED DIETARY CURCUMIN AND CAPSAICIN

Thriveni V[1], Ravikumar H R[2,] Manjunatha H[3]

[1]Department of Studies and Research in Biotechnology and Bioinformatics, Kuvempu University, Shankaraghatta, -577 451, India
[2]Department of Biochemistry, Bangalore University, JBCampus, Bangalore -560 056, India
[3]Department of Biochemistry, Bangalore University, JBCampus, Bangalore -560 056, India
Corresponding author:*Ravikumar H R, Department of Biochemistry, Bangalore University, JB Campus, Bangalore -560 056, India

ABSTRACT

The in vitro antioxidant activity of curcumin/capsaicin/combined curcumin and capsaicin was evaluated by employing various chemical and biochemical methods. The chemical methods involved reducing power and radical scavenging assays, while biochemical assays employed H_2O_2induced oxidative stress in erythrocytes, hemoglobin and DNA. Curcumin/capsaicin have shown 2, 2-diphenyl-1-picrylhydrazyl (DPPH), NO and OH radical scavenging property, whilst combined curcumin & capsaicin has shown additive antioxidant property. Further in biochemical assays, curcumin/capsaicin has shown protective activity against H_2O_2 induced oxidative stress in erythrocytes, hemoglobin and DNA. Conversely, combined curcumin & capsaicin yet again has shown additive property against H_2O_2 induced erythrocytes and DNA oxidation, while in hemoglobin assay it has become synergistic. The present study revealed additive as well as synergistic efficacy of combined curcumin and capsaicin.

Keywords: Curcumin, Capsaicin, Erythrocytes, Hemoglobin, DNA, Antioxidant property.

INTRODUCTION

Free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) are the products of normal cellular metabolism. They are continuously generated in the body in response to changes in intra and extracellular environmental conditions like aerobic respiration, by stimulating polymorphonuclear leukocytes, macrophages and exposure to various physico - chemical pollutants[1,2] However, during normal condition free radicals were continuously scavenged by natural enzymatic and non-enzymatic defense system[3]. The enzymatic defense system includes antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, catalase and many non-enzymatic antioxidant compounds such as polyphenols, tocopherols, ascorbic acid, uric acid, glutathione and other thiol protein groups [4]. Enhancement of ROS levels or less detoxification ability of the natural antioxidant system leads to the overproduction of these free radicals results in oxidative stress inducing damage to cell structures, including cellular lipids and membranes, proteins and DNA by inhibiting their normal function and this can increase the risk of mutagenesis[5]. Oxidative stress

has been implicated in a number of human diseases such as diabetes mellitus, inflammation, cancer, cardiovascular diseases, neurodegenerative diseases, hypertension, rheumatoid arthritis, asthma, brain dysfunction and in ageing process [5, 6].

Bioactive compounds present in fruits, vegetables, spices and herbs have been reported for their beneficial antioxidant activity without any side effects [7]. They play a crucial role in scavenging the active free radicals before they attack biologically vital molecules by donating hydrogen atoms to maintain the cellular homeostasis. Bio-molecules are more vulnerable to oxidative stress conditions, because they are considered as prime targets for free radical attack. Strong oxidants, such as H_2O_2 can easily enter cells and they can trigger the cellular oxidative cascade, such is the case in erythrocyte since it is used as a unique cell model having simple metabolism and more vulnerable to oxidative stress. Hemoglobin (Hb) is highly sensitive to oxidative damage and usually transforms into other products, such as Methemoglobin (MetHb), Heinz bodies and colorless hemichrome with the release of iron ions which are catalytically active in initiating free radicals production and lipid peroxidation [8,9].

Recent researches have shown that spices have active components called nutraceuticals that contribute to the plethora of properties [10]. Spices are extensively used in cooking to enhance the taste, flavor and color to the food including several health beneficial physiological effects. Curcumin, the yellow coloring principle of turmeric (*Curcuma longa*) and capsaicin the pungent principle of red pepper (*Capsicum annuum*) have been documented individually and in combination to possess significant antioxidant influence in *in vivo* systems [11,12]. The combined *in vitro* antioxidant activity of curcumin and capsaicin STILL TO be explored to a greater extent, since a growing consensus among scientists that a combination of antioxidants molecules rather than single entities could be more effective over the long term [13].

Therefore, the aim of the present study is to investigate the *in vitro* antioxidant property of combined curcumin and capsaicin using various chemical and biochemical methods.

2. MATERIAL AND METHODS

2.1 Chemicals

Curcumin, capsaicin, thiobarbituric acid (TBA), 2,2-diphenyl-1- picrylhydrazyl (DPPH), nicotinamide adenine dinucleotide (NADH), ferrozine, nitro blue tetrazolium disodium salt (NBT), phenazine methosulfate (PMS), O-dianisidine (ODA), 2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) and (α-napthyl)-ethylene diamine were obtained from Sigma–Aldrich. Butylated hydroxyl toluene (BHT), sulfanilamide, sodium nitroprusside (SNP), trichloroacetic acid (TCA) were purchased from Merck (Germany). Ascorbic acid, ferrous chloride, haemoglobin and potassium persulfate were procured from Himedia (India). pUC19 and agarose were procured from GeNei^{TM} (India). All the chemicals used were of analytical grade.

2.2. CHEMICAL ASSAYS

2.2.1. Total antioxidant capacity

Total antioxidant capacity of curcumin/capsaicin/combined molecules was analyzed by phosphomolybdenum method [14]. 300 µl of curcumin/capsaicin/combination at different concentration (20, 40, 80 & 160 µg) was mixed with 3 ml of reagent mixture (4 mM ammonium molybdate, 0.6M sulfuric acid

and 28 mM of sodium phosphate). Test tubes were kept in boiling water bath for 90 min. and allowed to cool. Absorbance of the content was measured at 695 nm. Antioxidant capacity of spice molecules was expressed as equivalents of ascorbic acid.

2.2.2. Total reductive capacity

Total reductive capacity of curcumin/capsaicin/combined molecules was determined according to the method of Oyaizu [15]. 1 ml curcumin/capsaicin/combined molecules at different concentration (40, 80, 120, 160 & 200 µg) was mixed with 2.5 ml of phosphate buffer (0.2 M pH 6.5) and potassium ferricyanide (2.5ml, 1%). The mixture was incubated at 50 ˚C for 20 min. and at the end of the incubation period; trichloroacetic acid (2.5ml, 10%) was added and centrifuged at 3000 rpm for 10 min. To the 2.5 ml of supernatant, 2.5 ml of millipore water and 0.5 ml of ferric chloride (0.1%) was added. The absorbance was measured at 700 nm and total reducing capacity was expressed as equivalents of standard BHT.

2.2.3. DPPH radical scavenging assay

DPPH radical scavenging potential of curcumin/capsaicin/combined molecules was conducted on the basis of scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical[16]. Different concentrations of curcumin/capsaicin/combined molecules were added to 3 ml of 0.004% DPPH in 95% methanol and the mixture was incubated at room temperature for 30 min. in dark condition. The scavenging activity of molecules against DPPH radical was determined by measuring the absorbance at 517 nm with BHT as standard. Radical scavenging activity was calculated using the following formula (1).

$$\% \text{ inhibition} = [(A_{control} - A_{test})/A_{control}] \times 100,$$

Where $A_{control}$ is the absorbance of the control (without samples) and A_{test} is the absorbance of the sample (with spice molecules) reaction.

2.2.4. ABTS radical scavenging assay

ABTS radical cation ($ABTS^{\cdot+}$) was produced by reaction of ABTS solution (7 mM) with 2.6 mM potassium per sulfate and incubated in dark at room temperature for 12-16 h before use[17]. Prior to assay, 1 ml of ABTS stock solution was mixed with ethanol (1:60 V/V) to get working solution. 150 µl of different concentration of curcumin/capsaicin/combined molecules were added and the final volume was made up to 3.0 ml with working ABTS solution and the reaction mixture was incubated at room temperature in dark condition for 2 h. The scavenging of ABTS radicals by molecules was determined by measuring the absorbance at 745 nm with BHT as standard. The free radical scavenging activity was calculated using the formula (1).

2.2.5. Superoxide radical scavenging assay

Superoxide anion radical scavenging potential of curcumin/capsaicin/combined molecules was determined according to the method of Nishimiki et al.[18] with slight modifications. All the reagents were prepared in phosphate buffer (pH 7.4). 1 ml of NBT (156 µM), 1 ml of NADH (468 µM) and 2 ml of curcumin/capsaicin/combined molecules at different concentration was added. The reaction was initiated by adding 100 µl of PMS (60 µM) and incubated at 25 ˚C for 5 min. followed by the measurement of absorbance at 560 nm against blank. Ascorbic acid was taken as reference standard. The percentage of inhibition was calculated using the formula (1).

2.2.6. Nitric oxide radical scavenging activity

Nitric oxide radical scavenging activity of curcumin/capsaicin/combined molecules was determined according to Marcocci et al [19]. The reaction mixture containing 2 ml of sodium nitroprusside solution (10 mM in 0.5M phosphate buffer, pH 7.4) and 500µl of curcumin/capsaicin/combined molecules at different concentration was incubated at 25 ˚C for 150 min. Aliquots of 250 and 500 µg/ml of reaction mixture was added to test tube having 1 ml of Griess reagent and incubated at room temperature for 30 min. Absorbance of the reaction mixture was measured at 546 nm. The percentage of nitric oxide radical scavenging was calculated using the formula (1).

2.2.7. Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity of curcumin/capsaicin/combined molecules was measured by deoxyribose method [20]. 0.2 ml of various concentration of curcumin/capsaicin/combined molecules was taken in different test tubes and 2.5 ml of 2-deoxyribose (3 mM), 200 µl of Ferric chloride (0.1 mM), 200 µl of EDTA (0.1 mM), 200 µl of ascorbic acid (0.1 mM) and 200 µl of H_2O_2 (2 mM) in phosphate buffer (pH 7.4, 20mM) was added. The mixture was kept in water bath at 37˚C for 30 min. 200 µl of chilled 15% trichloroacetic acid was added followed by the addition of 200 µl of 1% thiobarbituric acid in 0.25 N HCl. The mixture was incubated in boiling water bath for 30 min. and allowed to cool at room temperature. The absorbance was measured at 532 nm. The percentage of hydroxyl radical scavenging was calculated using the formula (1).

2.2.8. Metal chelating activity

Metal chelating activity of curcumin/capsaicin/combined molecules was measured according to the method of Dinis et al [21]. 3 ml of 5 µg/ml of curcumin/capsaicin/combined molecules were taken in different test tubes and 3 ml of EDTA, ferrous chloride (50 µl, 2mM) and ferrozine (20 µl, 5 mM) was added. Tubes were allowed to stand for 10 min. at room temperature. Absorbance of reaction mixture was measured at 562 nm with EDTA as standard. Percentage of metal chelating activity was calculated by using the formula (1).

2.3. BIOCHEMICAL ASSAYS

2.3.1. Lipid peroxidation inhibition assay

Thiobarbituric acid (TBA) reacts with malondialdehyde (MDA) to form a diadduct, a pink chromogen detected according to the method of Halliwell and Guttridge [20]. 10% of rat liver homogenate in 0.15 M potassium chloride was prepared. 0.5ml of liver homogenate and different concentration of 1 ml of curcumin/capsaicin/combined molecules was taken in different test tube. Lipid peroxidation was induced by adding ferrous sulfate (50µl, 0.07M) and incubated at room temperature for 30min. The reaction was stopped by adding chilled acetic acid (1.5 ml, 20%, pH 3.5) containing 20% TCA followed by the addition of TBA (50µl of 0.8% TBA in 1.1% SDS). The content was mixed thoroughly and incubated in boiling water bath for 60 min. After cooling, 5 ml of butanol was added and centrifuged at 3000 rpm for 10 min. Absorbance of the supernatant was measured at 532 nm. Percentage of inhibition was calculated using the formula (1).

2.3.2. Protective effect of curcumin and capsaicin on induced oxidative stress in erythrocytes

a. Preparation of erythrocytes

For erythrocyte isolation, 2 ml of human blood from healthy individual was collected

in heparinized tube using sterile syringe. Blood samples were centrifuged at 3000 rpm for 10 min at 4˚C, erythrocytes were separated from plasma and thicker buffy coat, washed 3-5 times by centrifugation at 3000 rpm (4˚C) for 5 min in 10 volumes of 10 mM phosphate buffer saline (pH 7.4). The supernatant and buffy coat of white cells was carefully removed with each wash. Washed erythrocytes were stored at 4˚C and used within 6 h for further studies. Erythrocytes were isolated and stored according to the method described by Yang et al.[22].

b. *In vitro* inhibition of erythrocyte hemolysis assay

The inhibition of H_2O_2 induced erythrocyte hemolysis by curcumin/capsaicin/combination was evaluated according to the method of Tedesco et al.[23] with slight modifications. 50 µl of curcumin/capsaicin/combined form at different concentration was added to a series of test tubes containing 100 µl of 5% (v/v) suspension of erythrocytes in phosphate buffer (PBS). To each tube 100 µl of 100 µM H_2O_2 (in 0.1M PBS pH 7.4) was added. Simultaneously, one negative control was maintained by adding erythrocytes suspension with 100 µl of 100 µM H_2O_2. The reaction mixture was gently shaken while being incubated at 37 ˚C for 3 h and diluted with 8 ml of PBS and centrifuged at 3000 rpm for 10 min. Absorbance of the supernatant was measured at 540 nm. The inhibitory effect of the molecules was compared with standard BHT. The percentage inhibition of erythrocyte hemolysis was calculated by using the formula (1).

C. Free iron release analysis

Free iron release upon oxidation was measured using ferrozine as described by Carter[24]. An inhibitory effect of curcumin/capsaicin/combination on free iron release upon Hb oxidation was estimated. 100 µl of hemoglobin solution (1%), 400 µl of phosphate buffer, 50 µl of curcumin/ capsaicin at different concentration (10 - 250µg), 100 µl each of H_2O_2 (10 mM) and ascorbic acid (100 mM) was taken in different test tube, mixed well and incubated at room temperature for 5 min. 50 µl of ammonium acetate (16%) and 50 µl of ferrozine (16 mM) were added. After 5 min of incubation at room temperature, the reaction mixture was centrifuged at 5000 rpm for 10 min at 4˚ C, and the absorbance was measured at 562 nm. The amount of free iron release was calculated by employing standard curve prepared by Mohr's salt. To assess possible Hb cleavage induced by curcumin/capsaicin/combined form, 100 µl of Hb alone was incubated with 250 µg of curcumin/capsaicin/combination and maintained as mentioned above.

d. Absorbance measurement

Absorbance measurement was done by using Ocean Optics DH 2000 UV-Vis spectrophotometer (USA) according to the method of Viollier et al.[25]. Reaction mixture was prepared in 1 ml of phosphate buffer mixed with Hb (0.63 µM), H_2O_2 (10M in phosphate buffer), O-dianisidine (0.02 M) and different concentration of curcumin/capsaicin/combination (10 - 60 µg). Reaction mixture was incubated for 10 min at 25˚ C. Absorbance spectrum was measured by scanning the reaction mixture from 200 to 800 nm.

2.3.3. Protective effect of curcumin and capsaicin on oxidative damage in hemoglobin

Protective effect of curcumin/capsaicin/combination on hydrogen peroxide free radical induced oxidative damage in hemoglobin was

assessed by free iron release analysis and absorbance measurement

2.4 pUC19 DNA NICKING ASSAY

DNA damage protective activity of curcumin/capsaicin/combination was determined by agarose gel electrophoresis using pUC19 DNA nicking assay[26]. 1 µg of pUC19 DNA with 5 µl of TAE, 10 µg of curcumin /capsaicin/combination was incubated for 30 min at room temperature with 5 µl of Fenton's reagent (30 mM H_2O_2, 50 mM ascorbic acid, 20 mM ferric chloride) as oxidizing agent. pUC19 was separated on 1% agarose gel with ethidium bromide at room temperature under constant voltage (50 V). Gel was documented and DNA damage protective activity was analyzed based on the mobility of DNA bands corresponding to control.

2.4.1. Preparation of carbon paste electrode

Carbon paste was prepared by grinding the 70% graphite powder and 30% silicon oil in an agate mortar by hand mixing for 30 min to get homogeneous bare carbon paste electrode. The paste was packed into a cavity of homemade carbon paste electrode and smoothened on weighing paper.

2.5 MTT ASSAY

Cytotoxicity of LPS on THP1 cells and its prevention by curcumin, capsaicin and combined molecules was examined using the ability of survived viable cells NAD(P)H-dependent cellular oxidoreductase enzymes to reduce the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide) to insoluble purple colored form formazan following the procedure as described earlier[27]. Briefly, the cells were aspirated into a 50ml centrifuge tube for centrifugation at 300 xg at 4 ˚C. Cell pellet obtained was suspended in RPMI medium containing 10% FBS (HiMedia, India) media and the cells count was adjusted such that 50µl of suspension contains approximately 10,000 cells. 50µl of the cell suspension was transferred into each well of the 96 well microtitre plate (Biolite, ThermoFisher Scientific). And incubated at 37 ^{o}C in 5% CO_2 atmosphere for 24 h. After 24 h, different concentrations of curcumin, capsaicin and combined curcumin and capsaicin were added to respective wells.

Spice principles treatment

Cells were pre-treated with curcumin (1, 5, 10 µM), capsaicin (1, 5, 10 µM) and their combination (Cur+Cap; 0.5+0.5, 1.0+1.0, 1.5+1.5 and 2.5+2.5 µM) in two groups for 1h and later 1 µg/ml LPS was added to all groups and incubated for 1h and 24h under optimum conditions. Together, normal control and negative control was maintained under parallel experimental conditions. After incubation period, 10µl MTT reagent (HiMedia, India) was added to each well to a final concentration of 0.5mg/ml, again plate was incubated at 37 ^{o}C in a 5% CO2 environment for 3 h. The culture medium was aspirated completely and carefully without disturbing the crystals. Then 100 µl of DMSO (HiMedia, India) was added and the plate was gently shaken in a gyratory shaker to solubilise the formazan. The absorbance was measured using a microplate reader at a wavelength of 570 nm and also at 630nm. The percentage growth inhibition was calculated, after subtracting the background and the blank, and concentration of test drug needed to inhibit cell growth by 50% (IC50) was generated from the dose-response curve for the cell line.

2.6. Statistical analysis

All the experiments were performed in triplicates and results are expressed as mean ± SEM. Statistical analysis was done by Graph pad prism 5.

3. RESULTS AND DISCUSSION

3.1. Evaluation of *in vitro* antioxidant activity by chemical methods

3.1.1. Total antioxidant and the total reductive capacity assay

The total antioxidant capacity of different concentration (20-160 µg/ml) of curcumin/capsaicin/combined form was expressed as equivalents of Ascorbic acid. Amongst, combined curcumin and capsaicin showed additive activity than individual curcumin and capsaicin (Fig. 1A).

The total reducing capacity of curcumin/capsaicin and their combined form (40, 80 & 160 µg/ml) was expressed as equivalents of standard BHT. The analysis has revealed that curcumin/capsaicin/their combined form have shown good total reducing capacity, however the combined form has shown higher reducing effect (Fig. 1B).

3.1.2. DPPH radical scavenging assay

DPPH radical scavenging property of curcumin/capsaicin/combined form was shown in commensurate with standard BHT in a concentration dependent manner (Fig. 2A). In this case, Keto form of curcumin can abstract a hydrogen atom from the highly activated carbon atom present between the two methoxyphenol rings, since phenolic hydrogen atoms are intramolecularly H-bonded to the adjacent methoxy groups making it difficult for abstraction of hydrogen atom from phenolic ring[28]. Whereas, absorption spectra of capsaicin revealed that the phenolic OH-group of capsaicin remained intact after reaction with DPPH radicals, indicating that the hydroxyl group is not associated with the radical scavenging reaction (hydrogen abstraction) and it was found to be C7-benzyl carbon group[29]. This mechanism revealing the scavenging of DPPH radical by curcumin (73.7±1.9; IC50-67.8) and capsaicin (57.8±1.66%; IC50-86.5) at 100 µg/ml respectively. This is in agreement with the earlier report of Tuba et al., (2008) whose research dwelt on concentration dependent scavenging of DPPH free radical by curcumin. While capsaicin also shown potential scavenging of DPPH radical [30]. Nevertheless, combined curcumin and capsaicin (87.8 ± 0.58; IC50-56.9) scavenging property (100 µg/ml) found to be more significant; as standard BHT shown (74.4 ± 0.67; IC50-67.2) at 100 µg/ml. Increased abstraction of electrons and hydrogen atoms of combined curcumin and capsaicin may be due to the activity of highly activated carbon atom present between the two methoxyphenol rings of curcumin and C7-benzyl carbon group of capsaicin.

3.1.3. ABTS radical scavenging assay

It has been reported that the curcumin [31] and capsaicin [32] can exhibit ABTS scavenging activity. The ABTS radical scavenging capacity of curcumin/capsaicin/combined form was increased consistently with the increase in the concentration is shown in Table. 1. ABTS radicals are more reactive than DPPH radicals and involve an electron-transfer process. The scavenging of $ABTS^{\bullet+}$ by these molecules and standard BHT is in the order of; combined form (IC50-5.096)>capsaicin (IC50-5.202)>BHT (IC50-5.213)>curcumin (IC50-5.813) (98.1±0.4, 96.1±0.17, 95.9±0.3 and 86.01±0.66%) respectively at 10 µg/ml. Overall, combined form ABTS scavenging property was indeed more evident and it is additive.

3.1.4. Superoxide radical scavenging assay

Major production of superoxide radical occurs within the mitochondria during electron transport state. Superoxide radicals

thus formed can further interact with other molecules to generate secondary ROS and contribute to redox imbalance and amplify pathophysiology of a variety of diseases [33].

The superoxide radicals were efficiently scavenged by capsaicin/curcumin/their combined form including standard ascorbic acid. The percentage scavenging of superoxide radical by capsaicin (IC50-2.590) and curcumin (IC50-2.983) at 5µg/ml were 96.5±0.40 & 83.8±0.48 respectively is shown in Table 1. These results are in conformity with the earlier report of Ki Hyeon et al.,[30] who had shown 66% scavenging activity of capsaicin, while Tuba et al.,[28] reported efficient superoxide radical scavenging activity of curcumin. Combined curcumin and capsaicin (IC50-3.001) and standard ascorbic acid (IC50-3.048) had shown 83.3±0.43, and 82.0±0.31% of scavenging respectively. The combined curcumin and capsaicin has shown additive property.

3.1.5. Nitric oxide radical scavenging assay

Nitric oxide role is well known in various inflammatory processes, as sustained higher level of NO radical are toxic to cells and tissues leading to vascular collapse associated with septic shock, whereas in chronic expression it causes various inflammatory conditions and carcinomas[34]. Curcumin [35] and capsaicin are found to have possessed good nitric oxide scavenging property. Curcumin/capsaicin/combined curcumin & capsaicin and standard BHT have all shown dose dependent inhibition of nitric oxide formation as results are shown in Fig. 2(B). Nitric oxide radical scavenging activity of curcumin and capsaicin at 500 µg/ml is found to be 84.8±0.31 & 56.6±0.7% respectively, while combined curcumin and capsaicin has shown 88.0±0.01% (500 µg/ml) scavenging activity. Although, curcumin, capsaicin, combined curcumin & capsaicin and BHT (44.5±1.32% at 500 µg/ml) have all shown nitric oxide scavenging property, it is observed that the nitric oxide scavenging by combined curcumin and capsaicin is found to be higher.

3.1.6. Hydroxyl radical scavenging assay

The interaction of free iron ions with hydrogen peroxide in a biological system can lead to formation of a highly reactive tissue damaging species viz, hydroxyl radicals [36]. Scavenging of these hydroxyl radicals by the antioxidant molecule can prevent deleterious effect to biomolecules. Antioxidant spice principles curcumin [37] and capsaicin [38] are reported to exhibit hydroxyl radical scavenging activity. In the present study, hydroxyl radical scavenging capacity of curcumin/capsaicin/combined curcumin & capsaicin were compared with standard BHT (Table 1.). At 6 µg/ml the percentage scavenging of hydroxyl radicals by curcumin/capsaicin/combined curcumin & capsaicin and standard BHT were found to be 71.7±0.73, 70.8±0.53, 73.1±0.9 and 67.8±1.46 respectively. Results indicated that curcumin (IC50-4.184)/capsaicin (IC50-4.327)/combined curcumin & capsaicin (IC50-4.103) and standard BHT (IC50-4.424) have all shown hydroxyl scavenging property. However, combined curcumin and capsaicin was more efficient scavenger of hydroxyl radicals indicating the potential contribution of both molecules together in the scavenging process.

3.1.7. Metal chelating activity

There are some reports clearly establish metal chelating property of curcumin [39] and capsaicin. The results show that capsaicin, curcumin, combined curcumin & capsaicin and standard EDTA have all shown the ferrous ion chelating property. Metal chelating activity of curcumin,

capsaicin and their combined form at 5 µg/ml results are presented in Table 1. Capsaicin exhibited higher ferrous ion chelating capacity (29.2±0.18 %) than curcumin, combined curcumin & capsaicin and standard EDTA (25.1±1.65, 24.1±1.72 and 23.0±3.95%) respectively. It is to be drawn to a conclusion that combined form still demonstrated higher metal chelating efficacy.

3.2. EVALUATION OF *IN VITRO* ANTIOXIDANT ACTIVITY BY BIOCHEMICAL ASSAYS

3.2.1. Lipid peroxidation inhibition assay

Dietary supplementation of spices contributes to increasing the antioxidant potential of organelle membranes of the liver [40] contributing to better inhibition of lipid peroxidation than the chemical antioxidants. The inhibition of lipid peroxidation by curcumin/capsaicin/ combined curcumin and capsaicin was performed using liver homogenate, in which the individual and combined curcumin and capsaicin have inhibited the lipid peroxidation by reducing the formation of MDA in relatively concentration dependent manner (Table 1).

Reportedly, capsaicin capable of inhibiting the formation of both active oxygen species and the radical chain reaction on the membrane surface and in the interior of membranes indicating potential anti-peroxidation activity[29], while curcumin as a potent lipid soluble molecule can integrate itself within the cell membrane for intercepting lipid radicals and converts to phenoxyl radical. Being more polar than curcumin, the phenoxyl radical can move to the surface of the membrane and repaired by any water-soluble antioxidants [41]. Inhibition of lipid peroxidation by capsaicin and curcumin is found to be 97.3±0.01 and 86.3±0.64% (100µg/ml) respectively, while combined curcumin & capsaicin has shown 98.3±0.19% inhibition. Related to this study, Manjunatha and Srinivasan have reported reduced liver lipid peroxides *in vivo* by curcumin, capsaicin and their combined form plausibly combined form has shown higher potency[11]. The results of our study on curcumin, capsaicin and their combined form exhibited inhibition of lipid peroxidation; nevertheless combined form has shown additive activity.

3.2.2. Inhibition of erythrocyte hemolysis assay

Erythrocytes are more convenient and reliable biological sample models to understand the oxidative damage induced by various pro-oxidants due to their finite life span, simplicity, easy accessibility and as one of the prime cells to be affected by the adverse conditions due to the presence of lipid membrane, oxygen and hemoglobin. RBCs have a plasma membrane rich in polyunsaturated fatty acids (PUFA) and the cytoskeleton part is composed of several proteins including spectrin, ankyrin, actin and protein considered crucial for the maintenance of cells rigidity and shape [42]. Even minimal changes in the surface area may lead to morphological and functional abnormalities.

The treatment of human erythrocytes with H_2O_2 led to100% hemolysis due to induced oxidative stress. However, they are found to be stable in presence of curcumin/capsaicin/ combined form. Combined curcumin and capsaicin has shown higher inhibition of erythrocyte hemolysis than curcumin/capsaicin/standard BHT (Fig. 3). The percentage (50 µg/ml) inhibition of erythrocyte hemolysis is found to be in the order of: combined curcumin & capsaicin>standard BHT>curcumin>capsaicin (87.2±0.19,

82.5±0.40, 77.0±0.70 and 72.9±0.25) respectively.

3.2.3. Inhibition of oxidative damage to haemoglobin

Hydrogen peroxide induced oxidative damage to hemoglobin is associated with the formation of Methemoglobin (MetHb) with higher oxidation state molecules such as ferryl hemoglobin (Hb-Fe^{IV}=O) and oxoferryl hemoglobin (Hb*-Fe^{1V}= O) respectively[43,44]. These higher oxidative products of hemoglobin are considered as prime sources for cellular and tissue damage.

Antioxidant molecules were considered as powerful scavengers of hydrogen peroxide. Scavenging power of individual and combined curcumin & capsaicin increases with increasing concentration (Fig. 4A), thereby decreasing the amount of free iron release from Hb. Curcumin/capsaicin/combined curcumin & capsaicin have all shown protective effect against hydrogen peroxide induced oxidative stress hemoglobin. However, combined curcumin & capsaicin has shown higher protective effect. Further, the protective effect of individual and combined curcumin & capsaicin against H_2O_2 induced oxidative damage to Hb was analyzed using UV-Visible spectra from 200 to 800 nm by keeping constant Hb-H_2O_2-ODA (*o*-dianisidine) complex and varying concentration of individual, combined curcumin & capsaicin (10-60µg). The absorbance increased with increasing concentration of curcumin/capsaicin/combined curcumin & capsaicin indicating the inhibition of free iron release by preventing heme degradation of Hb. From UV-Vis absorption spectra (Fig. 4.B, C and D), it is observed that individual/combined curcumin & capsaicin have shown concentration dependent protection against H_2O_2 induced Hb lysis by suppressing the formation of new reactive products. Overall, the combined curcumin & capsaicin has shown synergistic (2 fold) effect against H_2O_2 induced oxidation to hemoglobin.

3.2.4. DNA protection activity

Higher free radicals produced in cells due to imbalance in oxidant – antioxidant status induced DNA damage, dilapidated to neurological diseases, mutagenesis, carcinogenesis and ageing[45,46]. Wattenberg reported that flavonoids could inhibit the carcinogenesis and tumor promotional events by acting as blocking agents by preventing the production of reactive intermediates formation as well as by activating the detoxification enzymes for neutralizing carcinogens, thereby preventing interaction with DNA[47]. DNA protective activity of curcumin/capsaicin/combined form was evaluated against hydroxyl radical induced pUC19 DNA damage *in vitro*. Hydroxyl radicals generated by Fenton's reaction induced DNA damage seen in the form of smear in lane 2 (Fig. 5). The individual and combined curcumin & capsaicin effectively mitigated the DNA damage induced by hydroxyl radicals. Combined curcumin & capsaicin (lane 5) shown higher efficacy in scavenging OH radicals, protecting the DNA. These results are in conformity with the previous reports of Borra et al [35], where they have reported protective activity of curcumin against hydroxyl radicals induced DNA damage. Similarly ESR spin trapping technique by Kinetic competition studies revealed the protective activity of capsaicin against Fenton reagent induced DNA damage [38]. The present data indicate more beneficial influence of combined curcumin and capsaicin in protecting the hydroxyl radicals induced DNA damage.

3.3 MTT ASSAY

Curcumin treatment (1, 5 & 10 μM) for 24h decreased cell viability. Curcumin treatment (1 & 5μM) for 1 h showed some protective activity against LPS induced toxicity compared to cells treated with LPS (1μg/ml) alone. Curcumin treatment alone at 10 μM for 24h is found to be toxic to the cells and hence could not protect the cells against LPS (Fig 8).While, capsaicin treatment (1, 5 & 10μM) for 24h decreased cell viability. Capsaicin treatment (1, 5 & 10 μM) for 1 h showed some protective activity against LPS as compared to cells treated with LPS (1μg/ml) alone (Fig. 9).

Pre-treatment with Curcumin for 1h and then with Capsaicin for another 1h before treatment with LPS (1μg/ml) for 24h showed better protective activity of combined molecules than pre-treatment with curcumin/capsaicin (Fig. 10). Further, we obtained similar results about curcumin/capsaicin/combined form on LPS induced over expression of TNF receptor associated factor 6, COX-2, IL-6 and TGF-βin human peripheral blood mononuclear cells *in vitro*[48,49] as well as in LPS induced hepatotoxicity in mice[50] in which combined molecules have shown higher efficacy.

4. CONCLUSION

The chemical and biochemical assays (except hemoglobin assay) have revealed additive effect of combined curcumin & capsaicin. However, hemoglobin assay has shown synergistic effect of combined curcumin and capsaicin. Further, combined molecules are more protective to LPS induced toxicity in THP1 cell lines. The voltammetric study demonstrated higher binding property of combined curcumin & capsaicin with BSA than individuals. Re-emphasizing the potential applications of combined nutraceutical molecules in food fortification.

ACKNOWLEDGEMENT

This work was supported by a grant from Department of Science and Technology - Science and Engineering Research Board (DST-SERB) (SB/EMEQ-343/2013), Govt. of India, New Delhi. First author (Thriven V) and guide (H. Manjunatha) express sincere thanks to DST-SERB for funding under the program “Empowerment and Equity Opportunities for Excellence in Science”.

Table. 1. In vitro antioxidant activity of individual and combined curcumin and capsaicin

Sl no	Activity	Concentrations of spice principles curcumin (Cur) and capsaicin (Cap) in µg		Percentage of inhibition	
		Cur	Cap	Cur	Cap
1	ABTS radical scavenging assay	5	5	69.8 ± 0.18	79.8 ± 0.16
		10	10	86.01 ± 0.66	96.1 ± 0.17
2	Superoxide radical scavenging assay	5	5	83.8± 0.48	96.5 ± 0.40
3	Hydroxyl radical scavenging assay	2	2	61.1 ± 3.32	70. 1 ± 0.03
		6	6	71.7 ± 0.73	70.8± 0.53
4	Metal chelating assay	5	5	25.1 ± 1.65	24.1 ± 1.72
5	Lipid Peroxidation inhibition assay	25	25	19.4 ± 0.47	30.7 ± 1.6
		50	50	78.6 ± 0.58	87.3 ± .13
		100	100	86.3± 0.64	97.3± 0.01

Table 2-1: ABTS, superoxide, hydroxyl radical scavenging activity; metal chelating activity and lipid peroxidation inhibition activity of curcumin, capsaicin and their combination

Sl.no	Scavenging Activity	Concentration (µg)			Percentage of inhibition		
		Cur	Cap	Cur + Cap	Cur	Cap	Cur + Cap
1	ABTS radical scavenging assay	5 10	5 10	2.25 + 2.25 5 + 5	79.8 ± 0.16 96.1 ± 0.17	79.8 ±0.16 96.1 ± 0.17	86.7 ± 0.43 98.1 ± 0.4
2	Superoxide radial	5	5	2.5 + 2.5	83.8 ± 0.48	96.5 ± 0.40	83.3 ± 0.43
3	Hydroxyl radical scavenging assay	2 6	2 6	1+1 3+1	61.1 ±3.32 71.7 ±0.73	70.1 ± 0.03 70.8 ± 0.53	65.1 ±0.2 73.1 ±0.9
4	Metal chelating assay scavenging assay	5 25	5 25	2.5+2.5 12.5+12.5	25.1 ±1.65 19.4 ± 0.47	24.1 ±1.72 30.7 ± 1.6	29.2 ±0.18 44.3 ±0.76
5	Lipid peroxidation scavenging assay	50 100	50 100	25+25 50+50	78.6 ±0.58 86.3 ±0.64	87.3 ±0.13 97.3 ±0.01	89.4 ±0.96 98.3 ±0.19

The results shown are averages of three independent experiments, values are expressed as mean ± SEM. Cur: curcumin, Cap: capsaicin, Cur + Cap: curcumin + capsaicin

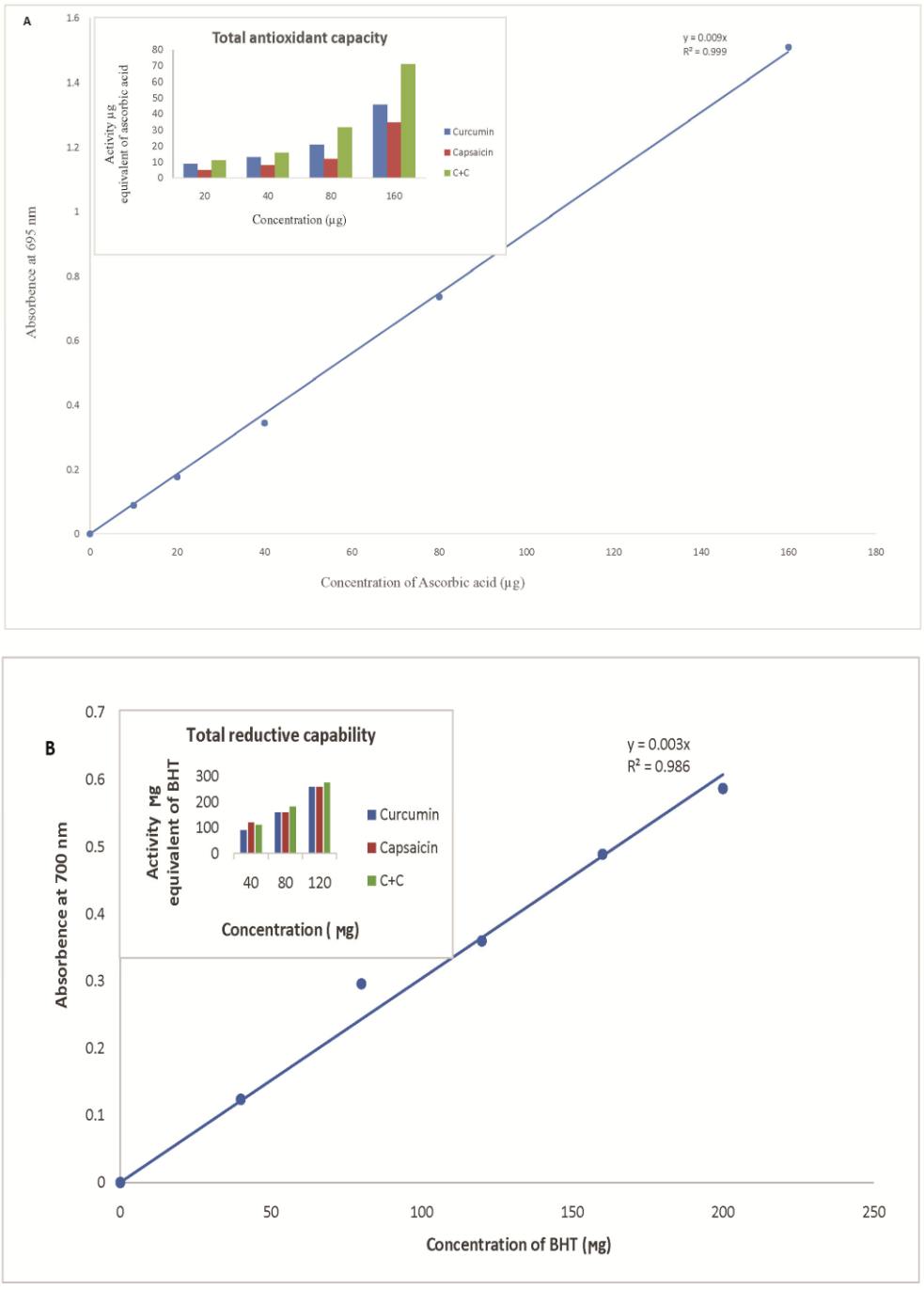


Figure 1: Plots of (A) total antioxidant activity and (B) total reductive capability of curcumin, capsaicin and their combination. The results are averages of three independent experiments

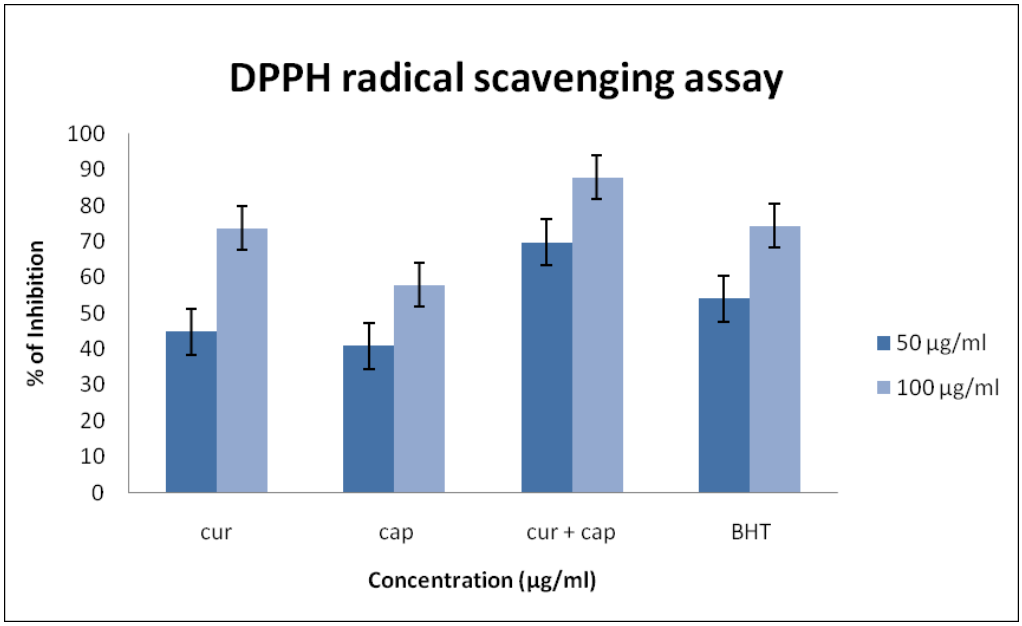


Figure 2: DPPH radical scavenging activity of curcumin/capsaicin and their Combination

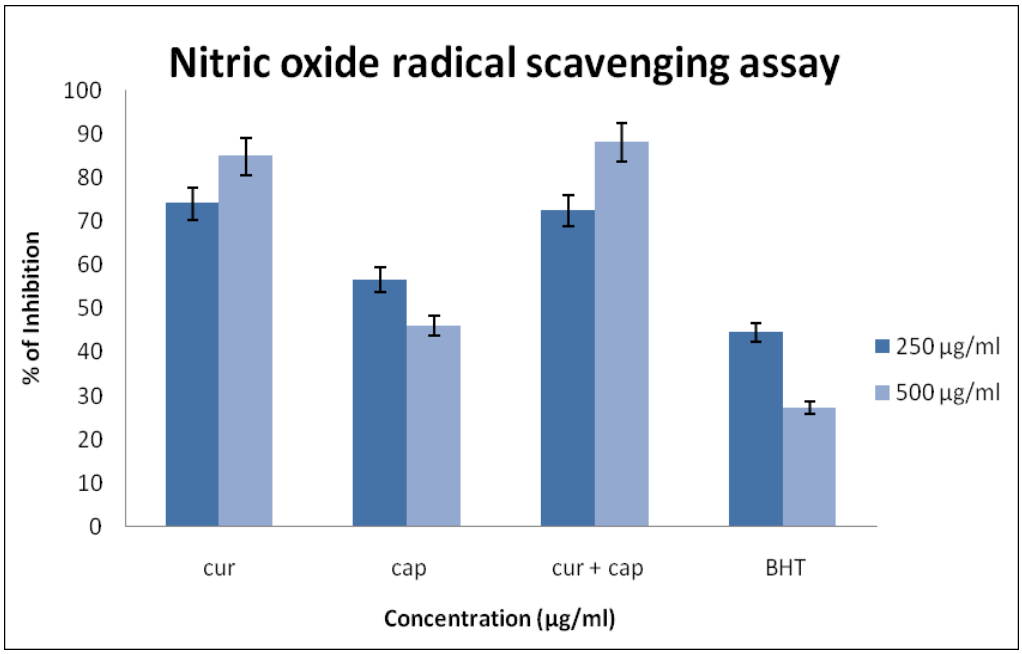


Figure 3: Nitric oxide radical scavenging activity of curcumin, capsaicin and their combination

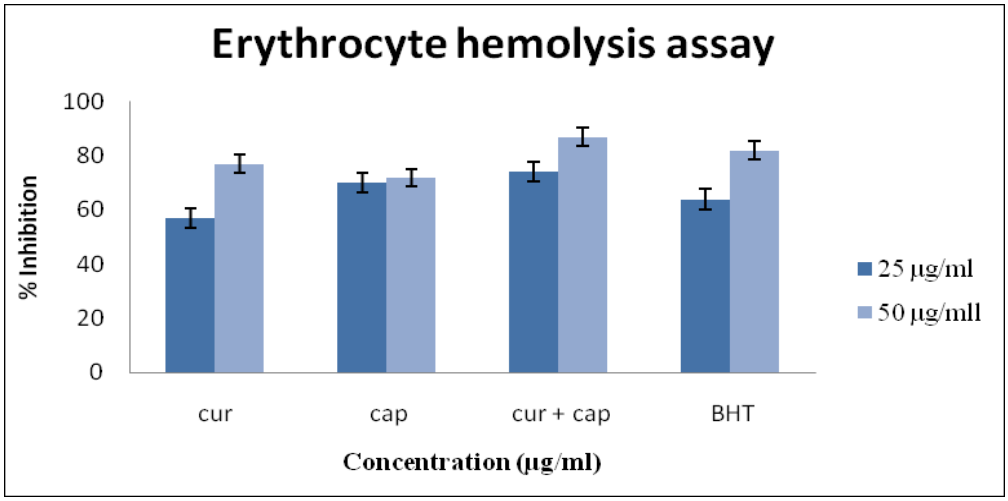
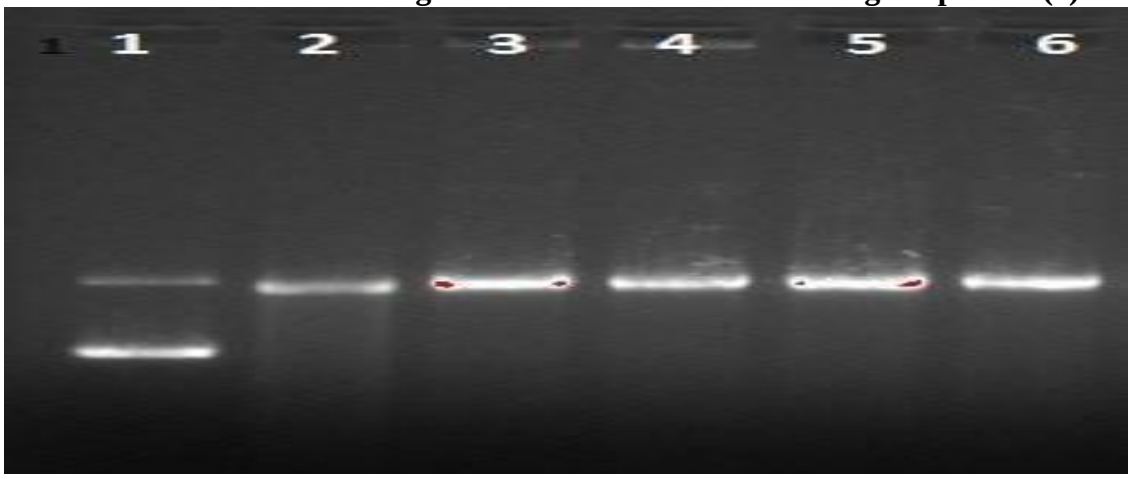


Figure 4: In vitro protective effects curcumin, capsaicin and their combination against H2O2 induced oxidative stress in human erythrocytes

Figure 5: Agarose gel electrophoresis showing DNA protective activity of curcumin, capsaicin and their combination against H2O2 induced DNA damage of pUC19.(1)



1 µg of native pUC 19, (2) DNA + 5 µl of Fenton's reagent, (3) DNA + 5 µl of Fenton's reagent + 10 µg of Curcumin, (4) DNA + 5 µl of Fenton's reagent + 10 µg of Capsaicin, (5) DNA + 5 µl of Fenton's reagent + 5 µg of Curcumin + 5 µg of Capsaicin, (6) DNA + 5 µl of Fenton's reagent + 10 µg of Quercetin.

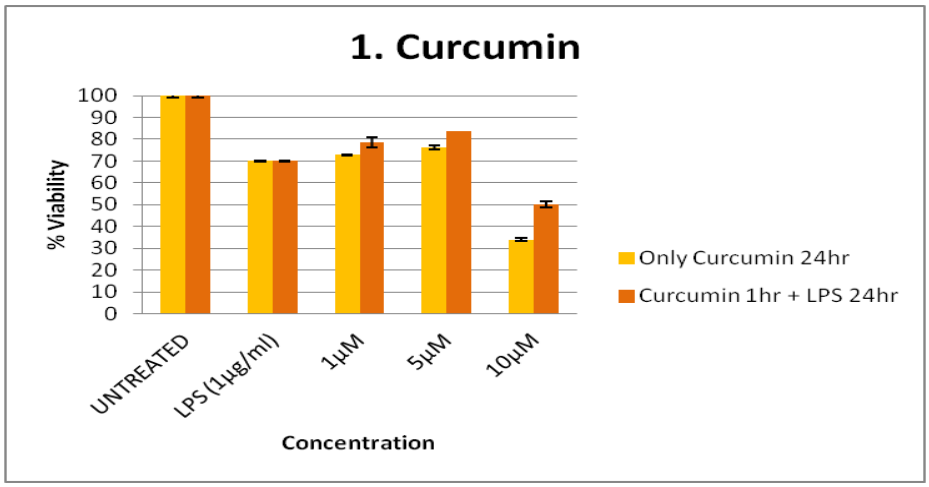


Fig.6 Effect of curcumin on LPS induced toxicity in THP1 cell lines

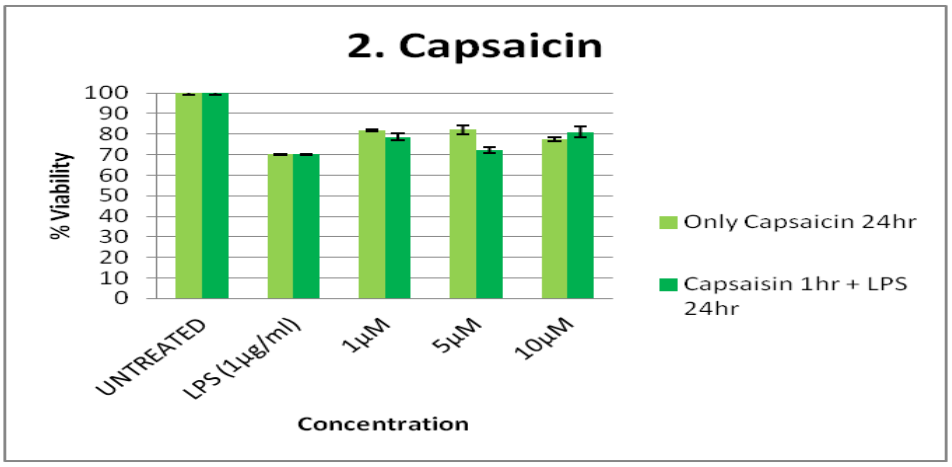


Fig.7 Effect of capsaicin on LPS induced toxicity in THP1 cell lines

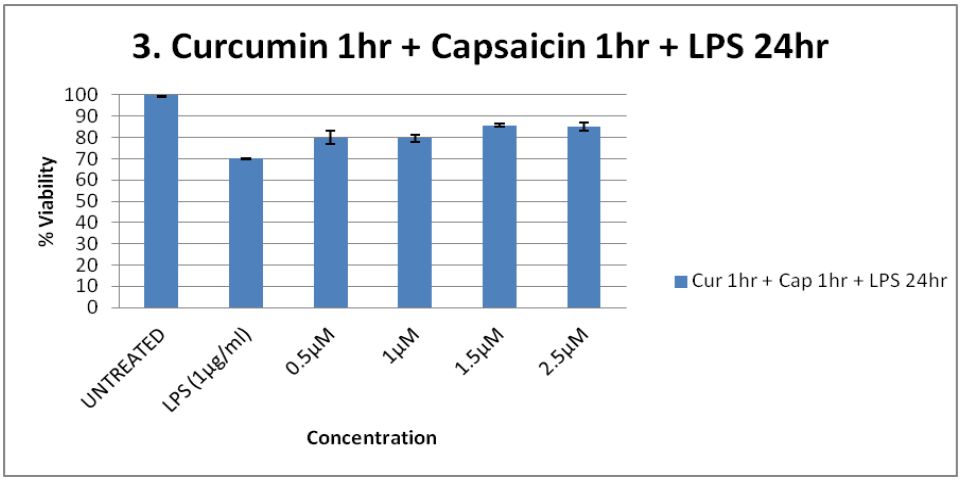


Fig.8 Effect of curcumin and capsaicin at various micromolar concentrations (Cur+Cap - 0.5+0.5; 1.0+1.0; 1.5+1.5; 2.5+2.5) on LPS induced toxicity in THP1 cell lines.

REFERENCES

1. Agnihotri N, Mishra PC. (2011) Scavenging mechanism of curcumin toward the hydroxyl radical: a theoretical study of reactions producing ferulic acid and vanillin. J Phys Chem A. 15; 115(49):14221-32.
2. Ak Tuba, Gulçin I.(2008) Antioxidant and radical scavenging properties of curcumin.Chem Biol Interact.10;174(1):27-37.
3. Anand BG, Dubey K, Shekhawat DS, Kar K. (2016) Capsaicin-coated silver nanoparticles inhibited amyloid fibril formation of serum albumin. Biochemistry 55:3345-48.
4. Anderson D, Phillips BJ (1999) Comparative *in vitro* and *in vivo* effects of antioxidants. Food Chemical Toxicology 37: 1015-1025.
5. Asai A, Nakagawa K, Miyazawa T. (1999) Antioxidative effects of turmeric, rosemary and capsicum extracts on membrane phospholipid peroxidation and liver lipid metabolism in mice. Biosci Biotechnol Biochem. 63(12):2118-22.
6. Bharath BR, Thriveni V, Manjunatha H & Bharath C (2017) Interaction of curcumin and capsaicin with LPS induced TRAF6 expression in peripheral blood mononuclear cells. Medicinal Chemistry Research 26, 2399–2409
7. Bourassa P, Kanakis CD, Tarantilis P, Pollissiou MG, Tajmir Riahi HA (2010) Resveratrol, Genistein, and Curcumin bind Bovine serum albumin. J. Phys. Chem. B 114: 3348-54.
8. Braca A, Tommasi N D, Bari LD, Pizza C, Politi M, Morelli I (2001) Antioxidant principles from *Bauhinia terapotensis*. Journal of Natural Products 64: 892–895.
9. Brieger K, Schiavone S, Miller FJ Jr, Krause KH. (2012) Reactive oxygen species: from health to disease. Swiss Med Wkly. 17; 142:13659.
10. Carter P (1971) Spectrophotometric determination of serum iron at the submicrogram level with a new reagent (ferrozine). Analytical Biochemistry 40: 450–458.
11. Cooper CE, Green ES, Rice-Evans CA, Davies MJ, Wrigglesworth JM. (1994) A hydrogen-donating monohydroxamate scavenges ferryl myoglobin radicals. Free Radic Res. 20(4):219-27.
12. Dinis TC, Madeira VM, Almeida LM (1994) Action of phenolic derivatives (acetaminophen, salicylate, and 5-amino salicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. Archives of Biochemistry and Biophysics 315 (1): 161–169.
13. Eman M, Alissa, Gordon A Ferns (2012) Functional Foods and Nutraceuticals in the Primary Prevention of Cardiovascular Diseases.Journal of Nutrition and Metabolism 1-16
14. Halliwell B, Dizdaroglu M (1992) The measurement of oxidative damage to DNA by HPLC and GC/MS techniques. Free Radical Research Communications 16: 75-87.
15. Halliwell B, Gutteridge JMC, Aruoma OI (1987) The deoxyribose method: A simple test tube assay for determination of rate constants for reactions of hydroxyl radicals.

Analytical Biochemistry 165: 215-219.

16. Jovanovic SV, Steenken S, Boone CW, et al., (1999) H-atom transfer is a preferred antioxidant mechanism of curcumin, J. Am. Chem. Soc. 121, 9677–9681.
17. Kannappan R, Gupta SC, Kim JH, Reuter S, Aggarwal BB. (2011) Neuroprotection by Spice-Derived Nutraceuticals: You Are What You Eat. Molecular Neurobiology 44:142-159
18. Ki Hyeon Sim, Ki Hyeon Sim, Han Young Sil (2008) Antioxidant activities of red pepper (Capsicum annuum) pericarp and seed extracts. International Journal of Food Science & Technology 43(10):1813 – 1823.
19. Kogure K, Goto S, Nishimura M, Yasumoto M, Abe K, Ohiwa C, Sassa H, Kusumi T, Terada H. (2002) Mechanism of potent antiperoxidative effect of capsaicin. Biochim Biophys Acta. 1573(1):84-92.
20. Kovacic P, Pozos R, Somanathan R, Shangari N, O Brien P (2005) Mechanism of mitochondrial uncouplers, inhibitors, and toxins: Focus on electron transfer, free radicals, and structure–activity relationships. Current Medicinal Chemistry 12: 2601-2623.
21. Lavorgna M, Orlo E, Nugnes R, et al. (2019) Capsaicin in Hot Chili Peppers: In Vitro Evaluation of Its Antiradical, Antiproliferative and Apoptotic Activities. Plant Foods for Human Nutrition. 74(2):164-170.
22. Lee JC, Kim HR, Kim J, Jang YS (2002) Antioxidant property of an ethanol extract of the stem of Opuntia ficus-indica var. saboten," Journal of Agricultural and Food Chemistry, V50(22), 6490–6496.
23. Manjunatha H, Srinivasan K (2006) Protective effect of dietary curcumin and capsaicin on induced oxidation of low-density lipoprotein, iron-induced hepatotoxicity and carrageenan-induced inflammation in experimental rats. FEBS Journal 273:4528-4537.
24. Manjunatha H, Srinivasan K (2007) Hypolipidemic and antioxidant effects of dietary curcumin and capsaicin in induced hypercholesterolemic rats. Lipids 42:11333-1142.
25. Marcocci L, Packer L, Droy-Lefaix MT, Sekaki A, Gardès-Albert M. (1994) Antioxidant action of Ginkgo biloba extract EGb 761. Methods Enzymol. 234:462-75.
26. Marx JL (1987) Oxygen free radicals linked to many diseases. Science journal 235:529-31.
27. Mary CPV, Vijayakumar S, Shankar R. (2018) Metal chelating ability and antioxidant properties of Curcumin-metal complexes - A DFT approach. J Mol Graph Model. 2018 Jan; 79:1-14.
28. Maslarova NVY (2001) Sources of natural antioxidants: vegetables, herbs, spices and teas (ed) Antioxidants in food. Practical applications. CRC Press, Washington, DC.
29. McCord JM, Day ED (1978) Superoxide dependent production of hydroxyl radical catalyzed by iron-EDTA complex. FEBS Lett 86:139-142.
30. Miral D, Pawel J, Birincioqlu M, Henry R (2002) Free radical induced damage to DNA: mechanisms and measurement. Free radical biology and medicine. 1102-15.

31. Mosmann T (1983) Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. Journal of lmmunological Methods 65:55-63.
32. Nagababu E, Rifkind JM (1998) Formation of fluorescent hemedegradation products during the oxidation of haemoglbin by hydrogen peroxide. Biochemical and Biophysical Research Communications 247:592−596.
33. Nishikimi M, Appaji N, Yagi K. (1972) The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. Biochem Biophys Res Commun. 31;46(2):849-54.
34. Okada Y, Okajima H, Shima Y, Ohta H (2002) Hydroxyl radical scavenging action of capsaicin. Redox Rep. 7(3):153-7.
35. Oyaizu M (1986) Studies on product of browning reaction prepared from glucose amine. Japanese Journal of Nutrition 44:307-15.
36. Panter SS, Sadrzadeh SMH, Hallaway PE, Haines JL, Anderson VE, Eaton JW (1985) Hypohaptoglobinemia associated with familial epilepsy. J. Exp. Med 161:748-754.
37. Prieto P, Pineda M, Aguilar M (1999) Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Analytical Biochemistry 269:337-341.
38. Puppo A, Halliwell B (1988) Formation of hydroxyl radicals from hydrogen peroxide in the presence of iron. Is haemoglbin a biological Fenton catalyst?. The Biochemical Journal 249: 185-190.
39. Rajesh KP, Manjunatha H, Krishna V, Kumara swamy BE (2013) Potential *in vitro* antioxidant and protective effects of *Mesua ferrea* Linn. bark extracts on induced oxidative damage. Industrial Crops and Products 47:186-198.
40. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine 26:1231-1237.
41. Rizvi SI, Maurya PK (2007) Alterations in antioxidant enzymes during aging in humans. Molecular Biotechnology 37:58-61.
42. Sai Krishna Borra et al (2013) Antioxidant and free radical scavenging activity of curcumin determined by using different in vitro and ex vivo models. Vol. 7(36), 2680-2690.
43. Saleh A. Mohamed, Reda M. El-Shishtawy,Omar A.M. Al-Bar &Ahmed R. Al-Najada (2017) Chemical modification of curcumin: Solubility and antioxidant capacity. International Journal food properties V20
44. Taylor BS, Kion YM, Wang QI, Sharpio RA, Billiar TR, Geller DA (1997) Nitric oxide down regulates hepatocyte-inducible nitric oxide synthase gene expression. Archives of Surgery 132, 1177-1183.
45. Tedesco I, Russo M, Russo P, Lacomino GG, Russo G L, Carrasturo A, Faruolo C, Mojo L, Palumbo R (2000) Antioxidant effect of red wine polyphenols on red blood cells. *Journal of* Nutritional Biochemistry 11, 114-119.

46. Thriveni V, Manjunatha H, Lakshminarayana R (2019) Curcumin and capsaicin modulates LPS induced expression of COX-2, IL-6 and TGF-β in human peripheral blood mononuclear cells. Cytotechnology 71, 963–976.
47. Thriveni V, Manjunatha H, Prabhakar BT, Santhosh KH (2017) Hepatoprotective effect of curcumin and capsaicin against lipopolysaccharide induced liver damage in mice. Pharmacogn J. 9(6), 947-951.
Aleksandra S, Dragan M, Dejan S, Marija T (2007) Electrochemical behavior and antioxidant and prooxidant activity of natural phenolics. Molecules 12: 2327-2340.
48. Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M (2006) Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chemico-Biological Interaction 160:1-40.
49. Viollier E, Inglett P W, Hunter K, Roychoudhury A N, Van cappellen P (2000) The ferrozine method revisited: Fe (II)/Fe (III) determination in natural waters. Applied geochemistry 15:785-790.
50. Wattenberg LW (1985) Chemoprevention of cancer. Cancer Research 45:1-8.
51. Yang HL, Chen SC, Chang NW, Chang JM, Lee ML, Tasi PE, Fu HH, Kao WW, Chiang HC, Wang HH, Hseu YC. 2006. Protection from oxidative damage using Bidens pilosa extracts in normal human erythrocytes. Food Chem. Toxicol. 44, 1513-1521
52. Yerra R, Senthil G P, Gupta M, Mazumdar U K, (2005) Studies on *in vitro* antioxidant activities of methanol extract of *Mucuna pruriens* (Fabaceae) seeds. European Bulletin of Drug Research 13:31.