

Evaluation of the antiradical and antioxidant properties of extracts from Indian red chili and black pepper by *in vitro* models

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Received July 31, 2009

Oxygen free radicals and lipid peroxides play an important role in increasing the risk factors for many chronic diseases and are also responsible for the deterioration of food products. The objective of this investigation was to evaluate the potentials of some extracts from chili and pepper to scavenge free radicals *in vitro* model systems such as: 1,1-diphenyl-2-picryl-hydrazyl (DPPH), N,N-dimethyl-*p*-phenylenediamine dihydrochloride (DMPD), 2,2-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid (ABTS), ferric cyanide reducing antioxidant power (FRAP), cuprum total antioxidant capacity (CUPRAC) assays and to exhibit antioxidant activities on the lipid peroxidation in rat liver homogenate. Alcohol extracts and oleoresins of chili and pepper showed significant radical scavenging activities and antioxidant potential in all the systems tested. These results can be exploited in preserving processed foods and at the same time get the benefits of medicinal properties of these spices.

Key words: Chili, pepper, oleoresin, radical scavengers, antioxidants, *in vitro* model systems

INTRODUCTION

Antioxidants can protect the human body from damages caused by free radicals mediated lipid peroxidation and deleterious effects of reactive oxygen species. They retard the progress of many chronic diseases [1]. Antioxidant compounds can scavenge free radicals and increase shelf life of food products by retarding the process of lipid peroxidation, which is one of the major reasons for deterioration of food products [2]. Normally synthetic antioxidants such as butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT) are used for preservation of processed foods. But indiscriminate use of these synthetic antioxidants can lead to tumor formation and liver damage as has been shown in experimental animals. Hence, a need for identifying alternative and safe source of antioxidants for use in food products has been advocated and the search for natural antioxidants especially of plant origin has notably increased in recent years [3].

Spices are dietary constituents used to enhance the flavor and taste of food. Many of them have been identified to possess potential chemo preventive action. Chili is one of the important spices used in varieties of Indian dishes for imparting color, flavor and pungency to the food. The traditional use of whole ground chilies in food and beverage indus-

tries is now very limited. Now a days, the use of value added chili products such as oleoresin, essential oil, capsaicin, capsanthin have gained importance in the global market as compared to use of whole or ground chili. The export of these products is increasing considerably every year from India.

Chilies contain Vitamin A, Vitamin C and capsaicin which are good antioxidants and antiinflammatory agents, which can also boost immune system. Antioxidants present in the chili are good free radical scavengers [4]. Chilies also act as detoxifier by removing the waste products from our body and increase the blood supply to the tissues. Chilies stimulate the release of endorphins that are natural pain killers. It has been noted that the Vitamin C, β -carotene and folic acid found in chili reduces the risk of colon cancer [5]. Vitamin A present in chili reduces the inflammation of lungs and emphysema resulting from cigarette smoking. Chilies enhance blood flow to the site of infection which helps in fighting infection.

Pepper is an important spice appreciated for both its aroma and pungency. It is valued for its distinct biting quality which is attributed to piperine and its isomers. Black pepper is used not only in diets as an adjunct but also for medicinal purposes and as a preservative. The pepper extract was also shown to enhance the bioavailability of a number of therapeutic drugs as well as phytochemicals [6].

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It is reported that black pepper has digestive stimulating activity. It improves appetite, cures cold, cough, diseases of the throat, intermittent fever, colic dysentery, worms and piles. As with many spices, it also possesses a broad spectrum of antimicrobial activity, analgesic, antipyretic and anti-inflammatory actions. It enhances the secretion of saliva and the activity of salivary amylase. The digestive stimulant action of spices is probably mediated through stimulating the liver to produce and secrete bile acids, which play a very important role in fat digestion and absorption [6, 7].

The pepper oleoresin containing the essential oil contributes to aroma. The oleoresins have many advantages such as convenience of handling and are free from microbial contamination. It also provides convenience for the preparation of processed foods. Unlike the essential oils, oleoresin of spices contains many natural antioxidants which make them more stable.

Chipault *et al.* [8] made a systematic study on antioxidant activity of some common spices and herbs. Since then a large number of studies examined the effect of plant extracts on lipid oxidative stability in order to find new sources of natural antioxidants [9]. India is one of the largest producers of spices in the world. Though they are mainly utilized for food, herbal medicine and flavorings, chili and pepper extracts can also be used as a food preservative (especially for preventing rancidity development in oils) [10]. With this background, the extracts of chili, pepper were studied for their antiradical and antioxidant activities and the results are reported in this paper.

MATERIALS AND METHODS

Chemicals

Tertiary butylated hydroxyquinone (TBHQ), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), gallic acid, N,N-dimethyl-*p*-phenylenediamine dihydrochloride (DMPD), 2,2-azino-bis-3-ethyl benzthiazoline-6 sulphonic acid (ABTS), 1,1-diphenyl-2-picryl hydrazyl radical (DPPH), neocuproine, cupric chloride and adenosine diphosphate (ADP) were obtained from Sigma (Moline, USA). Ascorbic acid, AlCl₃, ferrous sulphate (Fe₂SO₄), trichloroacetic acid, ammonium acetate was purchased from Sisco Research Laboratories, Mumbai, India. All other chemicals used were of analytical grade.

Plant materials and extraction procedures

Chili (Byadagi variety) and pepper (local variety) were procured from the local market of Mysore city. The cleaned spices were dried at 40°C in a labo-

ratory oven and then ground into a fine powder. The powder was extracted by using methanol as extracting solvent. Eight extractions with 3 volumes of the solvent for 4 h duration were carried out with either methanol or ethanol at room temperature (25°C). The extracts were pooled, desolventized and then dried in a dessicator overnight. Oleoresins of chili and pepper were prepared by methylene chloride extraction [11]. These extracts were used for antiradical and antioxidant assays. 10 mg of extracts were dissolved in 10 mL of methanol. Various concentrations of these extracts were used for evaluating antioxidant potentials by DPPH assay, DMPD assay, ABTS assay, reducing power assay, CUPRAC assay and by inhibition of ascorbic acid induced lipid peroxidation in rat liver homogenates.

Radical scavenging activity

Measurement of the radical scavenging activity by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) test. The DPPH radical scavenging activity was measured as described by Gulcin [12]. Briefly, 0.1 mM solution of DPPH radical was prepared in methanol. 0.5 mL of this solution was added to different concentrations of chili extracts or chili oleoresin or pepper extracts or pepper oleoresin in methanol as prepared above at different concentrations (100–2000 µg/mL). Final volume of reaction mixture was made up to 2 mL in methanol. These were mixed thoroughly and incubated in dark for 30 min. The absorbance was measured at 517 nm against appropriate blank samples:

$$\text{DPPH remaining} = (1 - \text{Abs}_0/\text{Abs}_t) \times 100, \quad (\%)$$

where Abs_0 is the absorbance of the control and Abs_t is the absorbance in the presence of test compounds.

*Measurement of radical scavenging activity by N,N-dimethyl-*p*-phenylenediamine dihydrochloride (DMPD) method.* DMPD radical scavenging activity of chili and pepper extracts and their oleoresins was performed according to Fogliano *et al.* [13]. Standard DMPD (100 mM) was prepared by dissolving 209 mg of DMPD in 10 ml of deionized water. 1 ml of this solution was added to 100 ml of 0.1 M acetate buffer (pH 5.25), the coloured radical cation (DMPD^{•+}) was obtained by adding 0.2 mL of ferric chloride (0.2%) and absorbance at 505 nm was measured. Freshly prepared solutions were used for each experiment. Different concentrations of antioxidants (100–2500 µg/mL) were added to test tubes and the total volume was made up to 0.5 ml with distilled water. One ml of DMPD^{•+} solution was directly added to the reaction mixture. Ten minutes later, the absorbance was measured at 505

nm. The scavenging activity was calculated using the following equation:

$$\text{DMPD remaining} = (1 - \text{Abs}_0/\text{Abs}_t) \times 100, \quad (\%)$$

where Abs_0 is the absorbance of initial concentration of the $\text{DMPD}^{\bullet+}$ and Abs_t is the absorbance of $\text{DMPD}^{\bullet+}$ in presence of test materials.

2,2-Azino-bis-3-ethyl benzthiazoline-6-sulphonic acid (ABTS) radical cation decolourization assay. ABTS forms a relatively stable free radical, which decolorizes in its non radical form. The $\text{ABTS}^{\bullet+}$ radical remaining was determined according to the method of Ree *et al.* (31). The $\text{ABTS}^{\bullet+}$ was produced by reacting 2 mM ABTS in H_2O with 2.45 mM potassium persulphate ($\text{K}_2\text{S}_2\text{O}_8$), kept in the dark at room temperature for 4 hr. The $\text{ABTS}^{\bullet+}$ was diluted with ethanol to give an absorbance of 0.750 ± 0.025 at 734 nm. 1 ml of $\text{ABTS}^{\bullet+}$ solution was added to 10 μl of chili and pepper extracts in ethanol at different concentrations (40–600 $\mu\text{g}/\text{mL}$). The absorbance was recorded after 30 min and the percentage of radical remaining was calculated. The extent of decolourization is calculated as percentage reduction of absorbance:

$$\text{ABTS remaining} = (1 - \text{Abs}_0/\text{Abs}_t) \times 100, \quad (\%)$$

where Abs_0 is the absorbance of a control without any radical scavenger and Abs_t is the absorbance of the $\text{ABTS}^{\bullet+}$ remaining in the presence of the anti-radical scavenger.

Estimation of antioxidant activity

Ferric cyanide (Fe^{3+}) reducing antioxidant power assay. The reductive potential of extracts was determined according to the method of Benzie and Strain [14]. Different concentrations of extracts (100–2100 $\mu\text{g}/\text{mL}$) in 1 mL of distilled water was mixed with 2.5 mL of 0.2 M sodium phosphate buffer, pH 6.6 and potassium ferricyanide [$\text{K}_3\text{Fe}(\text{CN})_6$] (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. Then the reaction was terminated by adding 2.5 mL of trichloroacetic acid (10%). The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and FeCl_3 (0.5 mL, 0.1%) and the absorbance was measured at 700 nm in a spectrophotometer against a blank sample. Reductive potential was measured with increase in absorbance when extract was added.

Lipid peroxidation by TBARS assay. Liver homogenates in 150 mmol/L KCl, 25 mmol/L, Tris HCl buffer pH 7.5, 2 mmol/L ADP, 10 $\mu\text{mol}/\text{L}$ Fe_2SO_4 were incubated at 37°C for 5min. The final volume of the reaction mixture was made up to 1 mL with Tris HCl buffer. The reaction was initiated by

adding 25 μL of ascorbic acid (100 mM). The tubes were incubated for 30 min at 37°C and the reactions were terminated by adding 2 mL of thiobarbituric acid. The tubes were heated in boiling water bath for 60 min. The malondialdehyde (MDA) formed was measured at 535 nm and quantitated using an extinction coefficient of $1.56 \times 10^{-5} \text{ M}^{-1}\text{cm}^{-1}$. The inhibition of lipid peroxidation in the presence of extracts was calculated according to Miller and Aust [15].

CUPRAC total antioxidant capacity assay. 1 ml of CuCl_2 ($1.0 \times 10^{-2} \text{ M}$), 1 mL of neocuproine alcohol solution ($7.5 \times 10^{-3} \text{ M}$) and 1 mL of ammonium acetate buffer solution (0.1 M, pH = 7) was added and mixed thoroughly followed by addition of extract at concentrations ranging from 100 μg to 1000 μg . To this deionized water was added to give a total volume of 4.1 mL and mixed well. After 30 min, the absorbance was read at 450 nm against a reagent blank. The antioxidant activity is expressed as mmoles of Trolox equivalent/mg of extract [16].

RESULTS

Effect of radical scavengers from chili and pepper extracts in model systems

DPPH radical scavenging activity. DPPH is usually used to evaluate the free radical scavenging activity of antioxidants. It is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. Figure 1 illustrates a decrease in the concentration of DPPH radical (column 1) due to the scavenging ability of chili and pepper extracts. This is compared with the effectiveness of synthetic antioxidants such as TBHQ and BHT to scavenge the radical. The free radical scavenging effect of extracts and standards are in the order of $\text{TBHQ} > \text{BHT} > \text{Chili oleoresin} > \text{Pepper oleoresin} > \text{Chili extracts} > \text{Pepper extracts}$. The effective concentration required to scavenge 50% radical (EC_{50}) were 6.8, 40, 800, 550, 900 and 750 μg for TBHQ, BHT, extracts of chili, chili oleoresins, pepper extracts and pepper oleoresins, respectively (Table 1). These studies indicated that oleoresins showed better radical scavenging activity compared to methanol extracts of spices.

DMPD radical scavenging activity. Spice extracts showed $\text{DMPD}^{\bullet+}$ radical scavenging activity in a concentration dependent manner (Figure 1, column 2). However, BHT and TBHQ showed better radical scavenging activity. EC_{50} value for chili extracts, pepper extracts, chili oleoresin and pepper oleoresin were 750, 500, 900 and 730 μg respectively (Table 2). The EC_{50} values with TBHQ and BHT were 8 μg and 35 μg respectively. These trends are similar to that observed with DPPH system.

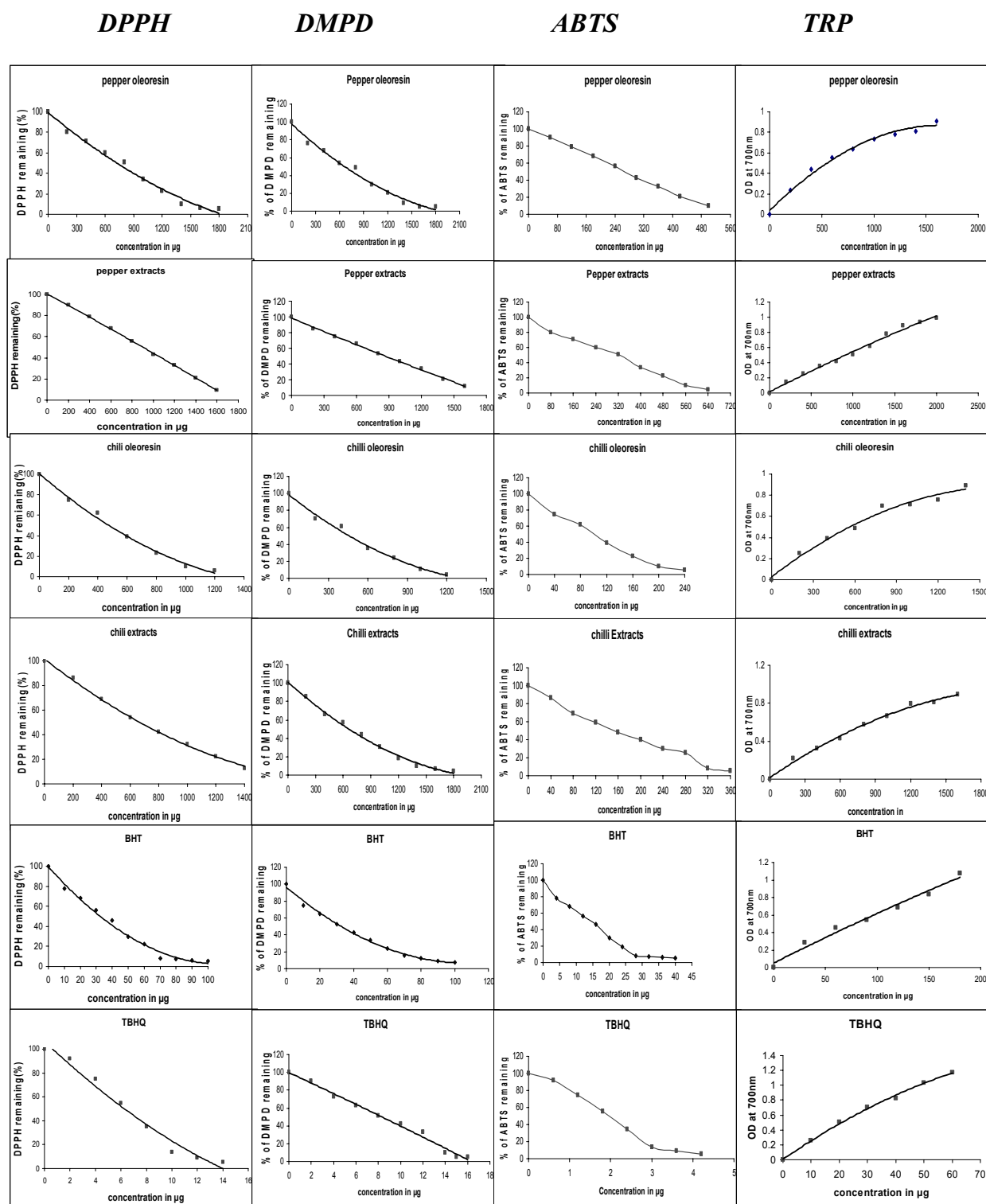


Fig. 1. Radical scavenging activity of spice extracts, BHT and TBHQ measured with DPPH, DMPD, ABTS and TRP (FRAP) assay as indicated in the methods.

ABTS radical cation decolourization assay. The extracts of chili and pepper and oleoresins of chili and pepper exhibited effective cation radical scavenging activity [17]. The EC₅₀ value for radical scavenging activity in the system were 2, 15, 210, 130, 330 and 270 µg for TBHQ, BHT, methanol extracts of chili, chili oleoresin, methanol extracts of pepper and pepper oleoresin respectively (see Figure 1, column 3 and Table 3). These studies indicated that oleoresins showed better radical scavenging activity compared to methanol extracts of spices.

Table 1. Antiradical activity of spice extracts in DPPH assay system.

Additions	EC ₅₀ (µg)
TBHQ	6.8 ± 0.4
BHT	40 ± 2
Chili extracts	800 ± 6
Chili oleoresin	550 ± 4
Pepper extracts	900 ± 5
Pepper oleoresin	750 ± 3

Values are mean ± SD, n = 4; DPPH – Diphenyl picryl hydrazyl; BHT – Butylated hydroxy toluene; TBHQ – Tertiary butylated hydroxy quinone.

Table 2. Antiradical activity of spice extracts in DMPD assay system.

Additions	EC ₅₀ (µg)
TBHQ	8.0 ± 0.2
BHT	35.0 ± 1.3
Chili extracts	750 ± 4
Chili oleoresin	500 ± 5
Pepper extracts	900 ± 4
Pepper oleoresin	730 ± 6

Values are mean ± SD, n = 4.

Table 3. Antiradical activity of spice extracts in ABTS assay system.

Additions	EC ₅₀ (µg)
TBHQ	2.0 ± 0.4
BHT	15.0 ± 0.2
Chili extracts	210 ± 2
Chili oleoresin	130 ± 1
Pepper extracts	330 ± 4
Pepper oleoresin	270 ± 5

Values are mean ± SD, n = 4.

Antioxidant activity

Reducing power assay using the potassium ferric cyanide reduction method. Figure 1 (column 4) shows the reducing power of the spice extracts and standards (TBHQ and BHT) in potassium ferric cyanide reduction method. The reducing power of extracts and standards increased with growing concentration of samples. Reducing power of extracts and standards were of the order: TBHQ > BHT > Chili oleoresin > Chili extracts > Pepper oleoresin > Pepper extracts when reducing power at equal concentrations of extracts were compared.

Inhibition of liver lipid peroxidation by spice extracts. Addition of TBHQ and BHT inhibited lipid peroxidation in rat liver homogenates (Table 4). The inhibition with TBHQ and BHT was 75 and 94% at 100 µg. With chili extracts, chili oleoresin, pepper extracts and pepper oleoresin the inhibition observed at 0.1% was 36, 41, 39 and 44%, respectively. At 1%, the maximum inhibition observed was 46, 55, 42 and 58%, respectively (Figure 2 and Table 4).

Table 4. Inhibition of the lipid peroxidation by spice extracts.

Additions	Concentration (µg)	Inhibition (%)
Chili extracts	100	36
	1000	46
Chili oleoresin	100	41
	1000	55
Pepper extracts	100	39
	1000	42
Pepper oleoresin	100	44
	1000	58
BHT	0.13	75
TBHQ	0.012	94

Control value in the absence of inhibitors: 19.41 ± 2.43 nmoles MDA/mg protein.

Total antioxidant capacity by CUPRAC method.

The CUPRAC antioxidant capacities of extracts were measured as Trolox equivalents. Trolox equivalent capacity obtained of chili extracts, chili oleoresin, pepper extracts and pepper oleoresin were 160, 213, 110 and 181 µg, respectively (Table 5).

Table 5. Total antioxidant activity of spice extracts by CUPRAC assay system.

Additions	µg of Trolox equivalents/mg of extract
Chili extracts	160 ± 2
Chili oleoresin	213 ± 2
Pepper extracts	120 ± 1
Pepper oleoresin	181 ± 2

Values are mean ± SD, n = 4.

DISCUSSION

The objective of the investigation was to evaluate the potentials of extracts from chili and pepper to scavenge free radicals and exhibit antioxidant activities which can be exploited in preserving processed foods or to prevent the rancidity of oils and at the same time get the benefits of medicinal properties of these spices. These spices are routinely used in Asian diets as food adjuncts. The medicinal values of these spices are known for many decades from traditional knowledge and by its use in Ayurvedic system of medicine [18]. Many studies in the recent past have also shown the action of these spices and their active principles in beneficially influencing physiological processes as well as controlling disease process.

One of the most thoroughly studied active components of chilis is capsaicin, which is the pungent component of chili. Capsaicin and dihydrocapsaicin inhibit iron mediated lipid peroxidation. It also inhibited copper induced oxidation of low density lipoprotein [19]. This effect is perceived to be mediated by its ability to form complexes with reduced metals and act as hydrogen donors [20].

Capsaicin reduced the generation of reactive oxygen species in activated macrophages and exhibited antiinflammatory effects [21]. It also augmented antioxidant systems by enhancing the activities of enzyme such as superoxide dismutase, catalase and glutathione peroxidase. Capsaicin was found to inhibit the growth of *Helicobacter Pylori*. It inhibited the release of gastrin by stimulating somatostatin. Dairam *et al.* [20] have examined the antioxidant, metal chelating properties and inhibitory effect on oxidative stress by capsaicin in rat brain homogenates. The neuroprotective effects of capsaicin through antioxidant and iron-binding properties of capsaicin was reported in this study. Thus, chilies and its bioactive components have beneficial effect in nullifying the potential damage caused by reactive oxygen species.

Pepper extracts contain piperine and other bioactive compounds [6]. Piperine has been demonstrated to give protection against oxidative damage by quenching free radicals, reactive oxygen species and inhibiting lipid peroxidation [22]. Reddy and Lokesh [23] have reported that piperine had inhibitory effects on ascorbate Fe^{2+} induced lipid peroxidation in rat liver microsomes at concentrations of 600 μM and above.

Piperine is shown to provide protection to human low density lipoprotein from copper induced lipid peroxidation [24]. The aqueous extract of black pepper as well as piperine have been examined for their effect on human 5-lipoxygenase, the key enzymes involved in biosynthesis of leukotrienes [25]. The formation of 5-hydroxyeicosatetraenoic acid was significantly inhibited in a concentration dependent manner with IC_{50} values of 0.13 mg for aqueous extracts of pepper and 60 μM for piperine. Thus, piperine in black pepper might exhibit antioxidant activity by modulating 5-lipoxygenase pathway.

Selvendiran and Senthilnathan [26] have investigated the effect of piperine on mitochondrial antioxidant system and lipid peroxidation in benzo-(α)pyrene induced lung carcinogenesis. Oral supplementation of piperine (50 mg/kg body weight) effectively suppressed lung carcinogenesis and decreased extent of mitochondrial lipid peroxidation with concomitant increase in the activities of enzymatic and

non enzymatic antioxidant levels. This suggests that piperine may exhibit its chemo preventive effect by modulating lipid peroxidation and augmenting antioxidant defense system.

Vijayakumar *et al.* [27] have examined the effect of supplementing black pepper or piperine on tissue lipid peroxidation, enzymatic and non-enzymatic antioxidants in rats fed a high fat diet. They observed that black pepper extract and piperine can reduce high fat diet induced oxidative stress. Significantly elevated levels of thiobarbituric acid reactive substances, conjugated dienes and lowered activities of superoxide dismutase, catalase, glutathione peroxidase, glutathione-*s*-transferase, reduced glutathione was observed in the liver, heart, kidney, intestine and aorta of rats fed high fat diet. Supplementation of diet with black pepper or piperine lowered lipid peroxidation and elevated antioxidant enzyme levels to that observed with control rats.

The effect of alcohol extract of piper longum and piperine was studied for immunomodulatory and antitumour activity [28]. The administration of extract (10 mg/animal) or piperine (1.14 mg/animal) inhibited solid tumor development in mice injected with Daltons lymphoma ascites cells. Pepper extracts also increased the survival of mice bearing Ehrlich ascites tumor cells [29].

Because of such varied effects of black pepper and its extracts, it has been used in Indian Ayurvedic system of medicine, folklore medicine of Latin America and West Indies its treatment of asthma, bronchitis, fever, arthritis and gastrointestinal disorders.

The spices and their active principles were found to be safe for consumption even at high doses. Black pepper, its oleoresin or its active principle piperine, fed to rats at doses 5–20 times normal intake levels in humans did not cause any adverse effect on growth, food efficiency ratio, organ weights, blood cell counts and blood constituents like hemoglobin, total serum proteins, albumin, globulin, glucose, cholesterol, activities of serum amino transferases and phosphatases, fat and nitrogen balance [30].

The antiradical and antioxidants from spices such as chili and pepper thus play a vital role in removing damaging free radicals and there by promote health and reduce risk factors for chronic diseases. Our unpublished results also indicated that there is a strong correlation between antioxidant activity of spice extracts and the total phenol content of these extracts. We have thus demonstrated the efficacy of spice extracts as radical scavengers in a number of in vitro models. Spice extracts being nontoxic and by its ability to stabilize the oils can be a useful alternative to synthetic antioxidant in oil and food

preservation, which is the objective of our next paper.

CONCLUSIONS

Comparable analysis based on the different *in vitro* assay systems: DPPH• and DMPD^{•+} radical scavenging assays, CUPRAC and ABTS decolorization assays, ferric reducing power assay demonstrated that alcohol extracts and oleoresins of chili and pepper have significant radical scavenging activities and antioxidant potential in all the systems tested. Their effects were compared with the reference antioxidants such as TBHQ, BHA and BHT. These studies indicated that chili and pepper extracts can be used for the medicinal properties as well as for food preservations by virtue of their ability to remove dangerous oxygen free radicals.

Acknowledgement: Partial financial support for this study was from the Department of Science and Technology, New Delhi, India (Grant Int/Bulgaria/B-16/2006) and of National Science Fund of Ministry of Education and Science of Bulgaria, Bilateral project with India (Grant BIn4/04) are gratefully acknowledged. The authors also thank Dr. V. Prakash, Director, Central Food Technological Research Institute, Mysore, India and Prof. Tchorbanov, DSc., Director, Institute of Organic Chemistry with Centre of Phytochemistry, Bulgarian Academy of Sciences, Sofia, Bulgaria for keen interest and encouragement for this work.

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ОЦЕНКА НА АНТИРАДИКАЛОВИТЕ И АНТИОКСИДАНТНИ СВОЙСТВА НА ЕКСТРАКТИ ОТ ИНДИЙСКО ЧЕРВЕНО ЧИЛИ И ЧЕРЕН ПИПЕР ЧРЕЗ „ИН ВИТРО“ МОДЕЛИ

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Постъпила на 31 юли 2009 г.

(Резюме)

Кислород съдържащите свободни радикали и липидните пероксиди играят важна роля в увеличаване на рисковите фактори за много хронични заболявания и са също така отговорни за влошаване на качеството на хранителните продукти. Целта на това проучване бе да оцени потенциала на екстракти от червено чили и черен пипер да улавят свободни радикали в *in vitro* моделни системи като: 1,1-дифенил-2-пикрил-хидразил (DPPH), N,N-диметил-р-фенилендиамин дихидрохлорид (DMPD), 2,2-азино-бис-3-етилбензтиазолин-6-сулфонова киселина (ABTS), ферицианиден антиоксидантен потенциал (FRAP), тотален антиоксидантен капацитет (CUPRAC) методи и да покаже антиоксидантните активности спрямо липидното перокисление. Алкохолните екстракти и олеорезиновите проби на червено чили и черен пипер показаха значителни активности като уловители на свободни радикали и антиоксидантна активност във всички тествани системи. Тези резултати могат да бъдат използвани за предпазване на храни при преработката им и в същото време показват предимствата на лечебните свойства на тези подправки.