African Journal of Biotechnology Vol. 6 (9), pp. 1136-1139, 2 May 2007
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2007 Academic Journals

Full Length Research Paper

Identification of a naturally occurring 2, 6-bis (1.1-dimethylethyl)-4-methylphenol from purple leaves of the halophyte plant *Mesembryanthemum crystallinum*

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Accepted 1 March, 2007

2, 6-Bis (1.1-dimethylethyl)-4-methylphenol (BHT) is a synthetic antioxidant used generally for food, cosmetics and pharmaceuticals. The leaf extract from the halophyte plant, *Mesembryanthemum crystallinum*, was fractionated by using semi-preparative HPLC. The different fractions were tested for their antioxidant activity using DPPH method. One fraction exhibited a high level of antioxidant activity. The molecule responsible for this antioxidant activity was identified as 2, 6-bis (1.1-dimethylethyl)-4-methylphenol) by gas chromatography/mass spectroscopy (GC/MS).

Key words: BHT, HPLC, GC/MS, antioxidant activity, DPPH

INTRODUCTION

Solar radiation is a prerequisite for life on earth as a major source of energy driving the photosynthetic process (Edreva, 2005). Plants have evolved a range of avoidance and tolerance strategies against excess light and UV-radiation (Jansen, 2002). Light stress may trigger pyrimidine dimerization (Britt, 1996) and cause peroxide-ation of lipids, which inevitably leads to membrane damage by oxygen radical formation (Comporti, 1989; Jansen et al., 1998). Naturally generated reactive oxygen species (ROS) are molecules that can attack cell compo-nents and create several types of biological damage (Hutadilok-Towatana et al., 2006). They play important roles in the pathogenesis of various diseases in human ranging from carcinogenesis to aging (Ames et al., 1993). Many plant species have been investigated in search for novel antioxidant compounds (Pourmorad et al., 2006). It has been mentioned that the antioxidant activity of plant might be due to their phenolic compounds (Cook and Samman, 1996). There are some synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), commonly used in processed foods (Yildrim et al., 2001). However, it was suggested that these compounds have some side effects (Branian, 1975; Ito et al., 1983).

The halophyte plant *Mesembryanthemum crystallinum* L. (ice plant) (Aizoacea family) has been established as an extremely stress tolerant model system (Ibdah et al., 2002). Upon irradiation with high light irradiance, *M. crystallinum* displays a rapid cell-specific accumulation of plant secondary metabolites in the upper leaf epidermis; a phenomenon that is not detectable with salt or drought stress. *M. crystallinum* induced betacyanin formation and simultaneous formation of flavonol conjugates (Ibdah et al., 2002).

A convenient, rapid and sensitive method for the anti-oxidant screening of plant extracts is a free-radical scave-nging assay using 1, 1 diphenyl-2-picryl-hydrazyl (DPPH) stable radical spectrophotometrically. In the presence of an antioxidant, DPPH radical obtains one more electron and the absorbance decreases (Koleva et al., 2002).

The aim of this study was to identify antioxidant mole-cules from purple leaves extract of the halophyte *M. crys-tallinum.*

MATERIAL AND METHODS

Plant material

Arial parts containing betalain pigment of *M. crystallinum* were coll-

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ected from their native biotope in Monastir (Tunisia) coasts in June 2005.

Extraction

Twenty five grams of freshly leaves were homogenized with 100 ml of 80% aqueous methanol, containing 50 mM ascorbic acid. After centrifugation at 14000 x g, 4°C, 10 min, the supernatants was removed and fractionated by semi-preparative HPLC.

Chromatography

Semi-preparative high performance chromatography (HPLC)

A Shimadzu UV detector HPLC was used to separate leaves extract with a gradient elution at 0 min 10% B, 20 min 50% B, 30 min 50% B, 35 min 80% B, 40 min 80% B and 45 min 10% B; solvent A: water (0.1% formic acid) and B: 30/70 water/acetonitrile (0.1% formic acid). (Injection volume: 20 µl; detection at 540 nm).

Gas chromatography / mass spectroscopy (GC/MS)

A Hewlett 5890 Packard series II gas chromatograph with a detector of mass spectroscopy 5972 series was used to separate the extract fraction which contains the maximum of antiradical activity. A HP-Innowax 30 m x 0.25 mm x 0.25 µm polar columns was used; hold 1 min, 5°/min to 250°C.

Radical scavenging activity

Free radical scavenging activity of plant extract was determined by using a stable free radical, (1, 1-diphenyl-2-picryl-hydrazyl) DPPH (Blois, 1958). DPPH solution was prepared at a concentration of 0.024 mg/ml in ethanol. During assay 1 ml of the crude extract was mixed with 1 ml DPPH solution. The mixture was incubated in the room temperature for 30 min; absorbance was recorded at 517 nm (Cam spec M230/330 UV visible spectrophotometer, United Kingdom). Synthetic butylated hydroxytoluene (BHT) was used as a standard for the investigation of the antiradical activity.

The percentage of remaining DPPH•(%$DPPH_{REM}$) at the steady state was determined as follows: $\%DPPH_{REM} = 100\ C_{DPPH}/C_{DPPH\ (t=0)}$. Where $C_{DPPH\ (t=0)}$ is the initial DPPH concentration and C_{DPPH} is the DPPH concentration at the steady state.

RESULTS AND DISCUSSION

In this study, we investigated the potential antiradical activity of extract from purple leaves of the halophyte plant *M. crystallinum.* Semi-preparative HPLC was used to obtain different fractions of the plant extract at 540 nm; the six peaks were tested for their antiradical activity (Figure 1). Our results (Table 1) showed a very high anti-oxidant level in fraction 1 (1 ml = 98 ± 0.5 % of DPPH inhibition). Further, it was important to identify the molecule present in fraction 1 which seemed to be responsible for the significant anti-oxidative activity observed. The molecule was inactivated in the total extract in the presence of other molecules and therefore it was extracted with 80% methanol at acidic pH (addition of ascorbic acid) with a maximum absorption at 540 nm. As an antioxidant molecule, it is volatile (total volatilization of the fraction after freeze-drying) with a low molecular mass.

Table 1. Radical scavenging activity of fraction 1. The results referred to the activity of the synthetic BHT.

Extract	Fraction 1	BHT
% of DPPH inhibition	98±0.5	90±0.4
Quantity	1 ml	1ml= 0.5 mg

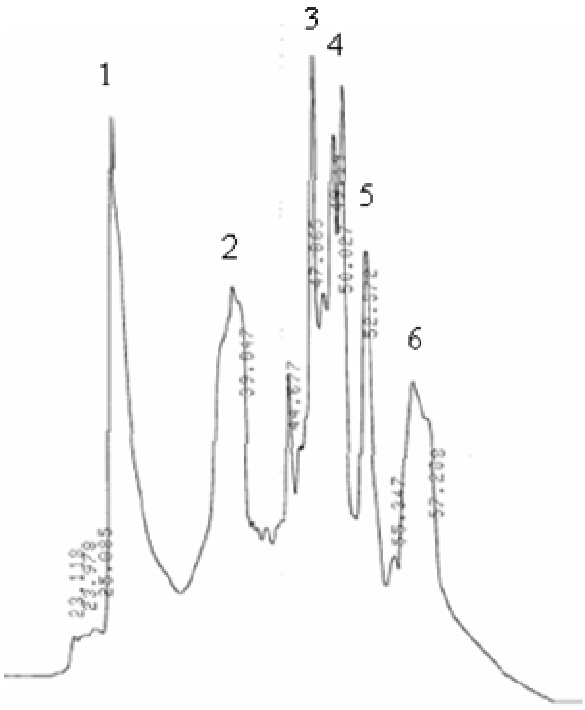

Figure 1. HPLC chromatograms of methanol extract at 540 nm.

The antioxidant molecule was identified as the 2, 6-bis (1.1-dimethylethyl)-4-methylphenol (BHT) using GC/MS (Figures 2 and 3). Synonyms: 2, 6-Di-tert-Butyl-4-Methylphenol; BHT; 2, 6-Di-tert-Butyl-1-Hydroxy-4-Methylbenzene; 3, 5-Di-tert-Butyl-4-Hydroxytoluene; 4-Hydroxy-3, 5-Di-tert-Butyltoluene; Dibutylated Hydroxytoluene; 4-Methyl-2, 6-di-t-butyl-phenol. Molecular Weight: 220.18 g/mol. Molecular Formula: $C_{15}H_{24}O$. Melting Point: 71°C. Boiling Point: 265°C. It was insoluble in water and in propylene glycol, but was freely soluble in alcohol.

Most studies on the halophyte plant, *M. crystallinum,* have focused on its physiological responses to abiotic stresses such as salinity, drought and high irradiance. This plant was found to accumulate bioactive compounds such as betacyanin and flavonol conjugates in response to light stress (Ibdah, 2002; Ibdah et al., 2002) and phenolic compounds (Ibdah et al., 2002). In our study, fractionation of the methanol extract from the purple

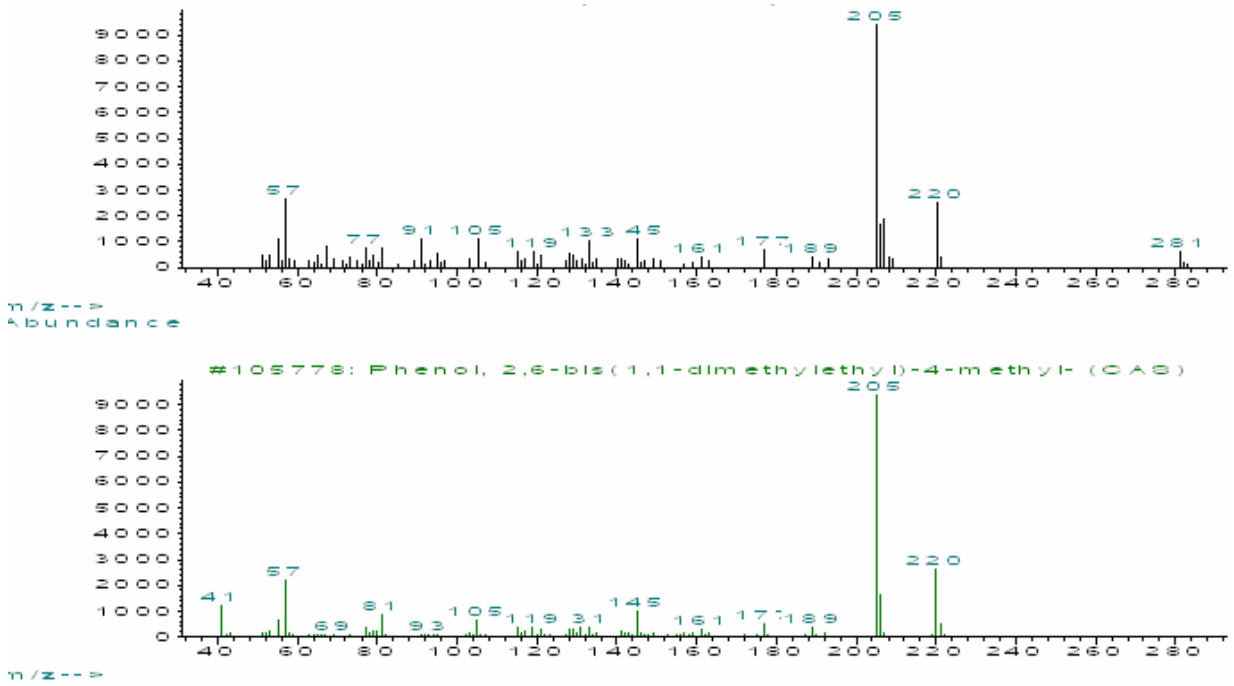

Figure 2. Mass spectra corresponding to fraction 1. The molecule identified by GC/MS was the 2,6-bis (1.1-dimethylethyl)-4-methylphenol.

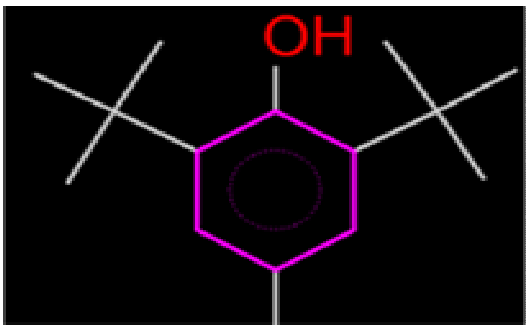

Figure 3. The 2, 6-bis (1.1-dimethylethyl)-4 methylphenol extracted from *Mesembryanthemum crystallinum* leaf tissue.

leaves of the ice plant demonstrated the presence of a phenol compound recognized as a butylated hydroxytoluene (BHT). To investigate the potential antioxidant activity of different fractions, we used the DPPH stable free radical method. It is a rapid method to survey the antioxidant activity of a specific compound or plant extracts (Koleva et al., 2002). The synthetic BHT was used as a standard; 0.5 mg/ml was needed for the scavenging activity by 90 ± 0.4%. Fraction 1 contained more than 0.5 mg/ml of BHT due to a very high potential for DPPH inhibition (1 ml = 98 ± 0.5 % of DPPH inhibition).

The synthetic (BHT) is generally used as a chemical antioxidant and a food additive. It is obtained by alkylation of *p*-cresol with isobutene or by monobutylation of *m*, *p*-cresol mixtures. This phenol derivative reacts with free radicals thereby slowing down the rate of autoxidation responsible for changes in the food color and taste. Antioxidant butylated hydroxytoluene (BHT) reduces the amount of reactive oxygen species (ROS) and prevents apoptosis in etiolated seedlings, prolongs coleoptile life span, and prevents the appearance of all apoptotic features mentioned (Bakeeva et al., 2001). In addition, BHT induces large structural changes in the organization of all cellular organelles and the formation of new unusual membrane structures in the cytoplasm (Vanyushim et al., 2004). BHT distorts mitosis resulting in the appearance of multi-blade polyploid nuclei and multinuclear cells. In roots of etiolated wheat seedlings, BHT induces differentiation of plastids with the formation of chloroplasts. Therefore, ROS controlled by BHT, seems to regulate mitosis, trigger apoptosis, and control plastid differentiation and the organization of various cellular structures formed by endoplasmic reticulum (Vanyushim et al., 2004).

It has been demonstrated that BHT is not only an antioxidant it but also possesses antiviral activity (Schwarz, 1996). BHT could inactivate herpes simplex and other lipid-coated viruses *in vitro* (Snipes et al., 1975). Studies have confirmed the activity of BHT against many different human and animal viruses, including such members of the herpes family as CMV (cytomegalovirus) (Kim et al., 1978), pseudorabies (Pirtle et al., 1986) and genital herpes (Richards et al., 1985). BHT appears to inhibit infectivity of HIV (Aloia et al., 1988). This is due to the fact that BHT is a highly potent, membrane-active antioxidant as well as a membrane fluidizer. It is known

that ROS play a role in the pathogenesis of viral infections including RNA viruses such as influenza, DNA viruses such as hepatitis B, and retroviruses such as HIV and it's been suggested that antioxidants may be useful as therapeutic agents in such infections (Schwarz, 1996).

CONCLUSION

The results of the present study are very important because we have shown that BHT, as an antioxidant, occurs naturally in leaf tissues of the halophyte plant *M. crystallinum*.

This phenolic compound exhibited a very high antioxidant activity (98 ±0.5 % DPPH inhibitions) as compared to the synthetic BHT which showed relatively less activity (90 ± 0.4 % of DPPH inhibition). Future studies need to focus on the potential medicinal proprieties of the halophyte plants. Because of their relatively high tolerance to ROS, halophyte plants are very important source of antioxidant compounds. Due to the presences of bioactive compounds in their tissues, halophyte plants can be used extensively for medical, cosmetic and industrial purposes.

ACKNOWLEDGMENTS

We are grateful to Pr. Ayadi (Center for Biotechnology, Sfax. Tunisia) and Pr. Hammami (Faculty of Medecine, Monastir. Tunisia) for their invaluable assistance.

REFERENCES

Aloia RC, Jensen FC, Curtain CC, Mobley PW, Gordon LM (1988). Lipid composition and fluidity of the human immunodeficiency virus. Proc Natl. Acad. Sci. U SA 85: 900-904.

Ames BN, Shigenaga MK, Hagen TM (1993) Oxidants, antioxidants, and the degenerative disease of aging. Proc. Natl. Acad. Sci. USA 90: 7915-7922.

Bakeeva LE, Zamyatnina VA, Shorning BY, Aleksandrushkina NI, Vanyushin BF (2001). Effect of the Antioxidant Ionol (BHT) on Growth and Development of Etiolated Wheat Seedlings: Control of Apoptosis, Cell Division, Organelle Ultrastructure, and Plastid Differentiation. Biochemistry 66: 850-859.

Blois MS (1958). Antioxidant determinations by the use of a stable free radical. Nature 181**:** 1199-1200.

Branian AL (1975). Toxicoly and biochemistry of butylated hydroxyanisole and butylated hydroxytoluene. JAOCS 52: 59-63.

Britt AB (1996). DNA damage and repair in plants. Ann. Rev. Plant Physiol. Plant Mol. Biol. 47: 75-100.

Comporti M (1989). Three models of free radical-induced cell injury. Chemico-biological Interaction. 72: 1-56.

Cook NC, Samman S (1996). Flavonoids-chemistry, metabolism, cardioprotective effects, and diatry sources. Nutritional Biochemistry 7: 66-76.

Edreva A (2005). The importance of non-photosynthetic pigments and cinnamic acid derivatives in photoprotection. Agric, Ecosyst. Environ. 106: 135-146.

Hutadilok-Towatana N, Chaiyamutti P, Panthong K, Mahabusarakam W, Rukachaisirikul V (2006). Antioxidative and free radical scavenging acivities of some plants used in Thai Folk Medicine. Pharm. Biol. 44: 221-228.

Ibdah M (2002). Lichtinduzierte Flavonoid und Betacyanakkumulation in *Mesembryanthemum crystallinum*. PhD thesis, University of Halle.Germany.

Ibdah M, Krins A, Seidlitz H, Heller W, Strack D, Vogt T (2002). Spectral dependence of flavonol and betacyanin accumulation in *Mesembryanthemum crystallinum* under enhanced UV radiation. Plant Cell Environ. 25: 1145-1154.

Ito N, Fukushima S, Hassegawa A, Shibata M, Ogiso T (1983). Carcinogenicity of butylated hydroxyanisole in F344 rats. J Natl. Cancer Inst. 70: 343-347.

Jansen MAK (2002). Ultraviolet-B radiation effects on plants induction of morphogenic responses. Physiol. Plant 116: 423-429.

Jansen MAK, Gaba V, Greenberg BM (1998). Higher plants and UV-B radiation: balancing damage, repair and acclimation. Trends in Plant Science 4: 131-135.

Kim KS, Moon HM, Sapienza V, Carp RI, Pullarkat R (1978). Inactivation of cytomegalovirus and Semliki Forest virus by butylated hydroxytoluene. J. Infect. Dis. 138: 91-94.

Koleva II, Van Beek TA, Linssen JPH, de Groot A, Evstatieva LN (2002). Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. Phytochem. Anal. 13: 8-17.

Snipes W, Person S, Keith A, Cupp J (1975). Butylated hydroxytoluene inactivates lipid containing viruses. Science 188: 64-6.

Vanyushim BF, Bakeeva LE, Zamyatnina VA, Aleksandrushkina NI (2004). Apoptosis in plants: specific features of plant apoptotic cells and effect of various factors and agents. Int Rev Cytol 233: 135-179.

Pirtle EC, Sacks JM, Nachman RJ (1986). Antiviral effectiveness of butylated hydroxytoluene against pseudorabies (Aujeszky's disease) virus in cell culture, mice, and swine. Am. J. Vet. Res. 47:1892-5

Pourmorad F, Hosseinimehr SJ, Shahabimajd N (2006). Antioxidant activity, phenol and flavonoid contents of some selected Iranian medicinal plants. Afr. J. Biotechnol. 11: 1142.

Richards JT, Katz ME, Kern ER (1985). Topical butylated hydroxytoluene treatment of genital herpes simplex virus infections of guinea pigs. Antiviral Res. 5: 281-290

Schwarz KB (1996). Oxidative stress during viral infection: a review. Free Radic Biol Med 21: 641-9.

Yildrim A, Oktay M, Bulaloglu V (2001). The antioxidant activity of the leaves of Cydonia vulgaris. Turk. J. Med. Sci. 31: 21-27.