Environmental Research Journal 6 (2): 94-99, 2012
ISSN: 1994-5396

In vitro Effect on the Antioxidative Properties of Crude Extract of *C. album* in Presence of the Organophosphate, Acephate

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Abstract: Acephate, an organophosphorus pesticide is a potent free radical generator. The aim of this study was to evaluate the role of plant phenolics in *Chenopodium album* against acephate toxicity *in vitro*, predominantly acting as a free radical scavenger. Experiments based on DPPH radical scavenging activity and reducing power of plant extract both in the presence and absence of acephate was analyzed using concentrations of 1, 2.5 and 5 μg mL^{-1} of polyphenolic compounds and 3 mg mL^{-1} of acepahte which showed that pesticides significantly reduced the activity of the plant extract and a higher concentration was required to quench the $ABTS^{+}$ radicals. No significant change was observed in case of lipid peroxidation in presence of acephate. Thus, from this study, it is evident that pesticides act as a serious free radical source whose effect can be reduced, if not totally but to a certain extent by green leafy vegetables.

Key words: Acepahte, plant polyphenols, free radicals, radical scavenging activity, lipid peroxidation, India

INTRODUCTION

Pesticides are used extensively to eradicate pests for the enhancement of food production. However, there continuous use is causing severe toxicity amongst mankind. Intoxication with pesticides even hampers animal health (Tuzmen *et al*., 2007, 2008; Banerjee *et al*., 1999). Organophosphorus pesticides have outnumbered the organochlorates in terms of their usage mainly due to their relatively low toxicity and persistence in mammalian systems (Joshi and Rajini, 2009). Long-term exposures to these pesticides cause neuronal disorders primarily through the inhibition of Acetylcholine Esterase (AchE) and also their lipophilicity affects the lipid bilayer thus interfering with the normal functioning of the membranes (Costa, 2006; Possamai *et al*., 2007). Such interactions have been mainly attributed to oxidative stress caused by these organophosphates (Kovasic, 2003).

Acephate (O, S-dimethyl acetylphosphoramidothioate), an organophosphorus insecticide is used against various insects like thrips, jassids, aphids, ants and wasps that infect paddy, wheat, etc. Free radicals are generated under normal conditions in the mitochondrial ETC and by enzymes such as lipoxygenases, peroxidases and dehydrogenases (Halliwell and Gutteridge, 1999). However, their excessive production can cause an imbalance between the prooxidant and antioxidant status of the body cells and tissues thereby causing tissue injury, DNA damage and enzyme inactivation (Poovala *et al*., 1998). Further, sulphur-containing organophosphates to which acephate belongs are S-oxidized to highly reactive intermediates within cells and tissues which may even lead to muatagenic changes in the body cells and tissues (Farag *et al*., 2000).

Green leafy vegetables are rich sources of many nutrients and their beneficial role has partly been attributed to the antioxidant components present in them of which the major portion is formed by the flavonoids, isoflavones, lignans, catechins and isocatechins (Subashree *et al*., 2009; Gupta and Prakash, 2009). Flavonoids are a large group of polyphenolic antioxidants that occur in several fruits, vegetables and beverages mainly as O-glycosides.

They are efficient antioxidants capable of scavenging radical species (peroxyl radicals, hydroxyl radical, superoxide radicals) forming a phenoxy radical (Rice-Evans *et al*., 1995; Rice-Evans and Miller, 1996). Plants of the Lamiaceae family, notably rosemary, oregano, sage, mint and thyme, contain a range of potential antioxidants such as carnosol, rosemanol and carvacrol which can contribute to the antioxidant potential of the diet (Lagouri and Boskou, 1996).

Thus, the antioxidative role of such leafy vegetables has been well studied proving to have good radical scavenging activities and capability of inhibiting lipid peroxidation. Investigations have proved their excellence

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in inhibiting lipid peroxidation even in heated oils when compared to artificial antioxidants like BHA, BHT etc. (Shayamala *et al*., 2005).

In vivo studies on how antioxidants in plants can protect against pesticide toxicity have been elucidated quite vividly till date. Their role in altering the body's defensive mechanisms has been highlighted in several studies (Khan *et al*., 2005). However, very few data is available on the activity of plant antioxidants *in vitro* in presence of pesticides. Hence, in this present study, an attempt has been made to evaluate the scavenging role of the antioxidative components present in the leafy vegetable *Chenopodium album* against the toxic effects of the organophosphate insecticide acephate *in vitro* based on DPPH and $ABTS^+$ radical scavenging activities reducing activity and lipid peroxidation.

MATERIALS AND METHODS

Chemicals: Methanol, ethanol, potassium persulfate, Folin-ciocalteau's phenol reagent, gallic acid, linoleic acid were purchased from E. Merck India (P) Limited; potassium ferricyanide, Ethylidene Diamine Tetra Acetic acid di-sodiumsalt (EDTA), ammonium thiocyanate, ferrous chloride were purchased from Sisco Research Laboratory (SRL), Mumbai India; Flavonoid Standards of rutin hydrate, rutin, quercetin, kaemferol, 1,1-diphenyl-2-picryl-hydrazyl stable radical, cation free radical and $ABTS^+$ from Sigma Chemical Co. (St. Louis, MO), acephate (Technical Grade-85% purity) was obtained from M/S Hindustan Insecticides Limited, Delhi, India.

Preparation of plant extract: The leafy vegetable, *Chenopodium album* was collected from the local market and was authenticated by the Department of Botany, University of Calcutta, Kolkata, India. The polyphenolic compounds were then extracted by following the methods developed in the laboratory (Mallick *et al*., 2009). Briefly, the whole plants (stem and leaves) of *C. album* were washed thoroughly, oven dried and ground to powder. It was then extracted using 80:20 methanol:water. The methanolic extract was used for the various *in vitro* assays. The concentrations were calculated on the basis of the total polyphenol content.

Determination of polyphenols: Total polyphenol content of the leafy vegetable was determined by the Folin-Ciocalteau Method as described by Matthaus (2002).

HPLC analysis of the plant extract: The hydrolyzed sample for HPLC detection of the plant polyphenols was prepared according to the method of Hertog *et al*. (1993). In brief, 0.5 g of the dried plant material was taken in a flask along with BHA (2 g L^{-1}) and sonicated for 5 min in 40 mL of 65% methanol. Total 10 mL of 6 N HCl was added and nitrogen purged for 60 sec. The mixture was then heated on a water bath at 90°C for 2 h stirring constantly. It was then cooled, filtered and sonicated for 5 min and then used for HPLC analysis. The components were then determined by HPLC according to Siddhuraju and Becker (2003). In brief, a gradient elution was employed for flavonoids other than catechins with a mobile phase consisting of 50 mM H_3PO_4, pH 2.5 (solution A) and acetonitrile (solution B) which is as follows: isocratic elution 95 A/5 B, 0-5 min; linear gradient from 95% A/5% B to 50% A/50% B, 5-55 min; isocratic elution 50% A/50% B, 55-65 min; linear gradient from 50% A/50% B to 95% A/5% B, 65-67 min; post-time 6 min before next injection. The system, WATERS 2487 is equipped with a C-18 column (Nova-Pak C_{18}, 3.9×150 mm). About 280, 340 and 370 nm wavelengths were selected for the detection. The flow rate of the mobile phase was 0.8 mL min^{-1} and the injection volume was 20 μL. The peaks were identified in comparison with authentic standards.

DPPH stable radical scavenging activity: In brief, different concentrations of samples were taken in different test tubes, volume adjusted to 1 mL by using distilled water. About 5 mL of 0.1-0.2 mM methanolic solution of DPPH was added to the test tubes and shaken vigorously. The tubes were allowed to stand at room temperature for half an hour. Changes in the absorbance of the sample were measured at 517 nm. Radical scavenging activity was expressed as the inhibition percentage and calculated using the following equation (Oktay *et al*., 2003):

$$\text{Radical scavenging activity (\%)} = \frac{[(\text{Control OD-Sample OD})]}{\text{Control OD}} \times 100$$

$ABTS^+$ radical cation scavenging activity: The activity was determined according to Re *et al*. (1999). Briefly, 5 mL 7 mM ABTS was reacted with 88 μL 140 mM potassium persulphate overnight in dark. Prior to use it was diluted with 50% ethanol for an initial absorbance of ≈0.7 at 734 nm. About 10 μL of sample extract was added to 1 mL of diluted ABTS and the change in absorbance was recorded for 5 min at 1 sec interval. The antioxidant capacity was expressed as EC_{50}, concentrations necessary for 50% reduction of ABTS.

Reducing activity: Different concentrations of extracts were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (2.5 mL, 1%). The mixture was

incubated at 50°C for 20 min. About 2.5 mL of 10% TCA was added to the mixture. About 2.5 mL of this solution was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%) and the absorbance measured at 700 nm. Increased absorbance of the reaction mixture indicates increased reducing power. All analysis were run in triplicates and averaged (Yen and Chen, 1995).

Inhibition of lipid peroxidation in a linoleic acid emulsion: It was determined by the method of Tomohiro *et al.* (1994). A reaction mixture containing 50 μg mL^{-1} of extract, 2.3 mL of phosphate buffer (pH 7.4) and 2.5 mL of linoleic acid emulsion was incubated in the dark at 37°C. About 0.1 mL of the mixture was added to 0.1 mL of 30% ammonium thiocyanate and 0.1 mL of ferrous chloride and mixed thoroughly. The absorbance was read at 500 nm after every 24 h.

Statistical analysis: The data was expressed as mean±SD. Differences among the experimental groups were analyzed using one-way ANOVA and the comparisons between the means were carried out using the Tukey test; $p<0.05$ was considered as statistically significant in all the experiments.

RESULTS AND DISCUSSION

Total polyphenol content and components: In the present study, the crude extract of *Chenopodium album* was used and the active components present in it were analyzed by HPLC (Fig. 1). It has been observed that the crude extract contain mainly flavonoids like rutin, rutin hydrate and quercetin as major components along with some unidentified materials.

DPPH stable radical scavenging activity: The DPPH radical scavenging activity showed a significant decrease ($p<0.05$) in the presence of acephate at all three concentrations of 1, 2.5 and 5 μg mL^{-1} of polyphenolic compounds when compared to those without pesticide treatment (Fig. 2).

$ABTS^{+}$ radical cation scavenging activity: A significantly high EC_{50} value i.e., the concentration required to quench 50% of the $ABTS^{+}$ free radicals was observed in case of the acephate treated tests (12.69±0.021) when compared to the control without any pesticide treatment (2.15±0.004).

Reducing activity: The reducing activity of the plant extract showed an increase in the presence of the pesticide at the concentration of 1 μg mL^{-1}. However, at

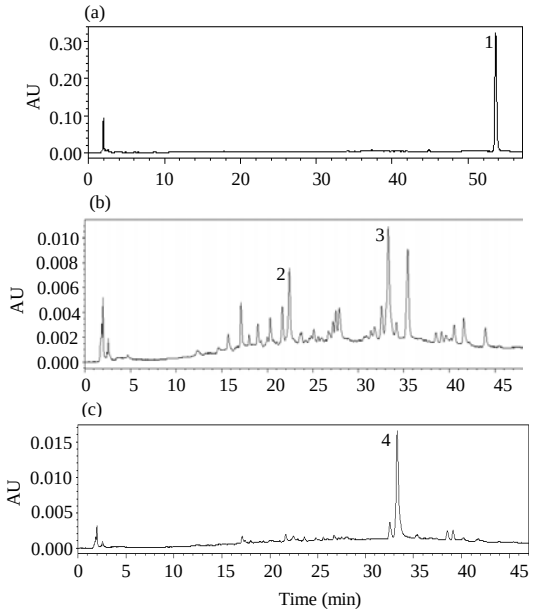

Fig. 1: HPLC chromatogram of *Chenopodium album* at a) 280, b) at 340 and c) 370 nm showing 1: Rutin hydrate, 2: Rutin, 3 and 4: Quercetin

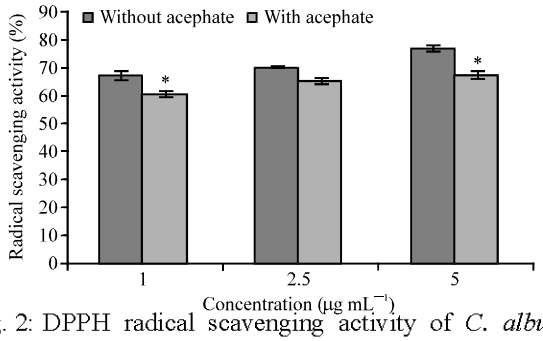

Fig. 2: DPPH radical scavenging activity of *C. album* extract at concentrations of 1, 2.5 and 5 μg mL^{-1} both in the presence and absence of the pesticide, acephate. The values are expressed as mean±SD, n = 3; *Significantly different from the sample without pesticide treatment at respective concentration, $p<0.05$

higher concentrations of 2.5 and 5 μg mL^{-1}, it showed a reduction in the activity with a sharp significant reduction ($p<0.05$) at the highest concentration (Fig. 3).

Inhibition of lipid peroxidation in a linoleic acid emulsion: No significant change was observed in the peroxidation levels in the tests containing acephate when compared to the tests containing only the plant extract. However, the tests treated with the plant extract alone did

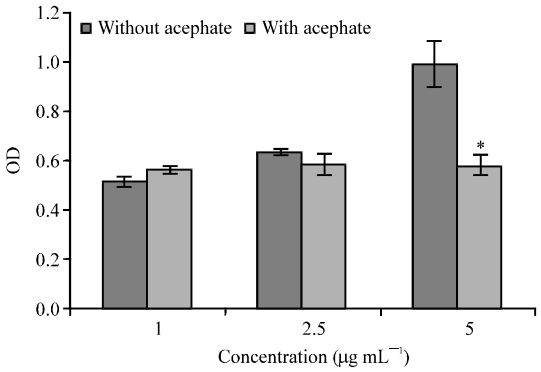

Fig. 3: Reducing activity of *C. album* extract at concentrations of 1, 2.5 and 5 μg mL^{-1} both in the presence and absence of the pesticide, acephate. The values are expressed as mean±SD, n = 3; *Significantly different from the sample without pesticide treatment at respective concentrations, $p<0.05$

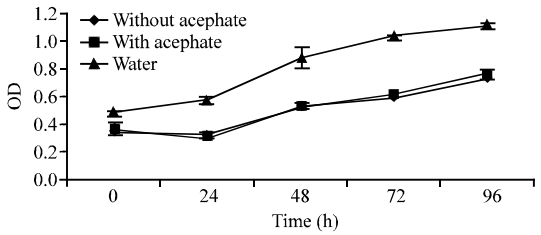

Fig. 4: Inhibition of lipid peroxidation in a linoleic acid emulsion by *C. album* extract in the presence of the pesticide, acephate taking water as the control. The values are expressed as mean±SD, n = 3

show a significant decrease ($p<0.05$) in lipid peroxidation rates when compared to the positive control containing no plant extract (Fig. 4).

In the recent years, the use of pesticides and their residual effects on the animal world has gained much importance. Pesticides have been used since ages indiscriminately in various forms for better food production where the role of organophosphates is vast due to their low soil persistence. Acephate, belonging to this group of organophosphates has been implicated as a potent generator of free radicals, their basic mechanism of action causing oxidative stress and damage to tissues (Rao *et al*., 1991).

Oxidation or the loss of an electron can sometimes produce these reactive substances known as free radicals that cause oxidative stress in the body. Oxidative stress has been implicated in the pathology of many diseases inflammatory conditions, cancer and aging.

Plant sources such as fruits, vegetables and whole grains have been proved to impart great health benefits. These observations have led to continuous research in the field of identifying specific bioactive components in foods such as antioxidants which may be responsible for improving and maintaining health (Packer *et al*., 2001; Fassina *et al*., 2004). Various forms of antioxidants such as vitamins, minerals, carotenoids and polyphenols have been reported to be present in food stuffs (Lau *et al*., 2006) of which an important and powerful food antioxidant is quercetin which is a flavonoid and more specifically a flavonol. Another flavonoid, rutin is recently gaining much interest in the research field and has been identified as a good antidiabetic factor (Kamalakkannan and Prince, 2006a, b).

Antioxidants are capable of stabilizing or quenching such free radicals before they can react and cause harm or can even minimize the effect if not nullify it totally. Because oxidation is a naturally occurring process within the body, a balance with antioxidants must exist to maintain health (Liu, 2004; Goodman *et al*., 2004; Rietveld and Wiseman, 2003) and hence the development of several defense mechanisms like superoxide dismutase, catalase, glutathione, glutathione reductase and so on. Antioxidants may offer resistance to oxidative stress primarily through free radical scavenging and inhibiting lipid peroxidation, thus preventing the onset of deadly diseases. Apart from the antioxidants synthesized naturally, the body requires a supplement of dietary antioxidants which can be obtained only by the consumption of an antioxidant-rich diet. The impoprtance of green leafy vegetables, most of which are underexploited are proven sources of essential nutrients like vitamin A and vitamin C, carotenoids, flavonoids, phenols, etc.

DPPH assay is one of the most widely used methods for screening the antioxidant activity of plant extracts. The assay is based on the evaluation of the ability of the antioxidants to scavenge the stable radical DPPH. DPPH is a stable nitrogen-centred free radical which produces violet colour in methanol/etahnol solution. DPPH radicals react with suitable reducing agents during which the electrons become paired off and the solution loses colour stoichiometrically depending on the number of electrons taken up.

This loss of colour or absorbance is measured spectrophotometrically at 517 nm (Blois, 1958). In the present tests, the solution progressively reduced to the yellow coloured product, diphenylpicryl hydrazine with the addition of the extracts in a concentration-dependent manner. The results obtained clearly indicate the potential

of the plant extracts in scavenging free radicals at high concentrations in the absence of the pesticide, acephate. However, it was also evident that acephate significantly reduced the scavenging activity of the *C. album* extract at all three concentrations possibly because of the production of the free radicals at a tremendous rate which the plant extract was unable to quench.

ABTS assay is based on the measurement of the absorbance produced by the inhibition of the $ABTS^{+\cdot}$ radical which has a characteristic long wavelength absorption spectrum (Sanchez-Moreno, 2002). The results obtained clearly imply that the methanol extracts of *C. album* inhibit the radical or scavenge the radical. However, the increase in the required concentration of the plant extract or the increased EC_{50} value in the presence of acephate proved that the antioxidant requirement in the system needs to be enhanced to deal with the large quantities of free radicals that are being generated by the pesticide.

Lipid peroxidation is the oxidative degradation of Polyunsaturated Fatty Acids (PUFA) involving free radicals and is a serious threat to lipid membrane structures. Lipid peroxidation by the ammonium thiocyanate method is based on the measurement of the red complex, ferric thiocyanate at 500 nm using linoleic acid as the substrate. Free radicals that were present in the system caused peroxidation of the lipid which was minimized to a certain extent, almost by 50% by the plant extract.

However, the presence of acephate did not show much variation in the measurements. This was probably because the pesticides themselves had abstracted protons or interacted with free radicals rather than Polyunsaturated Fatty Acids (PUFA), thereby protecting the latter against peroxidation and hence the antioxidative role of *C. album* could not be adjudged properly in this case.

CONCLUSION

This study shows that green leafy vegetables has an enormous scope as a natural antioxidant source. Their tremendous potential as dietary therapeutic agents needs extensive documentation. The deleterious effects of pesticides, a major prooxidant source in different forms and formulations are increasing in an alarming rate and have been identified with serious health hazards. Hence, inclusion of dietary polyphenols in the form of green leafy vegetables, containing flavonoinds like rutin, quercetin, etc. can avert various modern health problems.

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