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Responses of cucurbitacin A and B concentrations from fruits of *Cucumis myriocarpus* and *Cucumis africanus* to drying method

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ABSTRACT

Nemarioc-AL and Nemafric-BL phytonematicides, developed from fruits of wild cucumber (*Cucumis myriocarpus* Naude) and wild watermelon (*Cucumis africanus* LF.), respectively, are highly effective in suppressing root-knot (*Meloidogyne* species) nematodes in various crops. Fruits of *C. myriocarpus* and *C. africanus* contain cucurbitacin A and B, respectively, as active ingredients in the two phytonematicides. Due to the high incidence of post-harvest decays, chopped fresh fruits have to be dried soon after harvest, but there is scant information on the appropriate drying method. The objective of this study was to determine the comparative effects of four drying methods on concentration of cucurbitacins from fruits of the *C. myriocarpus* and *C. africanus* in order to allow for the selection of an appropriate method in initial preparation of inputs for the two phytonematicides. Chopped fresh fruits were subjected to oven-, sun-, freeze- and shade-drying methods. Relative to 52°C oven-drying method, other drying methods reduced the concentration of cucurbitacin A (46 to 81%) and cucurbitacin B (44 to 85%). In conclusion, oven-drying method at 52°C had the highest concentration of both the cucurbitacins in fruits of both plant species and should, therefore, be viewed as the preferred drying method in initial processing stages of inputs for the two phytonematicides.

Key words : Allelochemicals, biopesticides, *Cucumis africanus*, *Cucumis myriocarpus*, harvest time, *Meloidogyne* species

INTRODUCTION

Nemarioc-AL and Nemafric-BL phytonematicides consistently suppressed population densities of root-knot (*Meloidogyne* species) nematodes under various conditions (Pelinganga and Mashela, 2012; Pelinganga *et al.,* 2012). The two products are produced from fruits of *Cucumis myriocarpus* and *Cucumis africanus*, respectively, indigenous to Limpopo Province, South Africa (Kristkova *et al.,* 2003). *C. myriocarpus* and *C. africanus* have attained global prominence due to their potential medicinal benefits (Mphahlele *et al.,* 2012), especially in suppression of cancerous cells (Lee *et al.,* 2010). However, fruits of *C. myriocarpus* and *C. africanus* are highly prone to post-harvest decay, with *Penicillium simplicissimum* identified (Agricultural Research Council Depository No. M-48/377 & M-48/378) as the causal agent (Mphahlele *et al.,* 2012). Post-harvest decay is not unique to fruits of the *C. myriocarpus* and *C. africanus*, because in most botanicals the incident affects both quantity and quality of plant produce and its related products (Danso-Boateng, 2013).

Over many centuries, drying methods served as the initial process in preparation of botanicals and thereby, the preservation of the related active ingredients (Lusia *et al.,* 2015). Drying lowers moisture content preventing microbial degradation (Oztekin and Martinov, 2007). Drying methods can either be thermal (sun- and oven-drying) or non-thermal (shade- and freeze-drying) methods (Chan *et al.,* 2008; Lusia *et al.,* 2015). However, drying can result in unintended consequences by destroying potent chemicals through biochemical degradation and/or volatilization of targeted active ingredients (Sharma and Prasad, 2003). The unintended consequences could result in the reduction of bioactive compounds thereby

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affecting the quality of the products. The reduction of target active ingredients is undesirable because this may affect the doses and dosage, thereby compounding the unintended consequences. For example, at low concentrations, cucurbitacins consistently stimulate growth, whereas at high concentrations they inhibit growth of organisms (Damalas, 2011; Gobinda *et al.,* 2014).

The choice of a drying method is particularly important when the target chemical compounds are not thermo-stable (Hermann, 1995; Bravo, 1998). Cucurbitacins A and B are believed to be thermo-stable (Gry *et al.,* 2006; Shadung *et al.,* 2015). In most medicinal plants, low drying temperatures from 30 to 50°C are preferred in order to protect sensitive active ingredients (Muller and Heindl, 2006). Temperature for quantification of tannins in plant materials through ovens was optimised at 52°C (Makkar, 1991). Although cucurbitacins are not related to tannins, chopped fruits of the *C. myriocarpus* and *C. africanus* had been dried at 52°C in air-forced ovens (Mashela, 2002). The efficacy of oven-drying *C. myriocarpus* and *C. africanus* fruits at 52°C in relation to the concentration of cucurbitacins had not been compared with those of other available drying methods. The objective of this study was, therefore, to compare the relative efficacy of the oven-drying method to other drying methods on the concentration of cucurbitacins from fruits of *C. myriocarpus* and *C. africanus* in order to allow for the selection of an appropriate drying method.

MATERIALS AND METHODS

Study Site

Fruits from the *C. myriocarpus* and *C. africanus* from cultivated fields were harvested in April 2015 at the Green Technologies Research Centre (GTRC), University of Limpopo, South Africa (23°53′10"S, 29°44′15"E). The location was characterized by Hutton sandy loam (65% sand, 30% clay and 5% silt) containing 1.6% organic C, with electrical conductivity of 0.148 dS/m and pH of 6.5. Summers are hot and dry, with day maximum temperatures ranging from 28 to 38°C. The average annual rainfall is less than 500 mm.

Raising of Plant Materials

The *C. myriocarpus* and *C. africanus* were raised in parallel fields, each containing five plots (1 × 1 m) of both *Cucumis* species greenhouse-raised seedlings. Three days after transplanting, fertilizer application for each plant was 3 g 2 : 3 : 2 (22) NPK fertilizer mixture to provide a total of 186 mg N, 126 mg K and 156 mg P per ml water and 2 g 2 : 1 : 2 (43) fertilizer mixture which provided 0.35 mg N, 0.32 mg K and 0.32 mg P, 0.9 mg Mg, 0.75 mg Fe, 0.075 mg Cu, 0.35 mg Zn, 1.0 mg B, 3.0 mg Mn and 0.07 mg Mo per ml water. Plants were irrigated every other day using overhead sprinklers. Forty fruits from each plot were harvested, cut into pieces and divided into four equal portions, stacked in cooler boxes for further analysis.

Experimental Design and Drying

Portion per plot was plot in a completely randomized design within each drying method, with five replications. The materials on five plastic saucers were oven-dried in an air-forced oven at 52°C (EcoTherm, Labotech, Cape Town, South Africa) for 72 h. Under sun-drying, five separate portions of fruit were uniformly spread on plastic pot saucers, occasionally turned and left to dry under direct exposure to sunlight for three days. At night, the materials were covered to ensure that they were not moistened by dew. The remaining five portions were placed in a table top freeze dryer (Ilshin Lab Co. Ltd, Wilmington, Delaware, USA) and allowed to dry for three days at-45ºC. For shade-drying, the prepared materials were left in the shade for five days, but were also covered at night. After drying, the materials were ground in the Wiley mill to pass through 1 mm sieve.

Extraction of Cucurbitacins

A 4 g sub-sample of dried crude extracts of fruit was extracted with 100 ml methanol and dichloromethane [1:1 (v/v)] solution on a rotary evaporator (Rotavapor Model R-205, Buchi Labortechnik, Essen, Germany) set at 60 rpm at 40°C for 4 h. After extraction, sub-samples were concentrated by reducing the volume to 30 ml under reduced pressure on a rotary evaporator and then 1 ml aliquot centrifuged at 2422 gn for 10 min before

filtering through 0.22 µm-pore filter (Miller; Sigma-Aldrich, Johannesburg, South Africa). Concentrations of cucurbitacin were quantified using the isocratic elution Shimadzu high performance liquid chromatography (Prominence Model LC-10 AD VP; Shimadzu, Kyoto, Japan) with detection using a diode array detector (CTO-20A; Shimadzu). Quantification was performed in a wide pore reverse phase C18 (25 cm × 4.0 mm, 5 µm) discovery (Sigma-Aldrich, Milan, Italy) using 2:3 (v/v) methanol and deionised water as a mobile phase at a flow rate of 1.0 ml/min in an oven at 35°C, with wavelengths monitored at 230 nm for 43 min.

Data Collection

Quantification of cucurbitacins A and B was accomplished by comparing the retention times and peak areas under that of the samples to those of pure (98%) cucurbitacin A and B standards (Wuhan ChemFaces Biochemical Co. Ltd., Wuhan : China), which were dissolved in methanol and prepared in serial dilutions of 0.02, 0.04, 0.06, 0.08 and 1.0 µg/ml.

Data Analysis

Cucurbitacins A and B data were subjected to analysis of variance procedure using SAS software (version 9.2; SAS Institute, Carry, NC). When treatments were significant (P≤0.05), the sum of squares were partitioned to determine the percentage contribution of sources of variation to total treatment variation (TTV) in concentrations of the two cucurbitacins. Mean separation was achieved using Fisher's least significant difference test.

RESULTS AND DISCUSSION

Cucurbitacin A

The four drying methods had significant (P≤0.05) effects on cucurbitacin A, contributing 43% total treatment variation in cucurbitacin A (data not shown). Oven-drying at 52°C resulted in significantly (P≤0.05) higher concentration of cucurbitacin A than that under the shade-drying method but was not different from sun- and freeze-drying (Table 1). Relative to oven-drying method, sun-, freeze- and shade-drying methods reduced concentrations of cucurbitacin A by 73, 44 and 85%, respectively.

Cucurbitacin B

The four drying methods also resulted in significant (P≤0.05) effect on cucurbitacin B, contributing 40% total treatment in concentration of this variable. Similarly, relative to oven-drying, sun-, freeze- and shade-drying methods reduced cucurbitacin B by 81, 60 and 46%, respectively. In contrast to cucurbitacin A, the oven-drying method resulted in significantly higher concentration of cucurbitacin B than the sun-drying method, but both did not have significant effects when each was compared with freeze- and shade-drying methods. In all drying methods, a unit mass of fruit produced high concentrations of cucurbitacin B when compared to cucurbitacin A.

Cucurbitacins in fruits of *C. myriocarpus* and *C. africanus* resulted in the highest concentrations under the drying method, which was consistent with the view that cucurbitacins were thermo-stable (Chen *et al.,* 2014). This thermo-stability can also provide adequate explanation for the comparative reduced concentrations of both cucurbitacins under freeze- and shade-drying methods. Chen *et al.* (2014) observed that eight enzymes were involved in catalysing precursors of cucurbitacins through mevalonic acid pathway (MVA). Our view (Shadung *et al.,* 2015; Shadung *et al.,* 2016) had been that the eight

Table 1. Influence of four drying methods on cucurbitacins A and B from fruits of *Cucumis myriocarpus* and *Cucumis africanus,* respectively (n=20)

Drying method	Cucurbitacin A		Cucurbitacin B	
	Y-value (µg/ml)	Relative impact[y] (%)	Y-value (µg/ml)	Relative impact (%)
Oven	1.1780[a]±0.103	-	3.1980[a]±0.655	-
Sun	0.3200[ab]±0.081	-73	0.5860[b]±0.274	-81
Freeze	0.6560[ab]±0.418	-44	1.2740[ab]±0.814	-60
Shade	0.1660[b]±0.096	-85	1.7400[ab]±0.465	-46

Relative impact = (treatment/Oven-dried - 1) × 100.
Column means followed by same superscript were not different at P≤0.05.

catalytic enzymes were thermo-stable when drying occurred at 52°C and the activities of MVA remained inhibited so that stable molecules, namely, cucurbitacins were formed. Our proposed hypothesis could also explain the observed reduced concentrations of cucurbitacin under freeze- and shade-drying methods. Under the two methods, the related catalysing enzymes were relatively inactive at low temperatures. In contrast, under the sun-drying method, volatilization of constituents required for biosynthesis of various precursors in the cucurbitacins might have interfered with bioactivities of MVA.

Drying below 60°C has negligent effects in denaturing enzymes responsible for catalysing acetyl coA inside the mitochondrion (Sukrasno, 2014), which is an important input in the transformation process of the cucurbitacins (Chen *et al.,* 2014). All three pathways used by secondary metabolites, namely, shikimic-, malonic- and mevalonic-acid pathways, are intended to modulate the quantities of acetyl-coA that enter the Krebs cycle (Mashela and De Waele, 2015). Temperatures between 30 and 50°C had been recommended for drying most medicinal plants (Muller and Heindl, 2006), with the drying time being inversely proportional to increasing temperatures (Sharma and Prasad, 2003). During early stages of exposure to low temperatures, cellular respiration increased (Bowsher and Tobin, 2001), thereby increasing precursors for secondary metabolites in all pathways. However, high temperatures denature enzymes required to drive the secondary metabolite processes for thermo unstable compounds and also increase the volatilization of others, thereby reducing concentration of targeted active ingredients (Makkar, 1991). However, thermo-stable chemical compounds behave differently.

Cucurbitacin A ($C_{32}H_{46}O_9$) from *C. myriocarpus* fruit and cucurbitacin B ($C_{32}H_{48}O_8$) from *C. africanus* fruit (Chen *et al.,* 2005), had boiling points at 731 and 699°C, respectively, at 760 mmHg (Krieger, 2001). When fruits from *C. myriocarpus* and *C. africanus* were dried at 52°C for 72 h and stored at room temperature in air-tight sealed and unsealed containers, in support of the thermo-stability hypothesis, the concentrations of cucurbitacins A and cucurbitacin B continued to increase quadratically over a six-month-storage period (Shadung and Mashela, 2017). Similar increases were observed in sun-dried aerial parts of thyme (*Thymus daenensis* Celak), where thymol and carvacrol increased during a 3-month-storage period at room temperature (Rowshan *et al.,* 2013). Organs of thyme contained essential oils such as thymol ($C_{10}H_{14}O$) and carvacrol ($C_{10}H_{14}O$) as active chemical compounds, which are monoterpenes (Zarshenas and Krenn, 2015). In contrast, oven-drying at 45°C for 48 h decreased flavonoid contents in Centella (*Centella asiatica* L.) organs (Mohd *et al.,* 2009).

Quantitatively, regardless of the drying method, per unit mass *C. africanus* appeared to contain large quantities of cucurbitacin. In *C. africanus*, cucurbitacin B is accumulated in all organs of the plant, whereas cucurbitacin A in *C. myriocarpus* is accumulated in fruits and roots only (Jeffrey, 1978). The standards used in quantifying cucurbitacins A and B in the current study could out detect other chemical compounds in the sub-sample. Cucurbitacin A is generally not stable and disintegrates into two bioactive chemical compounds, cucumin ($C_{27}H_{40}O_9$) and leptodermin ($C_{27}H_{38}O_8$) (Rimington, 1938), which could explain consistent low values of cucurbitacin A under various drying methods.

CONCLUSION

Oven-drying method at 52°C for 72 h when compared to other drying methods, resulted in relatively high concentrations of cucurbitacins A and B. Therefore, the oven-drying method could be viewed as the appropriate drying method for preparing fresh fruits of the *C. myriocarpus* and *C. africanus* for the manufacturing of Nemarioc-AL and Nemafric-BL phytonematicides, as well as for use in other industries.

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