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Antioxidant capacity of fresh and dried rhizomes from four clones of turmeric (*Curcuma longa* L.) grown in vitro

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Abstract

Plant genotype, field conditions, and postharvest processing of turmeric (*Curcuma longa* L.) can alter the combinations of phytochemicals in rhizomes. We investigated the effects of drying upon the antioxidant potential of methanolic extracts from rhizomes of four clones of turmeric grown in vitro under controlled conditions. Antioxidant properties of microrhizomes were also compared with commercially available rhizome powder. The antioxidant capacities of extracts were assayed for their ability to scavenge the DPPH* radical and chelate ferrous iron. Tissue drying negatively affected the ability of extracts to scavenge the DPPH* radical in all four accessions tested, whereas the effect of tissue drying on ferrous iron chelating ability of extracts was cultivar specific. Fresh tissue extracts were more potent than extracts from commercially available turmeric powder in all cases for both assays. The iron chelation assay revealed that extracts from recently dried tissue were significantly more potent than extracts from aged commercially available turmeric powder. DPPH* scavenging capacity of the dried tissue was usually of similar intensity to the commercially available powder with only one clone showing a significant difference in potency. Commercial drying methods may have negative effects on the antioxidants present in the rhizomes of turmeric. Genotypic selection minimized this effect. In vitro prepared rhizomes are a readily available source of fresh turmeric tissue from controlled environments irrespective of uncertainty associated with international outsource of seasonal, agricultural products.

Keywords: Antioxidant; Chelating; Curcumin; DPPH*; Microrhizome; Turmeric

1. Introduction

Turmeric (*Curcuma longa* L.) has been used for preparing traditional Indian curries for hundreds of years as a flavor, color, and preservative. Commercially, it is traded as a dye, spice, and source of industrial starch. For example, the characteristic yellow-orange curcuminoids found in rhizomes are used to make a yellow food and textile dye. Recently, it has attracted much attention due to its significant medicinal potential.

India produces about 400,000 t of fresh weight per year (Wardini and Prakoso, 1999) or about 80% of the world's supply of commercial turmeric. This tonnage is produced in an area of approximately 50,000 acres (derived from Balachandran et al., 1990). The rhizomes are harvested and dried before shipment around the world (Bruneton, 1999). Ayurvedic medical systems have different uses for both the fresh and dried preparations with dried powders being used to treat distinctly

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different ailments from pastes or plant juices (Kapoor, 1990; Parrotta, 2001).

This plant boasts a plethora of medicinally active chemicals with over 500 distinct activities in animal systems from over 100 distinct secondary metabolites (Duke, 2004). Major activities of turmeric chemical constituents include significant antioxidant capability. Potent fungicidal activities against phytopathogenic fungi have been demonstrated in greenhouse settings (Kim et al., 2003). Chowdhury et al. (2000) showed insecticidal properties of turmeric extracts. Other activities of turmeric constituents that have been demonstrated include their capabilities to fight Alzheimer's disease, arthritis, allergies, digestive ailments, depression, and cancer.

Much work has been carried out on the antioxidant and related anticancer activities of compounds derived from turmeric rhizomes. The curcuminoids [i.e., curcumin (1,7-bis(4-hydroxy-3-methoxyfenil)-1,6-heptadiene-3,5-dione), demethoxy-curcumin, and bis-demethoxy-curcumin] are major antioxidative compounds of turmeric. However, they are readily decomposed when exposed to bright light (Schieffer, 2002), high temperature or oxidative conditions (Buescher and Yang, 2000). In addition to the curcuminoids, other compounds possessing antioxidant capabilities include: γ-terpinene, ascorbic acid, beta-carotene, beta-sitosterol, caffeic acid, campestrol, camphene, dehydrocurdione, eugenol, *p*-coumaric acid, protocatechuic acid, stigmasterol, syringic acid, turmerin, turmeronol-a, turmeronol-b and vanillic acid (Duke, 2004). Carolina et al. (2003) reported that volatile chemicals were easily lost as a result of high temperature during the process of steam distillation. Selvam et al. (1995) isolated "turmeric antioxidant protein" that was stable upon heating. Methanolic extracts of turmeric inhibited cyclooxygenase and nitric oxide synthase, enzymes responsible for production of prostaglandins and nitric oxide. These two species are often credited in controlling many detrimental processes such as inflammation and carcinogenesis (Hong et al., 2002). Further studies revealed that curcumin was a potent quencher of singlet oxygen species (Das and Das, 2002). Curcumin may suppress cancer development by helping inhibit enzymes that lead to tumor production (Surh, 2002). Curcumin also prevents cancer along with inflammation by inducing production of enzymes used to detoxify electrophyllic species produced in lipid peroxidation (Piper et al., 1998). Verma et al. (1997) demonstrated that curcumin inhibited the growth of human breast cancer cells. In addition, turmeric inhibited production of tumors induced by carcinogenic benzo(*a*)pyrene (Thapliyal et al., 2002).

Turmeric plants are sterile triploids, and their field plantings must be maintained by repeated vegetative propagation of the subterranean rhizome. In typical cultivation, the divisions are grown for 2 years, but field culture is reduced to 12 months when the entire mother rhizome is planted (Wardini and Prakoso, 1999). Replanting subterranean divisions in tropical soils spreads fungal and bacterial diseases including bacterial wilt, *Pythium* rot, and *Fusarium* yellows, along with various leaf spots and blights from nurseries to production fields. In vitro propagation by crown divisions of leafy shoots on semi-solid agar media has facilitated dispersal of clean planting stock. More recently, in vitro microrhizomes were developed in liquid medium. Increased sucrose levels correlated with rhizome size, and larger microrhizomes were capable of survival in the field without any acclimatization (Shirgurkar et al., 2001; Salvi et al., 2002). A system of larger culture vessels with slow, gentle agitation has been shown to produce large microrhizomes of turmeric with size related to uptake of sugar and water (Adelberg and Cousins, 2006).

The objective of this study was to examine in vitro grown microrhizomes for the presence of antioxidant properties. Microrhizomes were dried to estimate changes in potency during processing. We assayed four clones grown under identical conditions to determine whether the results could be generalized for different turmeric genotypes.

2. Materials and methods

2.1. Chemicals

Ferrozine, ferrous chloride ($FeCl_2$), Tris–HCl and 2,2-diphenyl-1-picrylhydrazyl (DPPH*) were purchased from Sigma Chemical Co. (St. Louis, MO). Butylated hydroxyl toluene (BHT) was purchased from ACROS (NJ). Methanol was obtained from Fisher Scientific (Suwanee, GA). Ethylenediaminetetraacetic acid (EDTA) was purchased from JT Baker Chemical Co. (Phillipsberg, NJ).

2.2. Plant material

Four accessions of turmeric, *C. longa* L. (L22-5, L35-1, L43-4, and L50-3) were obtained from The University of Arizona, Southwest Center for Natural Products Research and Commercialization. Stage I explants were prepared by dissecting the quiescent shoot tips from rhi-

zomes, immersing in full-strength commercial bleach, and plating on hormone free MS (Murashige and Skoog, 1962) media in Petri plates. Stage II shoot cultures were maintained for at least 6 months by sub-culture every 6 weeks in liquid medium-modified MS with addition of 170 mg NaH_2PO_4, 100.0 mg *myo*-inositol, 0.25 mg nicotinic acid, 0.25 mg pyridoxine hydrochloride, 0.05 mg thiamine hydrochloride, 60 g sucrose, and 1 μmol benzyladenine (BA) per liter. Media pH was adjusted to 5.7 before being dispensed. Three to four bud explants were placed in 180 ml glass vessels containing 30 ml of liquid tissue culture media and cultured on an orbital shaker (100 rpm) with 25–35 $\mu mol\,s^{-1}\,m^{-2}$ PAR provided by cool white fluorescent tubes with 16 h d^{-1} photoperiod at 24 ± 2 °C.

Following the 5 week period in the jars, the plants were moved to a thin-film rocker system (Adelberg, 2004). The media used was modified MS media of the same formulation as used in the shake flask culture system. Buds (12–18) were placed in each box along with 200 ml of media. Additional media (100 ml) formulated previously was added to the vessels twice during the experiment, once at 12 weeks and again at 15 weeks.

2.3. Tissue harvesting and processing

All plants were harvested after 17 weeks. Media volume, concentration of sucrose remaining in the media, and numbers of buds were measured. Elongated leaves, rhizomes, and roots were separated and massed fresh. Portions of tissue were dried (71 °C for 48 h) to calculate dry weight percentages. Twenty gram portions of rhizome tissue were separated from the bulk and frozen separately for use in fresh extractions. All rhizome tissue was stored in darkness at 20 °C prior to extraction. Following the harvest, 20 g portions of rhizome tissue were dried at 71 °C for 48 h for subsequent dry tissue extractions.

2.4. Extractions, processing, and storage

The 20 g tissue samples were thawed, shredded, and placed in cellulose extraction thimbles. The thimbles were then inserted into a Soxhlet apparatus equipped with a condenser. Methanol (250 ml) was placed in a 500 ml round bottomed flask attached to the base of the Soxhlet. The mantle was set to 40% of capacity and each extraction continued for 12 h. Extracts were cooled to room temperature and gravity filtered twice through 20 g portions of anhydrous Na_2SO_4 yielding a final extract volume of approximately 200 ml. A 50 ml aliquot for each extract was collected. Aliquots of fresh extracts were concentrated to 20 ml while aliquots of dry extracts were concentrated to 15 ml due to inherent differential solubilities. A vacuum rotary evaporator was used to concentrate the extracts. Each of the concentrated extract samples was serially diluted 50% until eight samples of differing concentrations were available for use in the assays. Concentrations ranged from approximately 26 g l^{-1} methanol to 0.2 g l^{-1} with fresh tissue extractions and approximately 35–0.3 g l^{-1} for dry tissue extractions. All concentrations were calculated on a dry rhizome mass basis. Extracts were stored in darkness at −5 °C until assays could be preformed (always less than 1 week).

2.5. DPPH* free radical scavenging

Free radical scavenging effect was determined using the free radical generator DPPH* (2,2-diphenyl-1-picrylhydrazyl) by a similar method to Yamaguchi et al. (1998). Aliquots (200 μl) of the serially diluted extract samples were placed in 12 mm × 75 mm culture tubes with 800 μl of Tris–HCl pH 7.4. One thousand microliters of 500 μM DPPH* solution were added to the resulting mixture. The reaction mixture was thoroughly mixed using a vortex and placed in the dark for 20 min. After dark treatment, the absorbance was measured with a spectrophotometer at 517 nm on a Spectronic 20 Genesys™ Spectrophotometer (Fisher Scientific, Fairlawn, NJ). Scavenging activity was calculated via Eq. (1).

$$\text{Scavenging activity (\%)} = \left(1 - \frac{\text{absorbance of sample at 517 nm}}{\text{absorbance of control at 517 nm}}\right) \times 100 \quad (1)$$

2.6. Fe^{2+} chelating effect

Ferrous iron chelating effects were measured using the method described by Decker and Welch (1990) with some modifications. This assay depended on the formation of a ferrous iron ferrozine complex to spectophotometrically monitor the iron chelating ability of the plant extracts observed in this experiment. Eight hundred microliters of the serially diluted extracts were placed in 12 mm × 75 mm culture tubes with 200 μl of 0.2 mM $FeCl_2$ and 1 mM ferrozine. The mixtures were then thoroughly shaken and allowed to stand for 10 min at room temperature. The absorbances were then measured at 562 nm on a Spectronic 20 Genesys™ spectrophotometer (Fisher Scientific, Fairlawn, NJ), and the chelating

effects were determined via Eq. (2).

$$\text{Chelating effect (\%)} = \left(1 - \frac{\text{absorbance of sample at 562 nm}}{\text{absorbance of control at 562 nm}}\right) \times 100 \quad (2)$$

2.7. Experimental design

Antioxidant activities of fresh and dried rhizome tissue from the four clones were compared in a completely randomized design. The commercially available powder served as a control and was compared with each treatment. Three reaction mixtures at each of the eight levels of serial dilution were analyzed for each of the treatments and controls. DPPH* free radical scavenging and iron chelating curves were established by fitting the data to a hyperbolic tangent function. Estimates of the EC_{50}s (extract concentration with 50% of maximum) were obtained using the maximum likelihood method, and Wald's z-test was used for all comparisons (Casella and Berger, 2001).

3. Results and discussion

3.1. Tissue harvest

Within 17 weeks, there was a more than 100-fold increase in tissue mass in vitro. Each vessel contained 165.1 ± 8.2 g of tissue, being 29.3% rhizome fresh weight irrespective of clone. Rhizomes were 10.4% relative dry weight (dry weight/fresh weight) compared with leaves being 6.9% and roots being 6.3% relative dry weight, indicative of an active partitioning of solids by the plants to the rhizome. Rhizomes had characteristic yellow-orange color and possessed a fresh aromatic odor when cut. Functional storage organs, rhizomes, had formed in vitro.

3.2. DPPH* free radical scavenging

Extracts had reduced ability to scavenge DPPH* free radicals following the drying process (Fig. 1). This negative effect of drying was common in all four accessions tested with L22-5, L35-1, L43-4, and L50-3 showing fresh EC_{50}s of 4.542, 3.150, 3.749, and 3.935 g l^{-1} and dry EC_{50}s of 9.258, 7.528, 7.122, and 5.820 g l^{-1}, respectively (Table 1). When commercially available powder was assayed against DPPH*, its EC_{50} was found to be 9.740 g l^{-1}, which was greater than the EC_{50} of any of the fresh extracts of in vitro rhizomes. Therefore, in all cases, extracts of fresh tissue were more potent. The scavenging effects of the dried in vitro extracts were similar to the scavenging effects of extracts from commercially available rhizome powder with respect to three accessions (clone EC_{50}s: L22-5—9.258, L35-1—7.528, and L43-4—7.122 g l^{-1} compared with a powdered turmeric EC_{50} of 9.740 g l^{-1}). L50-3 was significantly more potent as dried in vitro rhizome than the commercial product.

The decrease in scavenging activity of the extracts following the drying process is likely the result of several different occurrences within the tissue. Curcumin and its relative compounds have been shown to be sensitive to high temperatures (Buescher and Yang, 2000). Damage to these potent antioxidants would likely contribute to the decrease in scavenging effectiveness. Other antioxidant compounds might also be vaporized or degraded thermally through the heating process. In

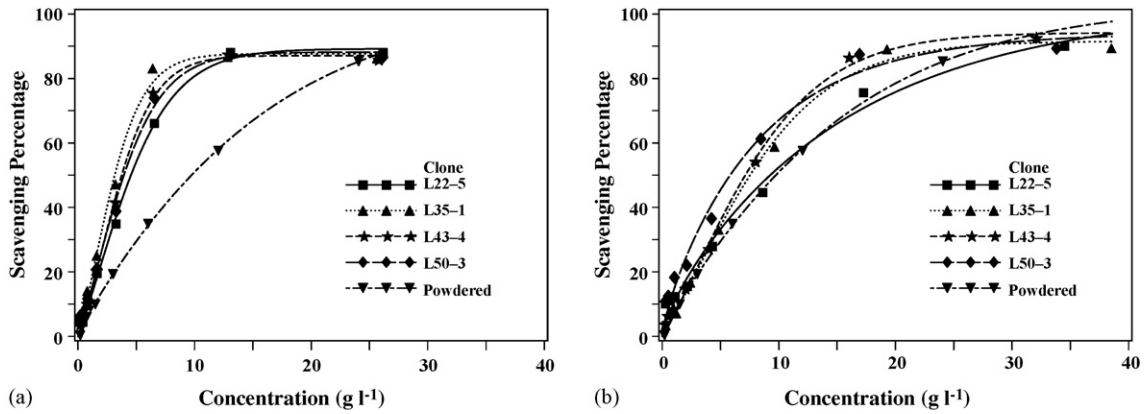

Fig. 1. DPPH* free radical scavenging curves of methanol extracts from fresh (a) and dry (b) tissue samples of four turmeric clones compared with commercially available turmeric powder.

Table 1
DPPH* free radical scavenging for four in vitro grown clones of turmeric, before and after drying, were compared to a commercially available powder by EC_{50} of methanolic extracts

Clone	Fresh		Dry		Fresh–dry	Fresh–powder	Dry–powder
	EC_{50} ($g\,l^{-1}$)	Variance	EC_{50} ($g\,l^{-1}$)	Variance			
L22-5	4.542	0.06784	9.258	6.80010	-4.716^{*}	-5.197^{***}	−0.482
L35-1	3.150	0.02229	7.528	0.13720	-4.378^{***}	-6.589^{***}	−2.212
L43-4	3.749	0.01648	7.122	0.19802	-3.373^{***}	-5.991^{***}	−2.617
L50-3	3.935	0.03975	5.820	0.72072	-1.886^{**}	-5.805^{***}	-3.919^{*}
Powder	N/A	N/A	9.740	3.49557	N/A	N/A	0.000

*, ** and, *** indicate differences significant at 0.10, 0.05, and 0.01 respectively as determined by Wald's z-test.

Table 2
Iron chelation for four in vitro grown clones of turmeric, before and after drying, were compared to a commercially available powder by EC_{50} of methanolic extracts

Clone	Fresh		Dry		Fresh–dry	Fresh–powder	Dry–powder
	EC_{50} ($g\,l^{-1}$)	Variance	EC_{50} ($g\,l^{-1}$)	Variance			
L22-5	1.046	0.00816	0.931	0.00110	0.115	-3.257^{***}	-3.372^{***}
L35-1	1.816	0.02122	0.961	0.00236	0.854^{***}	-2.487^{***}	-3.342^{***}
L43-4	1.551	0.00110	1.462	0.00494	0.089	-2.752^{***}	-2.841^{***}
L50-3	1.151	0.00239	1.227	0.00635	−0.076	-3.152^{***}	-3.076^{***}
Powder	N/A	N/A	4.303	0.29387	N/A	N/A	0.000

*, ** and, *** indicate differences significant at 0.10, 0.05, and 0.01 respectively as determined by Wald's z-test.

addition to damaging compounds directly responsible for radical scavenging, other compounds capable of providing a synergistic effect might have been damaged or removed further contributing to the decrease seen following the drying process. Yu et al. (2002) reported synergistic interactions between various isomers of linoleic acid when they were assayed together against DPPH*. Synergistic interactions have also been implicated in the still unexplained antioxidant behaviors of some Australian tea tree oil constituents (Kim et al., 2004).

3.3. Fe^{2+} chelating effect

Because elemental species such as ferrous iron can facilitate the production of reactive oxygen species within animal and human systems, the ability of substances to chelate iron can be a valuable antioxidant capability. The L22-5, L43-4, and L50-3 clones had fresh EC_{50}s of 1.046, 1.551, and 1.151 $g\,l^{-1}$ and dry EC_{50}s of 0.931, 1.462, and 1.227 $g\,l^{-1}$, respectively (Table 2). The iron chelating effect was not changed by tissue drying in the case of these three clones (Fig. 2). How-

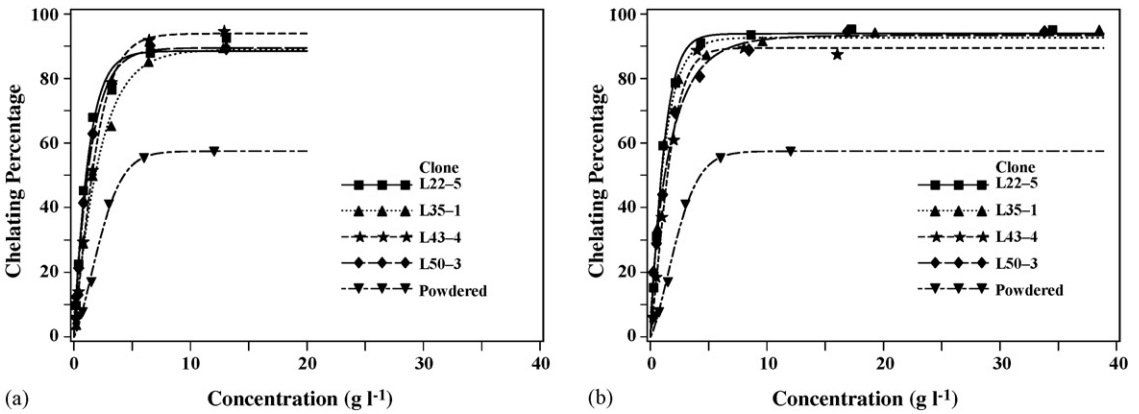

Fig. 2. Fe^{2+} chelating effect curves of methanol extracts from fresh (a) and dry (b) tissue samples of four turmeric clones compared with commercially available turmeric powder.

ever, in the case of L35-1 (fresh EC_{50}—1.816 and dry EC_{50}—0.961), tissue drying produced an extract more effective at chelating ferrous iron. These results show that the effects of tissue drying on the ability of extracts to chelate iron are dependent upon the genetics of the clone.

In all cases, fresh tissue extracts were significantly better iron chelators than extracts of commercial preparations. Extracts from commercial powdered turmeric (EC_{50}—4.303 g l^{-1}) had larger EC_{50}s (Table 2) than those from extracts of dried tissue cultured rhizomes. The powdered turmeric EC_{50} was more than 300% greater than all but one EC_{50} present for the in vitro derived tissue extracts. Thus, the commercially available powdered rhizome derived extracts were usually three times weaker than extracts of tissue cultured rhizomes.

Commercial harvest, curing, storage, and drying processes may have a detrimental effect on the ability of rhizome extracts to chelate iron. Our 2-day drying techniques, however, did not have the same negative effect. Genotypic differences are likely due to dissimilar phytochemical profiles of these tissues.

4. Conclusion

Clonal propagation in large (6-l) vessels allowed whole plants to form leaves, shoots, and rhizomes. The in vitro microrhizomes yielded potent phytochemicals following 17-weeks of culture. Turmeric, and ginger, *Zingiber officinale*, have very similar morphology, culture, and phytochemistry, and turmeric has an antioxidative activity approximately 10 times higher than that of ginger (Premavalli, 2005). In field-grown ginger, the oleoresin and (6)-gingerol content was highest at 16 weeks after planting (Ravindran et al., 2005) although economic harvest is done with 1 and 2 year old rhizomes. Despite much work in the area of in vitro secondary metabolite production in plant cells, only four commercial applications followed hundreds of attempted systems (Alfermann et al., 2003). Two plausible explanations for the disappointing results are: (1) cell culture fermentors are too expensive and (2) dedifferentiated cells do not accumulate adequate quantities of the desired compounds (Preil, 2005). We used a relatively simple device to grow potent storage organs as part of differentiated in vitro plants.

Drying fresh tissue reduced the ability of extracts to scavenge the DPPH* radical. The ability of extracts to chelate iron differs based on genetics of the plant. Many inherent variations in commercial processes across the production region including variation in drying techniques, time in the field, harvesting methods, and clone can lead to quality differences in the final products. Some researchers such as Sasikumar et al. (2004) reported that current practices designed to detect adulteration of turmeric powders were lacking in their ability to detect contamination of products with material from other *Curcuma* species and suggested alternative means for product quality control. The use of microrhizomes to prepare experimental quantities of fresh turmeric extract for preclinical testing may eliminate variation due to genotype, field environments, and processing techniques. We propose this as a unique method to obtain quality crude extracts.

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