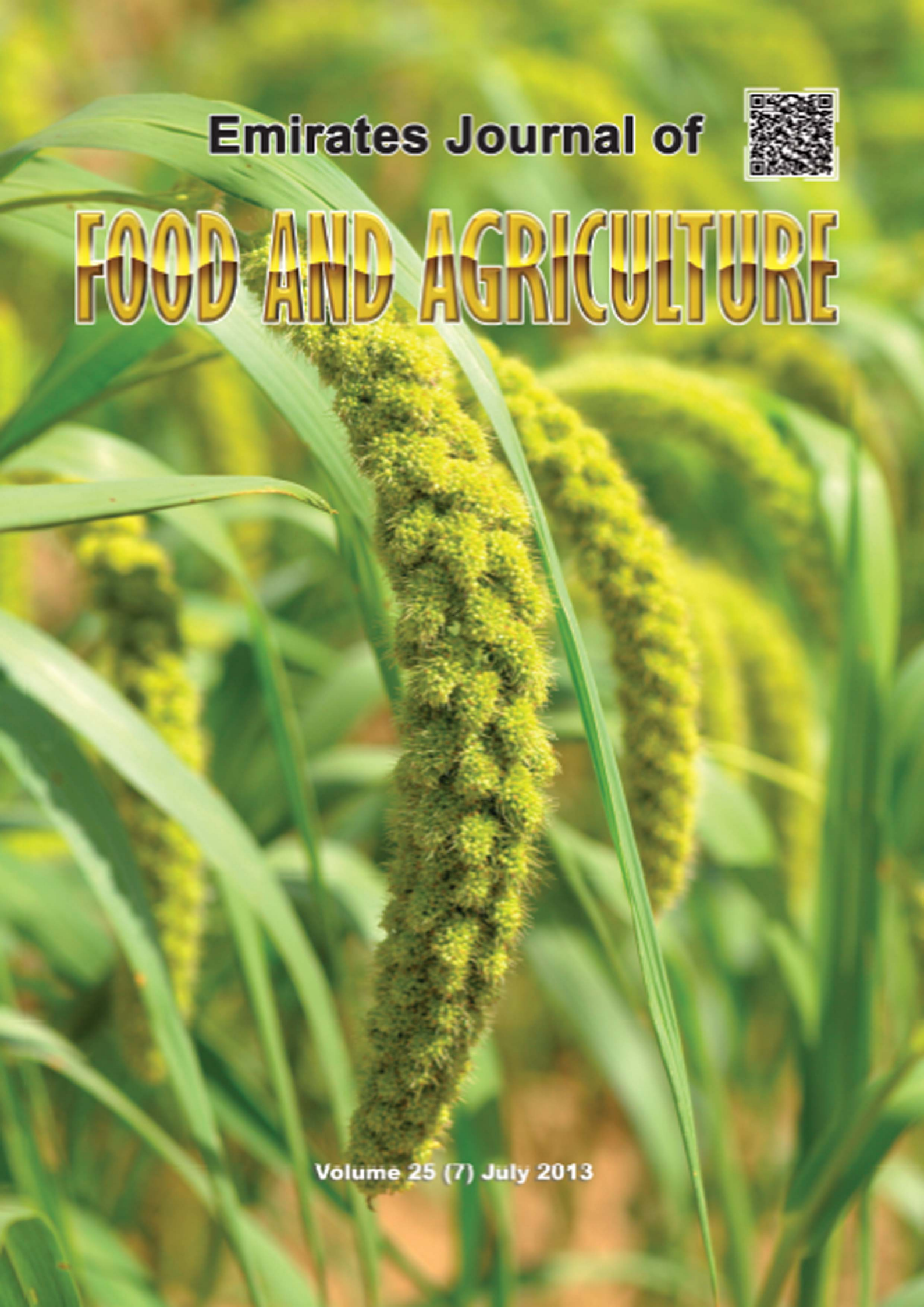
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FOOD SCIENCE AND NUTRITION

Seed composition of two chia (*Salvia hispanica* L.) genotypes which differ in seed color

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Abstract

The objective of this study was to investigate the relationship of seed color with protein, oil, fiber, amino acids, and antioxidants content composition of two chia (*Salvia hispanica* L.) genotypes. Study was carried out using chia genotypes known as Tzotzol and Iztac; the first has black-spotted seed, the second white seed. Results: the lack of significant ($p<0.05$) difference on biochemical compounds between Tzotzol and Iztac genotypes found in this study could be explained by the small genetic difference between these two genotypes. Conclusion: In summary, this paper showed no relationship of the seed coat color for all measured traits, protein, oil, fiber, amino acids, and antioxidants content composition. In addition, during this work it was found secoisolariciresorcinol diglucoside, an antioxidant not previously reported for this species which perhaps contributes to the stability of chia seed oil.

Key words: Antioxidants, Chia, Lignans, Phenolics, *Salvia hispanica*

Introduction

Salvia hispanica L., with the popular name chia, is a low water user plant and well adapted to arid and semiarid climates (Ayerza, 1995). It is currently not grown on a large scale. Nevertheless, due to the universal applicability of the products, the crop deserves high attention. The specific composition of chia seeds appears very attractive. Chia oil is highly unsaturated. The content of polyunsaturated fatty acids (PUFA) amounts to about 83%. The ratio of linoleic acid (18:2n-6)-(LA) with about 18%, and α-linolenic acid (18:3n-3)-(ALA) with about 64%, is unique between the common vegetable oils, such as soya (*Glycine max* L.) oil, sunflower (*Helianthus annuus* L.) oil, rape (*Brassica napus* L.) oil, olive (*Olea europaea* L.) oil, etc. Chia oil is also qualitatively different from the less common vegetable oils with a high content of PUFA such as flax (*Linum usitatissimum* L.) with the content of 53.3% ALA (Bhatty, 1993). In addition, chia seed has a significant content and composition of protein, antioxidants, and dietary fiber (Ayerza and Coates, 2005a).

In recent years chia seed has become increasingly important for human and animal health and nutrition because of its high content of α-linolenic fatty acid, and the beneficial health effects that can arise from its consumption (Ayerza and Coates, 2005b, 2007; Vuksan et al., 2007; Espada et al., 2007; Chicco et al., 2008; Jeong et al., 2010; Ayerza, 2011).

It is theorized that the chia seeds commercialized today were selected by Nahua botanists, but came into the twenty-first century as a mixed population. This mixture continues to be grown by the descendants of the Nahua and Maya nations living in the mountains of Southern Mexico, Northern Guatemala, and Nicaragua. Seed coat color in chia ranges from black and black spotted to white. However, chia commercialized today is mainly black-spotted, followed by a low but increasing percentage of white seeds (Ayerza and Coates, 2005a).

Recently, Ayerza (2011) reported no significant differences in oil content and fatty acids profile between spotted-black seeds and white seeds of chia grown in 5 different ecosystems. However, chia is also a good source of protein, antioxidants and fiber (Taga et al., 1984; Weber et al., 1991; Reyes-Caudillo et al., 2008) and the possibility of genotype variability for these seed components

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needs to be explored. The objective of this study was to determine if differences in protein, oil, fiber, amino acids and antioxidant concentrations occur for two chia genotypes which differ in seed color, and to identify the different antioxidants which exist in this seed.

Materials and Methods

This study was carried out using white and black-spotted chia seeds commercially grown in a Tropical Forest ecosystem located in Ecuador. The black-spotted and the white seeds belong to the Tzotzol and Iztac genotypes, respectively, as was reported by Ayerza and Coates (2009a).

Within the area where the chia was grown, representative commercial fields were selected for sampling (Figure 1). Samples were collected from the combine after mechanical harvesting, following the seed sample instructions of the Canadian Food Inspection Agency (2008). The samples were cleaned by hand and sent to the laboratory for analysis. The experimental design used was completely randomized, with six replications.

Figure 1. A view of *Salvia hispanica* L. commercial plantation.

Chemical analysis

Crude nitrogen of the chia seed samples was determined by standard micro-Kjeldahl method, and then converted to protein content using a 5.71 conversion factor (AOAC, 1995).

Lipids were extracted from the samples according to the method described by Folch et al. (1957). Total lipids were then converted into fatty acid methyl esters using the IRAM 5-560II method (IRAM, 1982), which is equivalent to ISO 5509-1978 item 6 (ISO, 1978). Fatty acid methyl esters were separated and quantified by automated gas chromatography (Model 6890, GC; Hewlett Packard Co., Wilmington, DE, USA) equipped with flame ionization detectors and 30m 9 530-lm i.d. capillary column (Model HP-FFAPFree fatty acid phase; Hewlett Packard Co., Wilmington, DE, USA). The temperatures of the oven, injector, and detector were set at 180, 290 and 330°C, respectively. The fatty acid composition of each sample was determined by integrating the recorded peaks using Hewlett-Packard Chem-Station Software. Results were expressed as percentage of total fatty acids.

The peroxide values were determined by ISO 3960/1977 procedure; results were expressed as meq oxygen/kg (AOAC, 2002).

Flavonoid analysis performed using HPLC by methodology adapted from Chang et al. (1997); utilizing water-acetonitrile (80:20) extract separated on a LiChrospher RP-18 column (Merck Chemicals, Basel, Switzerland), with mobile phase gradient elution of water-acetonitrile (0-10 min 80:20, 14-25 min 63:37) employing a flow rate of 1.0ml/min with detection at 270 nm. Caffeic acid analysis performed using HPLC by method adapted from Adzet and Puigmacia (1985); utilizing sample extracted into acetone and subjected to chromatography on a column (150x4.5mm) of Spherisorb C18 (µm) (Waters Corporation, Milford, MA, USA), eluted with a gradient mobile phase of 2.5% of acetic acid in aq. methanol with a linear gradient of 13 to 43% of methanol during 30 min, and detection by photo diode array (200-40nm) with UV detection at 325 nm. Isoresorcinol analysis performed using HPLC by method adapted from Charlet et al. (2002); utilizing acid hydrolysis, necessary for the release of lignan from their complex form to form free aglycone, subjected to separation on a Waters Symmetry C18 3µm column (150x4.6mm) (Waters Corporation, Milford, MA, USA) eluted with a gradient mobile phase of water (95%), acetonitrile (5%) changing linearly in 20 min to water (50%), acetonitrile (50%), with diode array detection. Amino acid analysis performed using HPLC following derivatization according to the AccQtag methodology (Waters Corporation, Milford, MA, USA) using 20mM HCl, Borate buffer, and AQC reagent in acetonitrile (1:3:1, v/v/v), followed by HPLC using Waters Extera C18 column (150x3.5mm, 3µm) (Waters Corporation, Milford, MA, USA), 40ºC, with isocratic mobile phase consisting of 20mM Potassium phosphate, pH3.0/Acetonitrile (95:5) 1.5ml/min with UV detection (254nm). Fiber analysis performed using AOAC 941.2 (1997) method. Insoluble fiber determined by difference following organic solute removal.

Statistical analysis

A one-way analysis of variance (ANOVA) was performed for oil, individual fatty acid content, individual amino acid content, protein content, soluble and insoluble fiber content, and peroxide value. When the F value was significant (p<0.05), means were separated using the least significant difference test (LSD) (Cohort, 2006).

Results and Discussion

Total water, protein content, oil content, peroxide value, and fiber contents are summarized in Table 1. The values of all measured parameters differed very little between the two genotypes. The differences in the analytical values were not statistically significant. All these parameters had results within the limits of values reported by Ayerza (1995, 2011) and Ayerza and Coates (2005b, 1999, 2004) for the same genotypes grown in a number of different sites of Argentina, Colombia, Bolivia, Ecuador and Peru.

Chia is a good source of dietary fiber (Table 1) and can be favored compared with traditional sources of fiber as barley (17.3%), corn (13.4%), wheat (12.6%), soybeans (15%), flaxseeds (22.33%), and sesame seeds (7.79%) (Dhingra et al., 2012). The importance of food fibres has led to the development of a large and potential market for fibre-rich products and ingredients and in recent years, there is a trend to find new sources of dietary fibre that can be used in the food industry (Chau and Huang, 2003).

Results of the fatty acid compositional analyses by genotype are presented in Table 2. Analysis of variance showed that the differences in fatty acid content were not statistically different between genotypes. The lack of significant (p<0.05) difference in fatty acid profile, between these two genotypes grown in five different ecosystems was recently reported. Ayerza (2010) demonstrated that the larger differences found in oil content and fatty acid composition are due to location (because of the environmental differences) rather than chia seed coat color. In addition, the fatty acid profile found herein are similar to that reported by a number of papers (Ayerza, 1995, 2010; Ayerza and Coates, 2009a).

Antioxidant content and composition in the Tzotzol and Iztac genotypes are presented on Table 3. Analysis results of the flavonols compounds showed the presence of myrcetin, quercetin, caffeic acid and chlorogenic acid in both white and black-spotted color seeds. Statistical analysis of the data showed that antioxidants content and composition differences between both genotypes were not significant. These flavonols compounds found in chia have been shown to posses consistently strong antioxidant properties (Castro-Martinez et al., 1986; Reyes-Caudillo et al., 2008; Taga et al., 1984).

Table 1. Water, protein, oil, fiber and peroxide values of seeds from two *Salvia hispanica* L. genotypes.

Genotype	Water %	Protein	Oil	Peroxide Value meq of O_2/kg	Fiber (g/100g) Soluble	Insoluble	Total
Tzotzol	5.7[a1]	19[a]	34.2[a]	0.61[a]	10.76[a]	12.43[a]	23.19[a]
Iztac	5.4[a]	18.8[a]	32.1[a]	0.82[a]	9.68[a]	16.27[a]	25.94[a]
SD[2]	2.541	5.082	5.08	1.91	8.334	10.765	4.275

1 Means in a column within a group with the same letter are not statistically different; 2Least significant difference for p<0.05.

Table 2. Fatty acid composition of *Salvia hispanica* L. Tzotzol and Iztac genotypes.

Genotype	Palmitic % of total fatty acids	Stearic	Oleic	Linoleic	Linolenic	ω-6:ω-3 rate	Linolenic mg/g of seed[3]
Tzotzol	6.5[a1]	3.65[a]	6.65[a]	17.5[a]	64.5[a]	0.29[a]	22.06[a]
Iztac	6.2[a]	4.1[a]	6.8[a]	18.4[a]	63.3[a]	0.27[a]	20.32[a]
SD[2]	2.541	1.27	1.272	2.541	7.624	0.032	5.886

1Means in a column within a group with the same letter are not statistically different; 2Least significant difference for p<0.05; 3Kilograms of seed by oil percentage by linolenic percentage.

Table 3. Antioxidant content and composition in the Tzotzol and Iztac genotypes.

Genotype	Flavonols (mg/g)					Lignans	Total
	Myrcetin	Quercetin	Kaempherol	Chlorogenic acid	Caffeic acid	SDG[3]	
Tzotzol	0.115[a1]	0.007[a]	0.025[a]	0.226[a]	0.139[a]	0.424[a]	0.934[a]
Iztac	0.121[a]	0.006[a]	0.024[a]	0.218[a]	0.149[a]	0.405[a]	0.924[a]
SD[2]	0.014	0.002	0.005	0.042	0.032	0.097	0.153

1Means in a column within a group with the same letter are not statistically different; 2Least significant difference for $p<0.05$; 3Secoisolariciresorcinol diglucoside.

The most significant finding in this chia trial was the detection of secoisolariciresorcinol diglucoside (SDG) compound in both Tzotzol and Iztac chia genotypes (Table 3). This lignan compound presence in chia seed composition was not reported before. Lignans are phenolic compounds linked to many health benefits, including cancer prevention (Crosby, 2005; Morris, 2001). Once ingested, lignans are deglycosylated and converted by bacteria in the large intestine to produce enterolactone and enterodiol, the mammalian forms of plant lignans (Kitts et al., 1999). The efficacy of SDG and particularly its mammalian lignans metabolites' enterodiol (ED) and enterolactone (EL) to act as antioxidants in lipid and aqueous in vitro model systems, at relatively low concentrations (i.e. 100 microM), potentially achievable in vivo, is an evidence of a potential anticarcinogenic mechanism of lignan SDG and its mammalian metabolites ED and EL (Kitts et al., 1999).

A number of studies have shown good oxidative stability of chia seed when used as animal feed or as food ingredient, with this being attributed to the high antioxidant activity of the flavonols compounds it contains (Ayerza and Coates, 2001, 2009b; Ayerza et al., 2002; Coates and Ayerza, 2009). This stability in animal products produced when diets containing up to 30% chia seeds were attributed to the quality and quantity of these antioxidants. However, the SDG content found herein suggests that the great oxidative stability of chia oil could be attributed not only to the flavonols compounds content but also to the SDG content.

Amino acids content and composition is presented in Table 4. Amino acids' analysis results showed no significant ($p<0.05$) differences on quantity and quality between Tzotzol and Iztac genotypes. An exception was leucine content which was 8% higher, but significantly different ($p<0.05$), in the Tzotzol seeds than in the Iztac seed's genotype. The reason for this finding is not evident from the available data. The protein quality of chia has been demonstrated to be higher than that of common cereals and oil seeds (Weber et al., 1991). The amino acids in chia have no limiting factors in adult diets (Fernandez et al., 2006).

Table 4. Amino acid concentration of two of chia seeds genotypes.

Genotype Amino acids	Tzotzol mg/g	Iztac	SD[2]
Alanine	7.41[a1]	6.93[a]	1.642
Arginine	16.34[a]	15.79[a]	1.613
Aspargine	12.29[a]	13.28[a]	1.931
Cystine	2.48[a]	2.39[a]	0.591
Glutamine	25.23[a]	25.95[a]	0.658
Glycine	6.58[a]	7.12[a]	0.687
Histidine	4.19[a]	4.19[a]	0.749
Isoleucine	5.34[a]	5.37[a]	0.795
Leucine	9.73[a]	9[b]	0.513
Lysine	7.65[a]	7.21[a]	1.216
Metionine	0.67[a]	0.63[a]	0.81
Phenylalanine	7.94[a]	7.91[a]	2.322
Proline	7.02[a]	7.25[a]	1.832
Serine	7.44[a]	6.89[a]	1.167
Threonine	5.54[a]	5.92[a]	1.208
Tryptophan	0.02[a]	0.02[a]	0.04
Tyrosine	4.73[a]	4.53[a]	0.212
Valine	8.76[a]	8.40[a]	0.773
Total	139.38[a]	138.78[a]	6.193

1Means in a row within a group with the same letter are not statistically different; 2Least significant difference for $p<0.05$.

The lack of significant difference on biochemical compounds between Tzotzol and Iztac genotypes found in this study could be explained by the reduced genetic variability between these two genotypes. Cahill (2004) using random polymorphic DNA markers, reported a loss of diversity accompanying chia domestication process and a near lack of diversity in modern commercial chia varieties.

Conclusion

In summary, this paper showed no effect of the seed coat color for all measured traits, protein, oil, fiber, amino acids, and antioxidants content and composition. In addition, this work found SDG, a diphenolic compound, which was not reported in chia seeds before, and suggest that this compound could be contributing to the chia seed oil stability.

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FOOD SCIENCE AND NUTRITION

Millets: Nutritional composition, some health benefits and processing - A Review

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Abstract

Millets are a major food source in arid and semi-arid parts of the world. Millets are good sources of energy. They provide protein, fatty acids, minerals, vitamins, dietary fibre and polyphenols. Typical millet protein contains high quantity of essential amino acids especially the sulphur containing amino acids (methionine and cysteine). Processing millet by milling removes the bran and germ layers that are rich in fibre and phytochemicals, causing significant loss. The millets are source of antioxidants, such as phenolic acids and glycated flavonoids. Millet foods are characterized to be potential prebiotic and can enhance the viability or functionality of probiotics with significant health benefits. The nutritional significance of millets demands for an examination of the nutritional characteristics and functional properties of different millet cultivars as well as developing value added products from millets.

Key words: Millets, Composition, Protein, Health benefits, Processing

Introduction

Millets are one of the cereals asides the major wheat, rice, and maize. Millets are major food sources for millions of people, especially those who live in hot, dry areas of the world. They are grown mostly in marginal areas under agricultural conditions in which major cereals fail to give substantial yields (Adekunle, 2012). Millets are classified with maize, sorghum, and Coix (Job's tears) in the grass sub-family *Panicoideae* (Yang et al., 2012). Millets are important foods in many underdeveloped countries because of their ability to grow under adverse weather conditions like limited rainfall. In contrast, millet is the major source of energy and protein for millions of people in Africa. It has been reported that millet has many nutritious and medical functions (Obilana and Manyasa, 2002; Yang et al., 2012). It is a drought resistant crop and can be stored for a long time without insect damage (Adekunle, 2012); hence, it can be important during famine.

Discrepancies exist concerning classification of family millet, with some references giving the family name *Gramineae*, and others classifying it in the family *Poaceae*. There are many varieties of millets. The four major types are Pearl millet (*Pennisetum glaucum*), which comprises 40% of the world production, Foxtail millet (*Setaria italica*) (Yang et al., 2012), Proso millet or white millet (*Panicum miliaceum*), and Finger Millet (*Eleusine coracana*). Pearl millet produces the largest seeds and it is the variety most commonly used for human consumption (Mariac et al., 2006; ICRISAT, 2007). Minor millets include: Barnyard millet (*Echinochloa* spp.), Kodo millet (*Paspalum scrobiculatum*), Little millet (*Panicum sumatrense*), Guinea millet (*Brachiaria deflexa = Urochloa deflexa*), Browntop millet (*Urochloa ramosa = Brachiaria ramosa = Panicum ramosum*), Teff (*Eragrostis tef*) and fonio (*Digitaria exilis*) are also often called millets, as more rarely are sorghum (*Sorghum* spp.) and Job's tears (*Coix lacrima-jobi*) (ICRISAT, 2007; FAO, 2009; Adekunle, 2012).

In 2007, global millet production reached about 32 million tonnes with the top producing countries being: India (10,610,000), Nigeria (7,700,000), Niger (2,781,928), China (2,101,000), Burkina Faso (1,104,010), Mali (1,074,440), Sudan (792,000),

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Uganda (732,000), Chad (550,000) and Ethiopia (500,000) (FAO, 2009). According to FAO (2005), pearl millet production attained approximately 54% of the global production in 2004.

Millets represent a unique biodiversity component in the agriculture and food security systems of millions of poor farmers in regions such as Sub-Saharan Africa. Pearl millet is an important food across the Sahel, although, India is the largest producer of pearl millet (Bhattacharjee et al., 2007). Millets are often ground into flour, rolled into large balls, parboiled, and then consumed as porridge with milk; sometimes millets are prepared as beverages. *Roti*, made from pearl millet has been the primary food of farmers in Gujarat India (FAO, 2009). There is an emerging need for the world to feed its growing population, therefore, it is important to explore plants such as millets that are grown locally and consumed by low income households in places like India and the Sahel zone (Obiana, 2003). Cereals, in particular, millet based foods and beverages are known worldwide and are still part of the major diet in most African countries (Obilana and Manyasa, 2002; Amadou et al., 2011). The present review summarizes the nutritional composition of millets some health benefits, and the use of millets in the food industry.

Nutritional composition of millet grains

Millets are unique among the cereals because of their richness in calcium, dietary fibre, polyphenols and protein (Devi et al., 2011). Table 1 represent amino acids content in different types of millets. Millets generally contain significant amounts of essential amino acids particularly the sulphur containing amino acids (methionine and cysteine); they are also higher in fat content than maize, rice, and sorghum (Obilana and Manyasa, 2002). In general, cereal proteins including millets are limited in lysine and tryptophan content and vary with cultivar. However, most cereals contain the essential amino acids as well as vitamins and minerals (Devi et al., 2011; FAO, 2009).

Plant nutrients are largely used in the food industry, and cereal grains constitute a major source of dietary nutrients worldwide (Amadou et al., 2011a; Izadi et al., 2012).

Modification of a protein is usually realized by physical, chemical, biological such as fermentation or an enzymatic treatment, which changes its structure and consequently its physicochemical and functional properties (Lestienne et al., 2007; Amadou et al., 2011b). Table 2 represent the content of different varieties of millet, foxtail, fonio, proso, pearl and finger millets.

Table 1. Amino acid profiles of different millet grains variety (Foxtail, Proso, Pearl and Finger millet).

Amino acids (g/100g)	Foxtail millet (defatted flour) (a)	Proso millet (Dehulled Grain) (b,c)	Pearl millet (true prolamine) (c)	Finger millet (native grain) (d)
Essential Amino Acid				
Isoleucine	4.59	4.1	5.1	4.3
Leucine	13.60	12.2	14.1	10.8
Lysine	1.59	1.5	0.5	2.2
Methionine	3.06	2.2	1.0	2.9
Phenylalanine	6.27	5.5	7.6	6.0
Threonine	3.68	3.0	3.3	4.3
Valine	5.81	5.4	4.2	6.3
Histidine	2.11	2.1	1.7	2.3
Tryptophan	NA	0.8	1.2	NA
Nonessential Amino Acid				
Alanine	9.30	10.9	8.1	6.1
Arginine	3.00	3.2	0.9	3.4
Aspartic acid	7.71	6.2	6.2	5.7
Cystine	0.45	NA	0.8	NA
Glutamic Acid	22.00	21.3	22.8	23.2
Glycine	2.91	2.1	0.7	3.3
Serine	4.56	6.3	5.4	5.3
Tyrosine	2.44	4.0	2.7	3.6
Proline	5.54	7.3	8.2	9.9
*PER[(b)]	0.80	1.10	1.60	2.00

*Protein Efficiency Ratio (PER). NA: not available. References: (a) Kamara, *et al.* (2009); (b) Bagdi et al., 2011; (c) Saldivar (2003); (d) Devi et al. (2011).

Table 2. Proximate composition of millet grain varieties.

Component (g/100g, dry basis)	Foxtail millet flour	Fonio whole grain	Proso millet dehulled grain	Pearl millet whole grain	Finger millet native grain
Protein	11.50	9–11	11.58	14.8	8.2
Ash	0.47	1–1.1	NA	1.64	2.7
Fat	2.38	3.3–3.8	4.9	4.86	1.8
Total CHO*	75.2	84–86	80.1	59.8	83.3
Crude fiber	NA	NA	0.7	12.19	3.5
References	Kamara et al. (2009)	Vodouhe et al. (2003)	Bagdi et al. (2011)	Taylor et al. (2010)	Devi et al. (2011)

*Carbohydrate (CHO). NA: not available.

Badau et al. (2005) reported that the phytic acid content of the unmalted pearl millet grain ranged from 2.91% to 3.30%. The total dietary fibre (22.0%) of finger millet grain were reported relatively higher than that of many other cereal grains (e.g. 12.6%, 4.6% and 12.8% respectively for wheat, rice, maize and sorghum (Shobana and Malleshi, 2007; Siwela et al., 2010). However, the dietary fibre content in pearl millet ranges between 8 to 9% (Taylor, 2004).

Bagdi et al. (2011) analyzed the composition of free and bound lipids in proso millet (*Panicum miliaceum*) flours and brans. In the free lipids, hydrocarbons, sterol esters, triacylglycerols, diacylglycerols, and free fatty acids were present (Bagdi et al., 2011). The predominant fatty acids in the free lipids were linoleic, oleic, and palmitic acids, though, in the bound lipids, monogalactosyl diacylglycerols, digalactosyl diacylglycerols, phosphatidylethanolamine, phosphatidyl serine, and phosphatidyl choline were tentatively identified (Bagdi et al., 2011). Saldivar (2003) studied the content of total lipids, lipid classes and fatty acids composition in small millets, such as foxtail millet (*Setaria italica*), proso millet (*Panicum miliaceum*), and finger millet (*Eleusine coracana*). They reported the total lipid content in the foxtail, proso, and finger millets ranged from 5.2 to 11.0% (dry basis), while it ranges from 5.1 to 8.3% in the little, kodo, and barnyard millets (Saldivar, 2003). In addition, examination of some millet cultivars from Tunisia and Mauritania by Ibrahima et al. (2004) showed that the fatty acid profile of the millet lipid is mainly characterized by the presence of high levels of linoleic, oleic and palmitic acid. However, palmitoleic acid (C16: 1), stearic acid (C18: 0) and linolenic acid (C18: 3) were less represented. Other fatty acids are identified in trace amounts (arachidic acid C20: 0, behenic acid C22: 0, erucic acid C22: 1). The work of Liang et al. (2010) presented the general properties of foxtail millet oil and its fatty acid profile. It is apparent that millet oil could be a good source of natural oil rich in linoleic acid and tocopherols (Liang et al., 2010; Amadou et al., 2011c). The main polyphenols in cereals are phenolic acids and tannins, whilst flavonoids are present in small quantities; they act as antioxidant and play many roles in the body immune system defence (Chandrasekara and Shahidi, 2010; Devi et al., 2011). Whole cereal grains are considered a rich source of fibre. However, foods from grains have marked differences in the amount and type of dietary fibre (Shukla and Srivastava, 2011). The dietary fibre content in cereal-based food varies greatly, depending on the extent of milling. Finger millet shows relatively higher than other cereals carbohydrate (72%) comprises of starch as the main constituent and the non-starchy polysaccharides which amounts to 15–20% of the seed matter as an unavailable carbohydrate dietary fibre content and complements which are the health benefits of the millet (Devi et al., 2011). The main function of dietary carbohydrate is to supply energy (Devi et al., 2011). Millets are good sources of magnesium and phosphorus. Magnesium has the ability to help reduce the effects of migraine and heart attacks, while, phosphorus is an essential component of adenosine triphosphate (ATP) a precursor to energy in the body (Badau et al., 2005; Liang et al., 2010; Devi et al., 2011).

Millet in probiotic and prebiotics foods

Probiotics aid the existing flora, or help repopulate the colon when bacteria levels are reduced by antibiotics, chemotherapy or disease. FAO/WHO stated that probiotics are “lives microorganisms which when administered in adequate amounts confer a health benefit on the host” though, this should also specify genus, species and strain level, as well as a safety assessment. Most of probiotic foods generate fatty acids, vitamins and other vital nutrients that improve the body's resistance against pathogens microorganisms (FAO/WHO, 2001; Abd El-Salam et al., 2012). Fermented foods are thus, important to human beings all over the world with between 20 to

40% of food supply being from fermented foods (Anukam and Reid, 2009; Rivera-Espinoza and Gallardo-Navarro, 2010; Amadou et al., 2011a). Lei and Michaelsen (2006) reported an interesting intervention study in Northern Ghana using spontaneously fermented millet product as a natural probiotic treatment for diarrhea in young children. Millet *koko* is an African spontaneously fermented millet porridge and drink, characterized by Lei and Jacobsen (2004) as a potential probiotic product as well as *Mangisi, Kunu-zaki and Uji* a thin, lactic acid-fermented porridge (Amadou et al., 2011a).

Prebiotics are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one, or a limited number of bacteria in the colon (Laminu et al., 2011; Abd El-Salam et al., 2012). Food ingredients classified as prebiotics must not be hydrolyzed or absorbed in the upper gastrointestinal tract, need to be a selective substrate for one, or a limited number of colonic bacteria, must alter the microbiota in the colon to a healthier composition and should induce luminal, or systematic effects that are beneficial to the host health (Lei and Jacobsen, 2004). Cereals, in particular millet-based fermented foods and beverages, have been extensively studied and form a major part of the diet in most African countries (Rivera-Espinoza and Gallardo-Navarro, 2010). Malting induces important beneficial biochemical changes in the millet grain. *Jandh* is just like *Koko* produced by steeping pearl millet overnight, discarding the steep water, wet-milling the millet grains together with spices such as ginger and chili pepper, water is added to the milled materials to make thick slurry. *Fura* is dough made from millet flour, it is hand molded into balls, placed inside a cooking pan containing boiling water, and cooked for 30 min at atmospheric pressure then the balls are kneaded again while still hot until a smooth, slightly elastic mass is obtained. *Mangisi* is a sweet-sour beverage made from naturally fermented millet mash. Moreover, unfermented millets foods and beverages such as *Dambu* a steamed granulated dumpling; *Masvusvu* a sweet beverage traditionally made from unfermented malted finger millet and *Ragi roti*, known as finger millet *roti* is an unleavened flatbread (Amadou et al., 2011a; Rivera-Espinoza and Gallardo-Navarro, 2010).

Some potential health benefits of millets

Millet is more than just an interesting alternative to the more common grains. The grain is also rich in phytochemicals, including phytic acid, which is believed to lower cholesterol, and phytate, which is associated with reduced cancer risk (Coulibaly et al., 2011). These health benefits have been partly attributed to the wide variety of potential chemopreventive substances, called phytochemicals, including antioxidants present in high amounts in foods such as millets (Izadi et al., 2012).

Millet is gluten-free, therefore an excellent option for people suffering from celiac diseases often irritated by the gluten content of wheat and other more common cereal grains. It is also useful for people who are suffering from atherosclerosis and diabetic heart disease (Gélinas et al., 2008). Choi et al. (2005) and Park et al. (2008) reported that protein concentrate of Korean foxtail millet and proso millet significantly elevated plasma adiponectin and HDL cholesterol levels and caused major decreases in insulin levels relative to a casein diet in type 2 diabetic mice. Furthermore, proso millet also improved glycemic responses and plasma levels (Park et al., 2008). In addition, proso millet protein concentrate has protective effects against D-galactosamin-induced liver injury in rats (Ito et al., 2008). Choi et al. (2005) and Park et al. (2008) concluded that proso millet protein could be a potential therapeutic intervention in type 2 diabetes. Devi et al. (2011) review the nature of polyphenols and dietary fiber of finger millet and their role with respect to the health benefits associated with millet.

Chandrasekara and Shahidi (2010) reported in their studies on free-radical quenching activity of finger millet (*Eleusine coracana*), that non-processed brown finger millet had the highest radical quenching activity than the processed one and postulated that tannins and phytic acid were responsible for the activity (Devi et al., 2011; Quesada et al., 2011; Kamara et al., 2012). Millets extract from the seed coat where reported to have shown high antibacterial and antifungal activity compared to whole flour extract due to high polyphenols content in seed coat (Viswanath et al., 2009; Xu et al., 2011). Free radical quenching potential of different millets kodo millet, finger millet, little millet, foxtail millet, barnyard millet (*kudiraivali*), great millet (*jowar*) and their white varieties were revealed to have significant antioxidant activity by 1, 1, Diphenyl -2-picrylhydrazyl (DPPH) method (Devi et al., 2011; Quesada et al., 2011; Kamara et al., 2012). Moreover, Kamara et al. (2012) reported different radical scavenging activities of fractionated foxtail millet protein hydrolysate.

Millet in the industry

Millets have good grain qualities suitable for processing. Processing of the grain for many end uses involves primary (wetting, dehulling and milling) and secondary (fermentation, malting, extrusion, glaking, popping and roasting) operations. Being a staple and consumed at household levels, processing must be considered at both traditional and industrial levels, involving small, medium and large-scale entrepreneurs (Obilana and Manyasa, 2002; Hamad, 2012). Dehulling is not favorable to millets due to their small grains sizes. In addition, dehulling causes nutrients loss. All the Millets can be milled by hand grinding (household level) or machine milling (cottage, small-to-medium scale service and large scale industrial). Millet and sorghum malt production is a traditional practice in Africa, where malt is used in lactic acid- and alcoholic-fermented beverages and infant food production (Adekunle, 2012). Traditional malting processes in many developing countries involve three main operations: soaking, germination, and drying. The duration and conditions of each operation are highly variable, resulting in highly variable malt and derived product quality (Vidya et al., 2012). *Burukutu* and *Pito* are traditional African beers differ from Western beer types in several ways: they are often sour less carbonated and have no hops; these beer are products of both at traditional and industrial level (Anukam and Reid, 2009; Amadou et al., 2011a). The emerging principal uses of millets as an industrial raw material include production of biscuits and confectionery, beverages, weaning foods and beer (Laminu et al., 2011; Anukam and Reid, 2009). Grits, flour, and meals from cereals such as millet, sorghum, and corn are now common items in the market. Soft biscuits and cookies are being made using sorghum, maize and wheat composites, while cakes and non-wheat breads have become a subject of increasing scientific and technological enquiry, showing encouraging results (Akeredolu et al., 2005; Hama et al., 2011; Laminu et al., 2011; Vidya et al., 2012).

In the infant weaning food sector, in spite of unlimited potential, progress has been slow, as the installed capacity for industrial malting is limited. Many brands of beer in the underdeveloped countries market have substantial content of local cereal such as millet to reduce the cost of imported barley. The industries are confronted with a number of problems which tend to diminish product qualities and affect overall utilization. For instance, in the nonalcoholic beverage and weaning food sectors, storage quality of the grain, nutritional losses after processing, high cost of imported equipment and variation among cultivars are some of the problems militating against improved utilization of millet in the developing countries (Akeredolu et al., 2005; Laminu et al., 2011; Adekunle, 2012). In a weaning process there is always the need to introduce soft, easily swallowed foods to supplement the infant's feeding early in life. A process weaning diet from pearl millet-conophor nut flour was found to promote growth in a clinical experiment (Akeredolu et al., 2005); whereas, weaning food blends prepared from fermented pearl millet/roasted cowpea in 70:30 and 60:40 ratios were reported to have resulted in lower levels of phytic acid and higher in vitro protein digestibility of the weaning food blends (Laminu et al., 2011).

Conclusion

Millets are still the staple food for millions of poor people in Africa and Asia. Like many other cereals, millets are high carbohydrate energy content and nutritious, making them useful components of dietary and nutritional balance in foods. Combination of millets with other sources of protein would compensate the deficiency of certain amino acids such as lysine. Successful improvement of these attributes would be a crucial key to expand the spectrum of applications of millet grains. Future trends should focus on the millet consumption in the developed countries that could help its industrial revolution.

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FOOD SCIENCE AND NUTRITION

Development of a non-commercial sugar-free barbecue sauce

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Abstract

There has always been a challenge in manufacturing a sugar free sauce. A basic barbecue sauce formulation was used to make 5 sugar-free preparations combining selected levels of xanthan gum, modified waxy maize starch, sucralose, and acesulfame-K. Physical, chemical, microbial and sensory properties were used to evaluate the product quality. Total Aerobic Plate Count was below detectable limits before and after the incubation period for all 6 products. Total Soluble Solids, water activity and pH −before and after incubation− of the control were significantly different from all 5 sugar-free treatments. Descriptive sensory analysis of prepared products showed that SU treatment (2.0% Starch+ 0.3% Sucralose) exhibited the best in all the sensory properties that were determined compared to all other treatments. One sugar free preparation was significantly more viscous than the control. This study will help producers in formulating a new a sugar free healthy barbecue sauce with properties closely comparable to a sugar containing control formula.

Key words: Sugar free, Barbecue Sauce, Quality Evaluation

Introduction

Barbecue sauce was not invented in America and its origins are not known. In the United-States, barbecue -or BBQ- originated in the late 1800's during Western cattle drives. The cowboys were fed a tough and stringy piece of meat that required five to seven hours of cooking to tenderize; marinades or sauces were often added to the meat to improve flavor. Today, there are four major types of barbecue sauce: Kansas City, North Carolina, Memphis, and Texas-style (Raichlen, 1998). They differ in terms of thickness and taste that goes best with the type and/or cut of meat. These barbecue sauces are sweet and contain a significant amount of sugars in the form of high fructose corn syrup, sugar, honey, molasses, etc. Some brand name barbecue sauces contain up to 50% sugar in their formulations. The calories contributed by these sugars do not mitigate some of the biggest health problems in the United States: obesity and diabetes. According to the American Diabetes Association, 18.2 million people in the United States have diabetes, and 5.2 million of them don't know it (ADA, 2005). Also, Type 2 diabetes and its associated long term complications can cost developed nations up to 10% of its financial budget, as well as decreasing quality of life and length of life by up to ten years (Khunti, 2012). In addition, 65% of Americans are overweight and 31% are obese (Flegal et al., 2002). The economic burden of diabetes and obesity in the United States is enormous. Direct and indirect medical expenditures attributable to diabetes in 1997 were estimated at $98 billion. Medical expenditures incurred by people with diabetes were $10,071 per capita, compared with $2,669 for non-diabetics (ADA, 2005). The total cost attributable to obesity was $99.2 billion (Wolf & Colditz, 1998). In a new study, it was found that in the USA the annual cost for the normal weight person was US$ 2578, overweight person was US$2262 while the obese person was $3042 (John et al., 2012). Providing obese and/or diabetic individuals with sugar free/low calorie products containing alternative sweeteners may alleviate these serious health issues. Gamonpilas et al. (2011) showed the

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possibility of producing a chili sauce by using xanthan-starch mixture to improve the rheological properties of the product. Also, Krystyjan et al. (2012) produced caramel sauces thickened with combinations of potato starch and xanthan gum. In their study, it was found that the treatment thickened with 0.3% potato starch and 0.02% xanthan gum, received the highest sensory score. There has always been a challenge in manufacturing a sugar free product that is comparable to its original formulation in terms of flavor, texture and shelf-stability. Today, new artificial sweeteners provide sweetness without the extra calories of natural sweeteners and without altering blood glucose levels. They do not count as a carbohydrate, fat, or any other exchange. They are excellent solutions for dieters, diabetics, or simply for new product development and line extensions. Five nonnutritive sweeteners with intense sweetening power have FDA approval (acesulfame-K, aspartame, neotame, saccharin, sucralose) and estimated intakes below the Acceptable Daily Intake. Nonnutritive sweeteners may assist in weight management, control of blood glucose, and prevention of dental caries. Over the years, the effects of nonnutritive sweetener use on health have been a concern among health professionals. FDA approved nonnutritive sweeteners are safe for human consumption, and are mainly used for children and pregnant women if taken in its permitted level. These permitted levels are: for Acesulfame-K is up to 15 mg/kg bw/day, and for Sucralose is up to 1.6 mg/kg bw/day (ADA, 2004). The focus of this research was to formulate a sugar free barbecue sauce with properties closely comparable to a sugar containing control formula.

Materials and Methods

Preliminary Work

Extensive preliminary work was conducted on basic barbecue sauce recipes, substituting brown sugar with different levels of the alternative sweeteners and/or thickening agents to determine their most suitable levels for the purpose of this research. Usage levels for xanthan gum and the modified food starch were determined according to recommendations from suppliers and after reviewing relevant research papers (Sanderson, 1996; Seib, 1998). Usage levels of the artificial sweeteners were originally determined according to their relative sweetness intensity as compared to sucrose: sucralose 600 times sweeter than sucrose and acesulfame-K, 200 times sweeter than sucrose (Aramouni et al., 1998). Substitution of the brown sugar resulted in color differences and proper adjustment was made using an artificial brown color. In addition to adequate usage levels, preliminary work proved that usage of tomato sauce Kroger's Brand (local Grocery Store) - rather than tomato paste− as a base resulted in a uniform product and substantially reduced syneresis.

Barbecue Sauce Ingredients and Preparation

The ingredients used in all barbecue sauce formulations were Xanthan gum: Colloides Naturals, Inc., (Bridgewater, NJ); Modified Food Starch: Cerestar USA(Hammond, IN); Acesulfame Potassium: American International Foods, Inc (Grand Rapids, MI); Sucralose: McNeil Specialty Products Co. (New Brunswick, NJ); Brown Sugar : Kroger's Brand (local Grocery Store); Artificial Color: Chr. Hansen, Inc., (Milwaukee, WI); tomato sauce: Kroger's Brand (local Grocery Store); vinegar: Kroger's Brand (local Grocery Store); onion powder, garlic powder: Kroger's Brand (local Grocery Store); salt: Kroger's Brand (local Grocery Store). Five treatments of sugar free barbecue sauce and a control were formulated (Table 1).

Table 1. Composition* of five different treatments of sugar free barbecue sauce and control (% by weight).

Treatment	Xanthan gum	Modified Food Starch	Acesulfame Potassium	Sucralose	Brown sugar	Artificial Color
XKSU	0.1	1.0	0.15	0.05	-	0.15
SK	-	1.50	0.25	0.15	-	0.15
XU	0.25	-	-	-	-	0.15
SU	-	2.0	-	0.30	-	0.15
XK	0.125	-	0.5	-	-	0.15
Control	-	-	-	-	32	-

* Common ingredients used were tomato sauce (2.8 Kg), vinegar (560 mL), onion powder (50 g), garlic powder (50 g), and salt (50 g). XKSU (0.1% Xanthan+1.0% Starch+0.15% Acesulfame-K+0.05% Sucralose), SK (1.5% Starch+0.25% Acesulfame-K), XU (0.25% Xanthan+0.15% Sucralose), SU (2.0% Starch+ 0.3% Sucralose), XK (0.125% Xanthan+ 0.5% Acesulfame-K), CONTROL (32% Sugar).

Barbecue sauce chemical and physical evaluation

Moisture content and water activity

The moisture content of the sauce was measured using AACC approved method 44-15A (AACC, 1995) and water activity (AOAC, 1980) was determined using an AQUA LAB CX-2 (Decagon CO.,Pullman,WA). A conventional oven (Fisher Scientific Isotope incubator, Pittsburgh, PA) was used.

pH Measurements and total soluble solids

The pH of the sauce was measured with a pH meter (Accumet portable AP63, Fisher Scientific, Denver, CO) with automatic temperature compensation, the Electrometric Method, AACC method 02–052 (AACC, 1995). The pH meter was calibrated with buffer solutions of 4 and 7. All measurements were recorded at ambient temperature when the readings were stable. Total soluble solids were determined by using a refractometer (Abb, Carnation, Washington).

Barbecue sauce physical evaluation

Color

A Hunterlab Ultrascan Sphere Spectro-colorimeter (Hunter Associates Laboratory Inc., Reston, Virginia) was used for color measurements. Fifty grams of the sauce were placed on a small white paper plate, the Spectrocolorimeter placed at approximately 1 cm from the surface of the product which was scanned to determine the L, a, and b values using illuminant C (at a 10º angle) which is equivalent to normal daylight.

Rheology

A steady shear test of the six different treatments of barbecue sauce was performed using a Bohlin VOR rheometer (Bohlin Reologi B, Lund, Sweden). Rheological testing was conducted using concentric cylinder geometry with a torque element of 91.1 g. The apparent viscosity was calculated within shear rates ranging from 0.92 s^{-1} to 91.9 s^{-1} at 23ºC (storage temperature)

Barbecue sauce microbial evaluation

Microbiological tests were performed using 3M Petrifilm^{TM} Plates (3M Microbiology Products, St. Paul, MN) for yeast and mold count and total aerobic plate counts. The total aerobic plate counts plates were held in an incubator (Boekel Industries Inc, Feasterville, PA) at 35°C and the yeast and mold plates were held at 20°C for 48 hours before evaluation.

Descriptive sensory analysis

The tested barbecue sauces were basic non-commercial formulations hence a complete descriptive profile was not needed. Product attributes were pre-determined by the author for their relevance and comparative value. A five-member panel was comprised of students and faculty members, who had previously served as descriptive panels. The panelists spent 6 hours (divided in 3 sessions) of training −conducted by the author− to become familiar with the definitions and references for the following attributes: color, thickness, sweet, sour, bitter aftertaste, and metallic aftertaste. Testing of the samples was done in 2 one-hour sessions. The panel evaluated the samples and reached consensus for each attribute. Attributes of the samples were identified and the intensities were quantified, utilizing a 15 point scale with 0.5 increments (0.0 = none; 0.5 – 5.0 = slight; 5.5 – 10.0 = moderate; 10.5 – 15.0 = extreme). Samples were presented monadically and coded with random 3-digit numbers. Three replications of each sample were evaluated.

Nutrition labeling

Nutrition analysis was performed using the Genesis R&D labeling program (ESHA Research, Salem, OR). Moisture content was adjusted for moisture loss during the processing of samples.

Statistical analysis

This research used a randomized complete block (RCB) design with 3 replications. Data were analyzed for significant differences by examining the appropriate least significant difference (LSD) values.

Results and Discussion

Moisture content and water activity

The moisture content value was calculated to adjust for the moisture loss during processing to accurately produce the nutrition label. The average of a total of 9 calculations (3 replications * 3 measurements) was 5%± 0.05. There were no differences in water activity (A_w) among the sugar-free treatments (Table 2). However, the control formula had a significantly lower A_w (0.951) than all the other treatments due to the water binding ability of the sugar present in the control. This difference in water activity could make the sugar free preparations more susceptible to mold and yeast growth.

The water activity of the barbecue sauce makes bacterial growth possible for all formulations. However, their low pH was reported to be a

limiting factor (Jay, 1992). Sauces are typically shelf stable, implying a shelf-life of several months at room temperature. This can be achieved by (i) a low pH, due to the presence of organic and inorganic acid or (ii) a salt concentration (Vermeulen, 2007).

Table 2. Mean °brix and water activity values of five sugar free treatments of barbecue sauce and control.

Treatments* [1]	Water Activity	°Brix
XKSU	0.976[a]	11.0[a]
SK	0.977[a]	10.8[a]
XU	0.977 [a]	10.9[a]
SU	0.974 [a]	11.2[a]
XK	0.974 [a]	10.7[a]
Control	0.951[b]	33.0[b]

[1]Means with different superscripts within the same column indicate significant differences between samples ($p<0.05$). *XKSU (0.1% Xanthan+1.0% Starch+0.15% Acesulfame-K+0.05% Sucralose), SK (1.5% Starch+0.25% Acesulfame-K), XU (0.25% Xanthan+0.15% Sucralose), SU (2.0% Starch+ 0.3% Sucralose), XK (0.125% Xanthan+ 0.5% Acesulfame-K), CONTROL (32% Sugar).

Total soluble solids

The absence of sugar in the sugar free treatments resulted in a 65% reduction in solid content when compared to the control formula (Table 2). The control contained 7 g of carbohydrates -per serving- more than all the sugar free treatments. However, there were no significant differences among the sugar-free treatments due to the very low amount of the other variable ingredients. This big reduction in carbohydrates content is beneficial for dietary intake planning especially for overweight and diabetic individuals – the sugar free barbecue sauces had approximately 25 less calories per serving than the control formula.

pH results

At both day 1 and day 31, the pH was not significantly different among the sugar free treatments; however, they all had a significantly lower pH than the control formula (Table 3). This is probably as a result of the lower proportion of acetic acid –contributed by vinegar, in the control formula due to the presence of sugar. At day 31, the pH of all the treatments was higher than their respective values at day 1 (Table 3). This is probably due to the buffering effect of some of the compounds present in the food products. The effect of the pH on the system components was not critical for several reasons: a- The modified waxy maize starch was chosen for optimal performance at this pH range (National, 2000); b- Sucralose degradation is minimal at this pH – 0.02% loss (Goldsmith and Merkel, 2001); c- Acesulfame-K degradation is also minimal at this pH – no loss observed (Lipinski and Hanger, 2001); d- Xanthan gum is also stable at this pH range (Whistler & BeMiller, 1993).

Table 3. Mean pH values of five treatments of sugar free barbecue sauce and control at days 1 and 31 after incubation at room temp.

Treatments*[1]	pH Day 1	Day 31
XKSU	3.77 [b]	4.01 [a]
SK	3.80 [b]	4.00 [a]
XU	3.78 [b]	4.00 [a]
SU	3.80 [b]	4.01 [a]
XK	3.78 [b]	4.01 [a]
Control	3.95 [a]	4.08 [b]

[1]Means with different superscripts within the same column indicate significant differences between samples ($p<0.05$). *XKSU (0.1% Xanthan+1.0% Starch+0.15% Acesulfame-K+0.05% Sucralose), SK (1.5% Starch+0.25% Acesulfame-K), XU (0.25% Xanthan+0.15% Sucralose), SU (2.0% Starch+ 0.3% Sucralose), XK (0.125% Xanthan+ 0.5% Acesulfame-K), CONTROL (32% Sugar).

Color

The measured L, a, and b color values did not show any significant differences between any of the formulations from day 1 or day 31. This was attributed to adequate preliminary work, the shelf-stability of the artificial color used, and the relatively short period of incubation.

Rheology

All treatments exhibited pseudoplastic behavior and the viscosity decreased with increasing shear rate (Figure 1). These results are in accordance with a study by Daget and Joerg (1988), using xanthan gum as a thickener in dessert creams and with the results obtained by Mandala et al. (2004). In this study, the xanthan gum was used as a thickener agent in white sauce. The values were statistically analyzed at a shear rate of approximately 50 s^{-1}, which is generally associated with the shear rate applied in the human mouth while consuming foods (Wood 1968). Although the viscosity increased at the lower temperature, the same pattern was observed between and within samples at both temperatures. Treatment SU (2.0% Starch+ 0.3% Sucralose) was significantly more viscous than the control and other treatments, while no other significant differences were noted. This could be due to the combination effect of these two ingredients. These results indicate that the usage levels of the modified waxy maize starch and/or the xanthan gum were successful in matching the viscosity of the control formula.

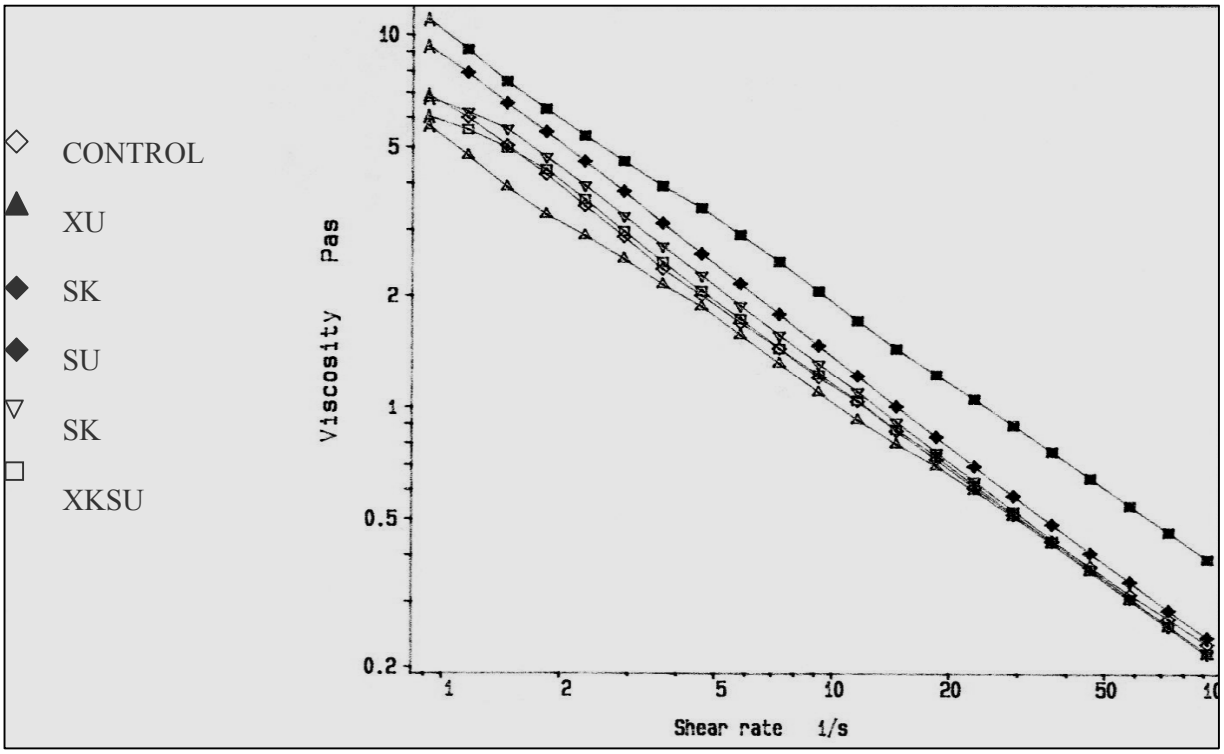

Figure 1. Viscosity measurements at various shear rates for five treatments of sugar free barbecue sauce and control.
*XKSU (0.1% Xanthan+1.0% Starch+0.15% Acesulfame-K+0.05% Sucralose), SK (1.5% Starch+0.25% Acesulfame-K), XU (0.25% Xanthan+0.15% Sucralose), SU (2.0% Starch+ 0.3% Sucralose), XK (0.125% Xanthan+ 0.5% Acesulfame-K), Control (32% Sugar).

Table 4. Sensory analysis results* of selected attributes of five treatments of sugar free barbecue sauce and control.

Treatment*	Color	Thickness	Sweet	Sour	Bitter Aftertaste	Metallic Aftertaste
XKSU**	11^{a}	6^{a}	7^{b}	4^{a}	2^{a}	3^{a}
SK	11^{a}	5^{b}	5^{c}	4^{a}	3^{a}	2^{a}
XU	11^{a}	6^{a}	7^{b}	4^{a}	2^{a}	2^{a}
SU	11^{a}	7^{a}	10^{a}	4^{a}	2^{a}	1^{b}
XK	11^{a}	6^{a}	6^{c}	4^{a}	3^{a}	3^{a}
Control	10^{a}	6^{a}	8^{b}	3^{a}	2^{a}	2^{a}

*Consensus among 5 panelists using a 1 to 15-point scale, one-point increments.

**XKSU (0.1% Xanthan+1.0% Starch+0.15% Acesulfame-K+0.05% Sucralose), SK (1.5% Starch+0.25% Acesulfame-K), XU (0.25% Xanthan+0.15% Sucralose), SU (2.0% Starch+ 0.3% Sucralose), XK (0.125% Xanthan+ 0.5% Acesulfame-K), Control (32% Sugar).

Total aerobic plate count

The microorganisms of concern in these conditions (pH 3.7-4.1) are yeasts, molds, and some spoilage bacteria (Jay, 1992). At both day 1 and day 31, the TAPC of all the treatments was below detectable levels (<10 CFU). This is a result of all the samples being processed to 195ºF or above-temperature recommended for 0.5 seconds to achieve pasteurization (Jay, 1992)- and every effort was made following the Good Manufacturing Practices as they would be applied in food manufacturing, packing, or holding (USCFR, 21CFR110.3-110.110). This is an encouraging result; however, we recommend a longer incubation period to have a more accurate assessment of the possible microbial load.

Descriptive sensory analysis

Both bitter and metallic aftertastes were very low (a score of 3 or less) for all six treatments (Table 4) and cannot be solely attributed to the artificial sweeteners (they might have originated from the metal can containing the tomato sauce).

As reported by Paulus and Braun, aqueous solutions with high concentrations of acesulfame-K exhibit a bitter taste. In foods with low concentrations of acesulfame-K, this effect is not of great importance (Paulus and Braun, 1988). However, it is worth noting that the sugar free treatment with sucralose had the lowest scores for both attributes, even lower than the control. This is in accordance with sensory studies on sucralose that show that bitterness was very low in the taste profile, even comparable to the bitterness perceived from sucrose (Goldsmith and Merkel, 2001). Brown color received the same score -11- for all the sugar free treatments which was one unit higher than the score -10- attributed to the control. The control formula, the two sugar free treatments containing xanthan gum, and the sugar free treatment combining both xanthan and starch had a thickness score of 6. Both sugar free treatments containing the modified waxy maize starch rated 5 and 7. These fairly close results are comparable to the instrumental color analysis and rheological measurements. In terms of sweetness, the control scored 8. The sugar free treatment containing 0.3% sucralose had the highest sweetness score of 10. Sugar free treatments with 0.25% and 0.5% acesulfame-K rated 5 and 6, respectively. The sugar free treatment containing a combination of sucralose (0.05%) and acesulfame-K (0.15%) had a sweetness score of 7. Treatment SU (2.0% Starch+ 0.3% Sucralose) exhibited the best in all the sensory properties that were determined compare to all other treatments. It is important to note that the sensory properties and sweetness intensities at different levels of artificial sweeteners are greatly influenced by the food or beverage system in which they are studied (Wiet and Beyts, 1988).

Nutrition labeling

The serving size for barbecue sauce indicated by the RACC — Reference Amounts Customarily Consumed — is 2 tbsp (USCFR, 21CFR101.12). Thirty mL — equivalent to 2 tbsp —of all 6 formulations weighed approximately 34g. The total carbohydrate content per serving of the sugar containing control was 10 g while that of the sugar free treatments was 3g originating mainly from the tomato sauce. The control formula had 9g of sugars while the sugar free treatments had only 1g. There were 40 calories per serving for the control while the sugar free treatments had only 15 calories. The substitution of sugar had a noticeable reduction of about 65% in the caloric and carbohydrate content of the sugar free treatments as compared to the control. The remaining carbohydrates are mainly from the tomato sauce.

Conclusion

The results of this research indicate the possibility of manufacturing a sugar-free barbecue sauce that is comparable to its sugar-containing original formula. However, the process of adjusting sweetness and thickness levels is lengthy and delicate. The sensory properties of a commercial barbecue sauce formulation are complex. Therefore, it is recommended to do a complete descriptive profile of the original formulation as well as the sugar-free formulations. In addition to that, a consumer acceptability/preference is also highly recommended to determine the most liked sugar-free formulation before the product is released to the market.

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FOOD SCIENCE AND NUTRITION

Effects of scale and skin on chemical and sensory quality of marinated sea bass filets (*Dicentrarchus labrax*, L. 1758) in sunflower oil during storage at 4ºC

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Abstract

In this study, the effects of descaling and skin removal on chemical changes and sensory attributes of marinated sea bass filets (*Dicentrarchus labrax*) in sunflower oil stored at 4ºC were investigated. In terms of overall acceptability, although skinless sea bass fillets reached consumable limit values at the 70th day, scaly and descaled samples did not reach limits even at the end of the 90-day storage. During 90-day storage, (Total Volatile Basic Nitrogen) TVB-N and (Trimethylamine–nitrogen) TMA-N values of sea bass fillets were found to be below consumable limits. Thiobarbituric acid (TBA) value reached the consumable limit of 8 mg malonaldehyde/kg at the 56th day for skinned fillets and at the 70th day for descaled fillets. Scaly fillets did not reach the TBA limit during 90-day storage time. According to obtained results, scaly and descaling sea bass fillets were found to be more appropriate for marination in terms of brightness, juiciness, tenderness, acid and salt transition and hygiene. It was detected that descaling has some disadvantages on texture (acid and salt transition) and hygiene of fish. Comparing the scaly and descaled fillets, it was found that scales effect the brightness of brine solution and also cause undesirable colour changes as the scales stick to the fish skin.

Key words: Sea bass, *Dicentrarchus labrax*, Marinade, Storage, Quality changes

Introduction

Marinating process is one of the oldest methods of conservation of fish and it is popular in Europe. Fatty fish like sardines, mackerel, herring, and anchovies, as well as several kinds of crustaceans and bivalves are usually used. The term ‘‘marinades’’ or ‘‘marinated fish’’ is used to define fish products which consists of fresh, frozen or salted fish or portions of fish processed by treatment with an edible organic acid, usually acetic acid, and salt and put into brines, sauces or oil (Duyar and Eke, 2008). Due to the increasing consumer demand for fresh refrigerated foods with prolonged shelf-life, many researches have focused on preservation techniques to control bacterial growth for safety purposes or for extending the shelf-life of the food (Sallam, 2007). Marination, a food-preservation technique, is based on treatment of muscle with solutions containing salt, spices, lemon juice, etc. and provides high sensory acceptability to a variety of meat products (Ozogul et al., 2009).

Marination is also used to tenderise or to change taste, textural and structural properties of raw material. Marinades stored at cooler temperatures (4–6°C) keep a long time such as four months. Marinades are semi-preserves; the preserving principal is the combination of acetic acid and salt. (Gokoglu et al., 2004; Sallam et al., 2007; Duyar and Eke, 2009). Salt and acetic acid uptake depends on many factors including species, muscle type, fish size, fillet thickness, weight, composition (lipid content and distribution), physiological state, salting method, brine concentration, duration of salting step, and fish-to-salt ratio, ambient temperature, freezing and thawing (Gallart-Jornet et al., 2007). The marinating process has many positive effects on the palatability and shelf-life of meat and seafood products. The main aims of marinating are to tenderize and enhance the flavour, safety and shelf-life of meat products owing to inhibition of microbial growth (Björkroth, 2005; Ozogul et al., 2008).

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Anchovies are generally used for marination fish production; however, use of some reared fish, like gilthead seabream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) has been recently proposed. Recently, in Italy, marination technology is also spreading to some reared fish, like gilthead seabream and sea bass (Giuffrida et al., 2007). Aquaculture of sea bass has major economic importance in Turkey and many European countries. Sea bass is one of the most widely farmed species with a total production of 105.900 tons in Europe in 2008 (FAO, 2008).

This study was aimed to determine the effects of scale, descaling and skinning on chemical changes and sensory attributes of marinated sea bass (*Dicentrarchus labrax*) in sunflower oil during storage at 4ºC.

Materials and Methods

Materials

A total of 50 kg, aquacultured sea bass samples (*Dicentrarchus labrax*) in the Eagean Sea coast were harvested by Kılıc Fisheries Holding (Mugla, TURKEY) and ice-chilled within 18h. Fish sample used in this study were degutted manually by the workers in the fish processing plant and were transported to the laboratory in polystyrene boxes with flaked ice within 90 minutes of purchasing. The mean weight of fish was 330±10 g.

Methods

Samples were divided into three lots. First lot of 18 kg sea bass was just filleted with no other treatment. Second lot of 18 kg was first descaled then filleted. Remaining lot of 14 kg was skinned and then filleted. Then, all three lots of fillets were dipped in an ice-water solution (1:4 v/v) for 60 minutes to provide physical cleaning and temperature stability of the fish samples. Then marinating solution composed of 2.5% acetic acid and 11% sodium chloride was added to fillets put in 5000 mL glass jars with the lids. Jars filled with sea bass fillets and marinating solution at a weight ratio of 1.5:1 were stored in a refrigerator at +4 (±1) °C for marinating. Marinating and brining processes were completed after 72 h. Fish samples were removed from the solutions and put into glass jars, filled with sunflower oil. During storage in oil, samples from all three groups were analysed at the first day 0 and the following days after 7, 14, 21, 28, 42, 56, 70, 90 days of the marinating.

Sensory analysis

Sensory analysis of marinated fillets was evaluated by 6 experienced panellists and the results were given as described by Sallam et al. (2007). Three representative fish samples were individually presented to each panellist. The judges were not informed about the experimental approach. Panellists were asked to evaluate the overall acceptability with regard to fish-like appearance, colour, texture, odour, rancidity, juiciness, tenderness, sour taste, salty taste and flavour-aftertaste. An eight-point hedonic scoring scale, with 8 = extremely intense/juicy/tender, 7 = very intense/juicy/tender, 6 = moderately intense/juicy/tender, 5 = slightly intense/juicy/ tender, 4 = slightly bland/dry/tough, 3 = moderately bland/dry/tough, 2 = very bland/dry/tough, 1 = extremely bland/dry/tough, was employed for odour and flavour intensity, juiciness, and tenderness, respectively, while a nine-point hedonic scale, ranging from extremely acceptable (9) to extremely unacceptable (1) was utilized for evaluation of the appearance. Sea bass samples receiving overall scores of more than 4 were considered acceptable, while a score between 3 and 4 was considered the borderline of acceptability. The parameters were tested throughout the storage period of 90 days.

Determination of quality changes

pH, Total Volatile Basic Nitrogen (TVB-N), Trimethylamine Nitrogen (TMA-N) and Tiobarbituric Acid (TBA) analyses were performed three times for each group of samples. The pH value was determined by dipping a pH electrode into homogenates of filleted muscle in distilledwater (1/1) (Manthey et al., 1988). All measurements were performed at room temperature using pH-meter (WTW Inolab, Weilheim, Germany). TVB-N was used as indices for the quality of fresh fillets of sea bass and determined according to the Antonocopoulus (1973). Volatile bases were seperated by steam distillation of homogenized samples, collected in 0.1 N HCl and titrated back with 0.1 N NaOH. TMA-N, which is produced by the decomposition of TMAO by bacteria or enzymatic reaction, is a spoilage indicator for fresh fish. TMA-N amount of the homogenized samples were determined according to the Schormüler (1968). Samples were extracted with trichloracetic acid. Bases in the extract were fixed with formaldehyde and after adding picric acid, the absorbance was measured at 410 ŋm. TBA was used as an indicator of lipid oxidation and calculated according to the Tarladgis et al. (1960). HCl were added to the fish samples and processed at condenser. TBA solution prepared with 90% glacial acetic acid was added to the distilled solution and was left in a water bath. The

absorbance was determined by a spectrophotometer at 538 ŋm. For chemical analysis, 6 fillets were used for each replicate.

Determination of proximate composition

Crude protein content was calculated by converting the nitrogen content determined by the Kjeldahl method (6.25xN) AOAC (1998). Lipid was determined by using the method described by AOAC (1998).

Statistical analyses

SPSS 14 for Windows was used to test the differences between mean values of the different analysed parameters. Differences between means were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey multiple comparison test, when a significant difference was detected between the days of storage ($P<0.05$).

Results and Discussion

There were significant increases especially for crude fat, TMA-N and TBA values and also there were considerable decreases in overall acceptability, pH and TVB-N values after marination (Table 1). No significant change was detected in crude protein values.

As shown in Table 2, when sensory score changes of marinated seabass fillets under refrigerator conditions were examined, it was seen that panellists found the scaly fillet group more fit and the skinned fillet group less fit in terms of appearence, color, rancidity, juiciness, acetic acid rancidity and salinity. Also, descaled fillet group was found to be more fit and the skinned group was found to be less fit in terms of odour, texture, softness, taste and flavour. It was detected that according to average acceptability assessment, skinned samples reached consumable limit value at day 70 where scaly and descaled samples still could not at day 90. Only for appearence, descaled samples were detected to be under limit values at day 90, skinned samples were detected to be under limit values at day 70; for colour, texture and softness assessment, skinned group was detected to be under limit values at day 70; for rancidity, scaly and descaled samples at day 70, skinned samples were detected to be under limit values at day 56; for acetic acid rancidity and salinity skinned samples were detected to be under limit values at day 56. A similar sensory analysis results has been reported by Baygar et al. (2002) for marinated sea bass. It was concluded that TBA values depending on the level of fatty oxidation and sensory evaluation determined the shelf life of marinated anchovies under vacuum and MAP conditions during storage at 2±2°C (Gunsen et al., 2011). It was reported that there was a correlation between the TBA value and sensory evaluation in many researches (Ramanathan and Das, 1992).

Protein and lipid contents

In our study, it was determined that as the crude fat content of fresh sea bass increased with the marination and storage in sunflower oil (Figure 1). We suggest that salt amount of the marination solution cause that artificial increase of crude fat. Although there was not an important change in crude fat contents of groups during oil storage, there were little decreases for all three group samples during the oil storage. A similar proximate composition (72.10% moisture, 19.87% protein, 4.84% fat, 1.54% ash) has been reported by Arık et al. (2002) for marinated fish.

Table 1. Sensory scores and chemical analyses results of raw seabass and marinated seabass in sunflower oil during stored at 4±1 °C.

Product		Overall acceptability*	Crude protein	Crude lipid	pH	TVB-N	TMA-N	TBA
Raw sea bass		8.40±0.23^{a}	20.18±0.19^{a}	6.34±0.19^{a}	6.35±0.02^{a}	16.89±1.45^{a}	2.86±0.1^{a}	0.30±0.01^{a}
Marinated sea bass (After 90-day)	Scaly	6.66±0.32^{b}	19.15±0.35^{b}	9.87±0.23^{b}	4.32±0.04^{b}	9.82±0.87^{b}	3.21±0.12^{b}	7.83±0.06^{b}
	Descaling	6.75±0.17^{b}	19.04±0.21^{b}	8.98±0.46^{c}	4.17±0.01^{b}	9.62±0.43^{b}	2.91±0.09^{a}	8.50±0.10^{c}
	Skinning	4.90±0.10^{c}	19.93±0.11^{a}	10.57±1.10^{d}	4.13±0.01^{b}	10.83±0.70^{c}	3.03±0.21^{a}	9.68±0.03^{d}

Data is expressed as mean ± standard deviation of three determinations. Mean with different letters within the row are significantly different ($P < 0.05$), [(n=3), *(n=6)].

Table 2. Sensory scores of sea bass filets marinated in sunflower oil during stored at 4±1°C.

Sensory parameters	Sample type	Storage time (day) 0	7	14	21	28	42	56	70	90
Appearance	Scaly	6.73	7.37	7.22	7.24	7.28	7.09	6.52	5.76	5.37
	Descaled	7.22	7.11	7.18	7.09	7.03	6.54	6.12	5.52	4.65
	Skinned	6.55	6.84	6.70	6.56	6.42	5.25	5.28	4.72	4.03
Colour	Scaly	7.28	7.19	6.94	6.92	6.89	6.65	6.78	6.17	5.42
	Descaled	7.00	7.27	7.11	7.08	7.02	6.87	6.02	5.66	5.09
	Skinned	7.08	6.58	6.25	6.12	5.89	5.67	5.35	4.78	4.52
Odour	Scaly	7.12	7.34	7.17	7.09	7.21	6.06	5.95	5.57	5.34
	Descaled	7.00	7.15	7.02	7.13	7.23	6.24	6.06	5.61	5.94
	Skinned	6.85	7.09	7.01	6.87	6.74	5.91	5.66	5.52	5.21
Texture	Scaly	7.50	6.97	6.88	7.04	7.01	7.14	6.96	6.75	6.61
	Descaled	7.43	7.05	7.13	6.91	6.93	6.78	6.81	6.88	6.79
	Skinned	7.13	7.09	7.01	7.11	6.87	6.56	6.41	6.35	6.44
Rancidity	Scaly	7.13	7.12	6.55	6.72	5.87	5.60	5.45	4.87	4.35
	Descaled	7.02	7.11	6.38	6.05	5.75	5.65	5.02	4.32	3.92
	Skinned	6.03	5.83	5.45	5.36	5.12	5.24	4.55	3.84	-
Juiciness	Scaly	7.12	6.80	6.54	6.41	5.87	5.52	5.44	5.67	5.32
	Descaled	7.31	6.74	6.31	6.07	6.21	5.89	6.11	5.63	5.28
	Skinned	7.13	6.58	6.20	6.03	5.56	5.72	5.12	5.45	5.07
Tenderness	Scaly	7.16	6.78	6.43	6.25	6.12	6.29	5.89	5.67	5.42
	Descaled	7.00	6.97	6.85	6.56	6.63	6.45	6.27	6.03	5.88
	Skinned	7.07	6.24	5.78	5.55	5.63	5.21	5.07	4.56	4.72
Sour taste	Scaly	7.52	6.65	6.39	6.25	5.87	5.43	5.75	5.68	5.32
	Descaled	7.42	5.56	6.71	6.48	6.24	6.03	5.78	5.56	5.11
	Skinned	6.45	6.12	5.95	5.62	5.55	5.30	4.42	4.17	-
Salty taste	Scaly	7.08	6.67	6.45	6.21	5.88	5.67	5.19	5.32	5.58
	Descaled	7.41	6.94	6.56	6.34	6.12	5.85	5.61	5.42	5.54
	Skinned	7.04	6.34	6.01	5.76	5.23	5.21	4.74	4.25	-
Flavour and after taste	Scaly	7.07	6.45	6.66	6.54	6.27	6.63	6.32	6.13	5.78
	Descaled	7.44	7.47	7.34	7.02	6.89	6.56	6.61	6.34	6.03
	Skinned	7.25	6.35	6.25	6.14	5.78	5.82	5.43	5.21	-
Overall acceptability	Scaly	7.25	7.07	7.03	6.95	6.92	6.67	6.74	6.81	6.66
	Descaled	7.34	7.22	7.12	7.05	7.09	7.11	6.99	6.83	6.75
	Skinned	6.88	7.18	7.07	6.55	6.72	5.62	5.34	4.90	-

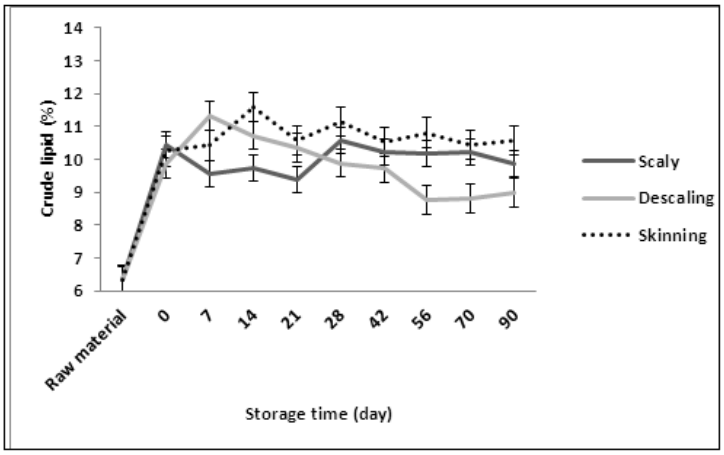

Figure 1. Changes in crude fat value of marinated seabass in sunflower oil during stored at 4±1 °C (Values are mean ± SD (n=3)).

Such as the crude fat content, crude protein content also showed an artificial increase during the beginning of oil storage. The protein content decreased during the oil storage and again reached its initial value (Figure 2). Erkan ve Özden (2007) determined crude protein level as % 20.35 ± 0.41 for sea bass. This protein results are consistent with initial values in our study.

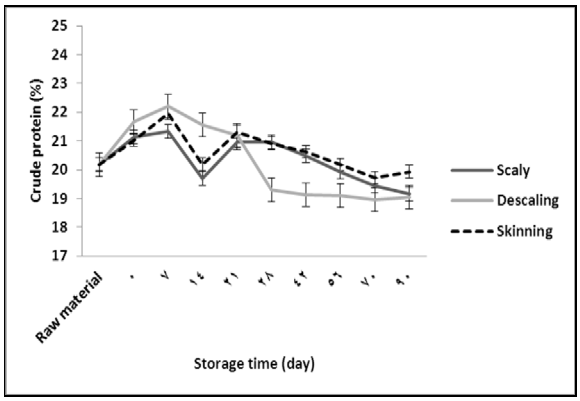

Figure 2. Changes in crude protein value of marinated seabass in sunflower oil during stored at 4±1°C (Values are mean ± SD (n=3)).

pH value

An important intrinsic factor related to fish flesh is the very high post-mortem pH (>6.0). Most fish contain only very little carbohydrate (<0.5%) in the muscle tissue and only small amounts of lactic acid are produced post-mortem (Sallam et al., 2007). pH value in raw fish flesh was 6,35. During marination process, scaly, descaling and skinning pH values dropped down to 4.32, 4.17 and 4.13, respectively. There was a significant difference ($p < 0.05$) in pH value between samples in sunflower oil. Lower pH values were found skinning samples in sunflower oil (Figure 3). Gokoglu et al. (2004) reported insignificant changes in pH levels of marinated fish.

TVB-N analysis results

TVB-N value is a quality index for unprocessed fishery products indicative of fish spoilage as a result of metabolic activity of fish spoilage bacteria and endogenous enzymes action (Gunsen et al., 2011). In marine fish, TVB-N values of 15-20 mg N/100 g show good quality, whereas TVB-N values of 50 mg N/100 g show poor quality (Connell, 1980). However, upper levels from TVB-N values of 28 mg N/100 g have been reported as "unacceptable" in processed fish, according to the Turkish Manual of Seafood Quality Control Limits (Anon., 2008). There was a significant change in TVB-N value of all three groups during marination then an increase occured after storage in oil (Figure 4). Although the highest TVB-N value was seen for skinned samples, all groups were under the consumable limit. TVB-N value is useful in assessing the degree of fish deterioration than in evaluating the changes occurring during the first stages of storage (Gokoglu et al., 2009), Ludorf and Meyer (1973) suggested levels of 30– 35 mg TVB-N/100 g as the upper limit of acceptable freshness. The samples did not reach such limit value throughout storage.

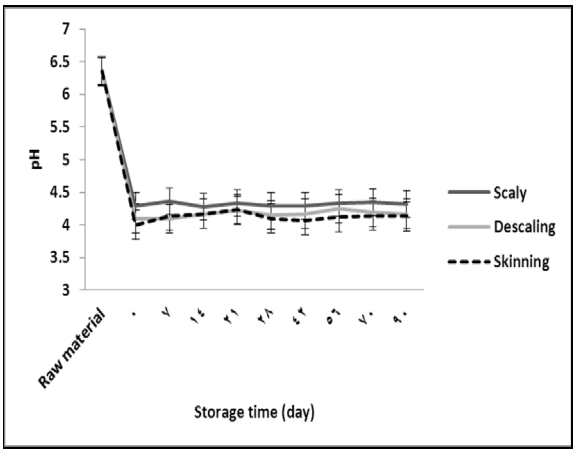

Figure 3. Changes in pH value of marinated seabass in sunflower oil during stored at 4±1°C (Values are mean ± SD (n=3)).

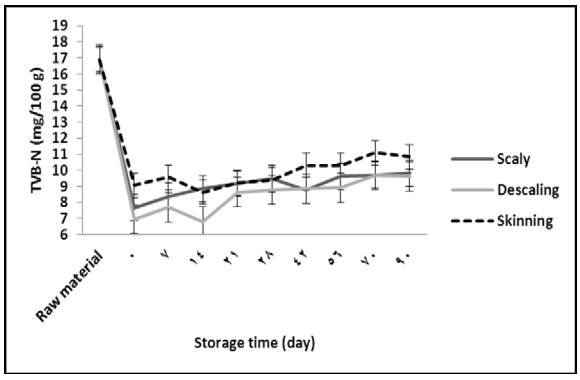

Figure 4. Changes in TVB-N (mg/100g) value of marinated seabass in sunflower oil during stored at 4±1°C (Values are mean ± SD (n=3)).

TMA-N analysis results

TMA-N is also used as an index of quality for assessing the state of freshness of fish and considered as a valuable tool in the evaluation of the quality of fish. At the first 7 days of oil storage, there was an important increase at TMA-N value of all groups and after the decrease at 14th day, it proceeded as a slight increase (Figure 5). Higher TMA-N values were found skinning samples. A TMA-N value of 5–10mg/100 g sample was reported as the limit for acceptability of fish (Sikorski et al., 1989). All TMA-N levels for samples in marination conditions of the present study were below the limit level. Similar results were reported in the previous researches (Dokuzlu, 2000; Gokoglu et al., 2004).

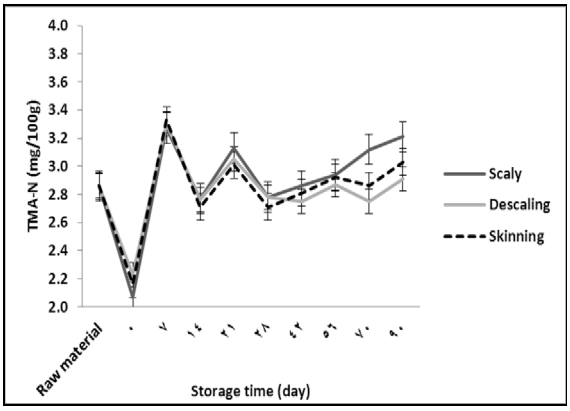

Figure 5. Changes in TMA-N (mg/100g) value of marinated seabass in sunflower oil during stored at 4±1°C (Values are mean ± SD (n=3)).

TBA analysis results

The initial TBA value of raw fish was found as 0.30 + 0.01. At the end of the 90 days storage period, the TBA values recorded for marinated scaly, descaled and skinned fillets were 7.82, 8.50 and 9.68 mg malonaldehyde/kg fish, respectively. The acceptable TBA limit value of 8 mg malonaldehyde/kg fish muscle was reached on days 70 and 56 for both the marinated skinned fillets and descaled, respectively (Figure 6). Sallam et al., (2007) found the raw TBA value of pacific saury (*Cololabis saira*) which they marinated in different acid solutions to be 0.37 mg MA/kg and emphasized that marinades were of good quality although no steady increase had happened during the 90 days of storage period. Baygar et al. (2012) reported after the marinating process, the TBA values recorded for marinated scaly, descaled and skinned fillets were 4.25, 5.74 and 5.95 mg malonaldehyde/kg fish, respectively. Olgunoglu (2007) found out that the TBA value of anchovy (*Engraulis encrasicolus*) marinades at the beginning of storage period was 1.16 mg MA kg^{-1} and it steadily increased and reached 4.20 mg MA kg-1 at the end of the 7 months of storage period. Varlik et al. (2007) and Kaya et al. (2010) maintained that the TBA number should not be <3 in a very good material and it should not be >5 in a good material.

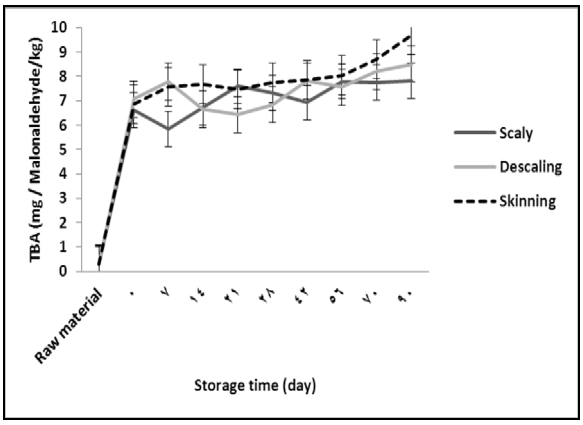

Figure 6. Changes in TBA (Mg malonaldehyde/kg) value of marinated seabass in sunflower oil during stored at 4±1°C (Values are mean ± SD (n=3)).

Conclusion

After storage in oil for 90 days, scaly and descaled fillets were found to be more suitable than skinned fillets for marinating and oil storage. Skin and scales effect relatively the acid and salt transition, especially at tail parts of thin fish flesh during storage in brine solution and oil. Variability in acid and salt taste in different parts of skinned fillets was not approved by panellists during the oil storage. Likewise changes at juiciness, tenderness and texture of skinned fillets were less acceptable than other samples. These problems of skinned samples affected the overall acceptability and TBA value so 70 days was determined as the maximum storage time of skinned fillets. Both of the scaly or descaled sea bass fillets are suggested to those companies who will do sea bass marination. Also, when scaly fillets will be used for marination, it is advised to clean the scales carefully before taking the fillets into oil.

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PLANT SCIENCE

Dissuasive effect of an aqueous extract from *Enterolobium cyclocarpum* (Jacq) Griseb on the drywood termite *Incisitermes marginipennis* (Isoptera:Kalotermitidae) (Latreille)

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Abstract

Heartwood aqueous extracts from *Enterolobium cyclocarpum* (Jacq.) Griseb. exhibited chronic toxic lethal effect against *Incisitermes marginipennis* (Latreille) with more than 60% mortality at a concentration of 56.63 mg. Feeding rate correlated positively with the mortality of termites. The termite hindgut cellulolytic activity, the symbiotic microorganism population in termite hindgut and fungal cellulase activity were not inhibited by the aqueous extract. Two major chemical groups were detected in aqueous extract by gas chromatography; they were monoterpenes (46.5%) and phenols (18.7%). Fifty seven constituents, including, D-limonene, terpineol and eugenol were identified; these terpenes have repellent activity against insects. These results suggest that essential oils from *E. cyclocarpum* contain a slow acting toxin with a dissuasive effect on termites.

Key words: *Enterolobium cyclocarpum*, Hindgut, Antitermite activity

Introduction

One drywood boring insect is *Incisitermes marginipennis,* this termite lives in small colonies into drywood almost all its life. Each year these insects emerge from wood to infest other drywood and establish a new colony. A small number of termites can cause a big damage to low density and durability of drywood from *Pinus* spp or *Abies* spp when they have not been treated with conventional preservatives, such as boron, chrome, copper and arsenic salts or phenolic derivatives and organophosphates. These preservatives produce environmental damage, e.g. they have forced a great selective pressure on insects and others organisms that have generated resistance to the pesticides.

Research has been conducted to find a new, environmentally friendly alternative to control the drywood termites. The secondary metabolites of plants with natural resistance to pests and pathogens are a source of potential insecticides for wood preservation exhibiting new mechanisms of biological action capable of replacing the present ones. The understanding of the natural resistance to biodegradation that some heartwood possess and the secondary metabolites extracted from them, begins to be applied to the treatment of wood showing low resistance to biodegradation and in strategies for the rational utilization of pesticides in the pest management. It has been reported that extracts of heartwood from *Thuja plicata* and *Chamaescyparis nootkatensis* applied to softwood of the same tree species avoided degradation by termites and fungi (Taylor et al., 2006).

The tree *E. cyclocarpum* (native name parota) is a Central American native species that grows in central Mexico, from the Pacific Ocean coasts and the Gulf of Mexico to the North of Brazil and Colombia (Martínez Pacheco et al., 2012). In this region, it is used to obtain aromatic rubber freed from the bark (latex) which is used for pulmonary and bronchial affections treatment, also in cabinetmaking and production of other furniture, as well as a specie capable of restoring the environment and as fodder (Carranza Montaño et al., 2003). It was observed that antique furniture, household articles and the housing construction industry of heartwood derivatives from *E.*

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cyclocarpum in use nowadays, show a high durability, indicating that wood possesses a natural resistance to the attack of drywood boring insects and to degradation by fungi.

It is believed that, in part, this is due to secondary metabolites present in the tree (Carter et al., 1975; Chudnoff, 1984; Grace and Yamamoto, 1994). All this evidence is a motivation to study the potential anti-termite property from aqueous extract of *E. cyclocarpum* heartwood.

Materials and Methods

Volatile constituents extraction

One tree sample from each sampling location was collected. *E. cyclocarpum* heartwood logs from four mature trees more than 15 years old which had recently fallen were used to obtain the aqueous extracts. The extracts were elaborated with 100 g of heartwood sawdust in 500 ml of hot deionized water during 20 min at a temperature of 45-50 ºC (Raya-González et al., 2008). A reddish brown color extract was obtained, filtered and concentrated by liophylization (14.157 g) and stored under vacuum cooling to 0ºC until its use.

Biological material

Drywood termites of the species *I. marginipennis* from blocks of infested dry pine wood were cultivated in a container (40x50x150 cm), and maintained in the laboratory in darkness at 25ºC for a month for acclimatization prior to the test of toxicity (Raya González, 2013).

Oral toxicity assay

Whatman number 3 filter paper disks (diameter at 8.5 cm), were previously impregnated with the aqueous extract (0, 0.57, 5.66, 28.3 and 56.63 mg) and then dried to a constant weight. The treated papers were placed in a Petri-dish with 25 termites and covered to protect them from light. It was verified that the termites fed with cellulose impregnated with the aqueous extract by visual inspection of the pigmentation of its abdomen and they were contrasted with the control termites.

Every week, for eight weeks both the live and the dead termites were counted. Other toxic effects leading to death, such as those that suffered a reduction on the dimensions of the abdomen were not taken into account. To determine the percentage of feeding, each week the weight of the control filter paper and the impregnated paper were registered. The controls were termites fed with filter paper without aqueous extract (Martínez Muñoz et al., 2009).

Aqueous extract effect on micro flora and cellulytic enzymes of hindgut termite. Termites were starved for 48 h, then, several groups of fifteen termites each, were fed for 24 h with the following diets: 1) filter paper impregnated with (56.63 mg) *E. cyclocarpum* heartwood aqueous extract and, 2) a plain filter paper (control). After a period of 24 h post treatment, each termite was extracted by suctioning the hindgut to measure cellulolitic activity, presence and viable count of bacteria, protozoa and spirochaetes.

Cellulases preparation (crude enzymatic extract) from termite was done pool the hindgut of 15 termites and macerating them with liquid nitrogen on saline solution. Crude enzymatic extracts were used to measure the cellulolytic activity. Also, cellulases obtained from the phytopathogen fungi *Colletotrichum lindemuthianum,* the causal agent of *Phaseolus* (common bean) anthracnose, which was obtained by fungus growing on cellulose and salt medium were used.

The extracellular enzymes were precipitated with 40% NH_4SO_4, then desalted by dialysis and concentrated by liophylization and refrigerated until their use.

The enzymatic activity determinations of the β-glycosidase and endo cellulase were made using the method reported by Habu et al. The assay mixture contained 10 U (enzyme activity units) of enzymes in 20 to 80 µg protein (enzymatic extract), the substrates were *p*-nitrophenyl glycoside (PNPG) and carboxymethyl cellulose (CMC) for the determination of β-glycosidase and endo cellulase activities, respectively. The specific activity is reported as *p*mol·min^{-1}·mg^{-1} protein.

Phytochemical analysis

Test of total phenolics was done by the method reported by Swain and Hillis, 1995, using the Folin-Ciocalteu´s Phenol reagent and cinnamylic alcohol as standard. Test of total flavonoids was done by the method reported by Nieva Moreno et al. (2000), using aluminum nitrate and quercitin as standard. Test of reducing sugars was done as reported by Miller, using dinitrosalicylic acid (DNS) and glucose which was used as standard and was reported as glucose equivalents (Miller, 1959).

Gas chromatography-mass spectrometry analysis (GC-MS)

The GC-MS analysis was performed using an Agilent 6850 Series II with a mass selective 5973 MS detector gas chromatographer. The run conditions were: HP-5 MS column (30 m x 0.25 mm x 0.25 µm thick film), Helium was used as carrier gas at 0.935 Kp·cm^{-2}, column flow rate of 1 ml·min^{-1}. One microliter of sample extract was injected at a split ratio 1:10.

Table 1. Natural variability of extractable, phenolics and flavoniods from an aqueous extract of *E. cyclocarpum* heartwood.

Sampling locations	Extractables (g/g dw)	Phenolics (mg/ml)	Flavonoids (mg/ml)
Apatizingan, Mich (19º16' N and 102º22' W)	1.20	0.36 ± 0.009^{c}	11.62 ± 0.003^{b}
Tacambaro, Mich (19º14' N and 101º28' W)	1.84	0.77 ± 0.024^{a}	12.4 ± 0.007^{b}
Tuzantla, Mich (19º12' N and 100º34' W)	1.42	0.31 ± 0.017^{d}	19.4 ± 0.008^{a}
Veracruz, Ver (17º09' N and 98º39' W)	1.33	0.54 ± 0.025^{b}	8.25 ± 0.026^{c}

The oven temperature was held at 150ºC for 3 min with increments of 5ºC·min^{-1} to 278ºC. Other chromatography conditions were; injector temperature, 270ºC; transference line temperature, 300ºC; scanning range, 50 to 600 a.m.u.; voltage ion source 70 eV, scanning speed 1.9 scan·s^{-1}; source pressure at 50 mTorr.

Constituents identification of aqueous extract was done by comparing the mass spectral data with those reported in NIST Rev. D.04:00 2002 mass spectral database.

Data analysis

Five independent experiments were done using three independent replicates. Feeding and survival rates were measured each time. The percentages of

The biological variability is a natural factor that depends on the genome and life history of the organism, e.g. Lewis et al. (2004) observed twice the amount of extractables in *Arabidopsis* extracts from individual plants grown under controlled environmental conditions compared to a pool of combined material extracts from all 24 plants in a tray. To reduce the variation of extractables quality and amount from individual tree in the extract preparation procedure, the sawdust from all trees samples were mixed to obtain a single aqueous extracts from the heartwood of *E. cyclocarpum*.

The concentration dependent toxic effect of an aqueous extract from *E. cyclocarpum* on the survival and feeding rates of *I. marginipenis* (termite) is summarized in Table 2. Termites were susceptible to 56.63 mg of extract with survival rate of 38 % after five weeks, the termites were fed with paper impregnated containing aqueous plant extract in a dependent concentration way.

Table 2. Survival and feeding rates of *I. marginipennis* exposed to aqueous extract of *E. cyclocarpum* after five weeks of exposure.

Heartwood extract (mg)	Survival rate (%)	Feeding rate (%)
0.00	97	100
0.57	96	92
5.66	77	63
28.30	44	29
56.63	38	26

mortality were calculated according to the Abbot equation (Abbot, 1925). The percentage of feeding was calculated on the basis of the consumption of the paper in the control test as 100% of feeding.

All the cases reflect the differences of values among the treatments and the control according to one way ANOVA, the statistical analysis of correlation was done with the Statistic 7.0 software.

Results and Discussion

The heartwood aqueous extracts from four trees with different harvest origin and development stage had a natural biological variability in the amounts of extractables, phenolics and flavonoids, therefore aqueous extract induced different feeding and mortality rates on termites, see Table 1.

Worker termites were susceptible to the plant aqueous extract; inducing a 62% termite mortality rate after five weeks, following oral feeding rate at 26%; these values did not change during the next three weeks. A strong positive correlation was observed between termite feeding and mortality rates. The range of termite feeding values was small and termite mortality matching the sample was positive by linear regression analysis, see Figure 1.

The regression line slope ($r = 0.967$, d.f. = 1, $F = 48$, s.e = 2.2) represents the efficiency of termites for eating filter paper impregnated with aqueous extract. *E. cyclocarpum* heartwood aqueous extract exhibited a toxic effect against *I. marginipennis* which was dependent on the time and the amount of extract consumed. The survival rate of termites in starvation was similar to the subterranean termite *Coptotermes formosanus* Shiraki and different from the termites of the genus *Reticulitermes* (Boue and Raina, 2003).

Nevertheless, the toxic effect was less effective than the one caused by tannins and modified tannins against *C. formosanus* (Yamaguchi et al., 2002).

Also, it was observed that in termites fed with paper impregnated with the extract, the size of the abdomen diminished visibly and finally led to their death. The termite population with these symptoms was roughly 20% after four weeks.

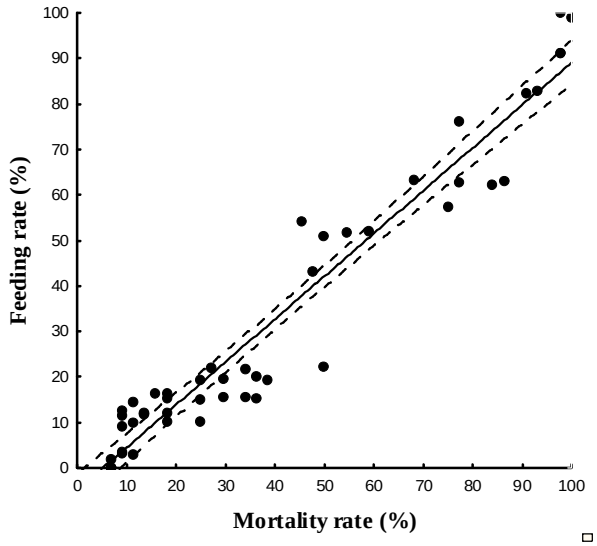

Figure 1. Consumption correlation of cellulose impregnated with aqueous extract of *E. cyclocarpum* and the mortality rate. The data of mortality and feeding rates were used, the line is a linear regression with r^2 = 0.94 and $\alpha = 0.05$ as a consequence the correlation is significant.

The no-selection food assays showed that concentrations over 28.3 mg of the plant aqueous extract caused a decrease in the consumption of cellulose due to a slow acting toxin with a lethal toxic effect. This reduced the number of termites, and no increase in the consumption of the filter paper was detected, similarly to the chlordane and chlorpyrifos effect on formasan subterranean termite or the imidinohydrazone effect on American cockroaches (*Periplaneta americana* Lin.) and German cockroaches (*Blotella germanica* Lin.) (Su et al., 1987; Lovell, 1979). From these results it was concluded that death from termites correlated with the consumption of increasing concentrations of the aqueous extract.

E. cyclocarpum aqueous extract presented a lethal toxic effect against *I. marginipennis* for a long therm. The understanding of the factors involved in the toxicity of aqueous extract on termites is difficult because of the presence of complex mixtures of secondary metabolites in the aqueous heartwood extract, however, it is possible that some of these affected the symbiotic relationship between the gut microbiota and the termite, in analogy to the sulfuramide effect on termite cellulases (Zhou et al., 2008).

It is thought that some constituents of the aqueous extract exhibit inhibitory activity on termite hindgut cellulases (β-glycosidase and endocellulases) or have a microbiocide activity that affects the digestive function of termites. A change on activity in termite hindgut was taken as an indicator of the kind of toxic effect caused by hardwood aqueous extract on termites. The β-glycosidase and endocellulase enzymes were taken as representatives of intestinal cellulolytic activity on termites. Administration of cellulose impregnated with heartwood aqueous extract did not alter the intestinal cellulolytic activity compared to that observed in termites fed with only cellulose (Table 3).

Similarly, *in vitro* assays of cellulases partially purified from fungus *C. lindemuthianum* were not affected by any chemical component of heartwood aqueous extract from *E. cyclocarpum*, see Table 3. Also, it was observed that termite symbiotic hindgut microorganisms were not severely affected by aqueous extract. The protozoa and spirochetes abundance did not show any change, and bacteria were weakly affected. The termite hindgut cellulolytic activity (cellulases secreted by protozoa, fungi, bacteria and probably by the insect) was not affected by any component of heartwood aqueous extract nor inhibited by *in vitro* activity of fungal cellulases.

Chromatographic analysis of the heartwood aqueous extract allowed detecting some groups of chemical, which were clustered in three groups; major abundance, monoterpenes (46.5%) and phenols (18.7%); intermediate abundance, alkanes (6.8%), alcohols (4.17%), aromatic alkenes (3.36%), aromatics (4.26%), ketones (6.55%), sesquiterpenes (5.82%) and minor constituents, carboxylic acids (0.39%) and quinones (0.74%).

Table 3. Effect of *E. cyclocarpum* heartwood aqueous extract on cellulolytic activity present in *I. marginipennis* hindgut.

Enzymatic source	β-Glycosidase (*p*mol·min^{-1}·mg^{-1} prot.)	Endocellulase (*p*mol·min^{-1}·mg^{-1} prot.)
Hindgut of termite fed without *Ec*	93 ± 12	2.23 ± 0.07
Hindgut of termite fed with *Ec* (56.63 mg)	102 ± 13	2.98 ± 0.11
Cellulase-*Cl* (10 U)	152 ± 10	7.02 ± 0.75

Cl, C. lindemuthianum; Ec, E. cyclocarpum aqueous extract

Table 4. Relative abundance of volatile constituents identified from *E. cyclocarpum* heartwood.

Constituents	Retention time (min)	Relative abundance (%)
α-pinene	3.119	0.15
D-limonene	6.538	17.83
p-cymene	8.720	10.93
exo-brevicomin	10.349	0.18
Methylheptenone	12.242	0.87
Tetradecane	13.533	0.50
2-butoxy ethanol	15.362	0.40
α,*p*-dimethylstyrene	15.690	2.14
Farnesane	16.602	0.21
Octen-3-ol	17.061	0.15
trans linalool oxide	17.679	0.22
Acetic acid	17.908	0.37
Pentadecane	18.062	1.89
Octyl alcohol	18.592	3.41
Linalool	20.859	0.44
α-bergamotene	21.081	1.69
Caryophyllene	21.213	1.08
(+)-fenchol	21.911	0.98
Aristolene	22.000	1.01
Hexadecane	22.328	2.75
4-terpeneol	22.583	0.99
2,5-dimethylhydroqinone	23.421	0.70
β-damascone	23.621	1.09
β-terpineol	23.845	0.51
Menthol	24.109	0.22
Acetophenone	24.494	0.53
γ-valerolactona	25.299	3.38
Pristane	25.549	0.23
2,6,10,14-tetramethyl hexadecane	25.601	0.20
trans-*p*-2,8-menthadien-1-ol	25.927	0.43
(-)-α-terpineol	26.310	5.60
β-bisabolene	26.756	1.55
Napthalene	27.364	0.62
Epizonarene	27.608	0.30
Veratrole	27.718	1.90
Carveol	28.279	0.28
Acetophenone	28.935	0.68
methoxy-phenyl oxime	29.594	0.52
Octadecane	30.022	0.65
Methyl veratrole	30.579	0.51
methyl napthalene	31.296	0.54
p-cymen-8-ol	31.892	4.84
Guaiacol (natural)	32.332	2.77
Paeonal	33.045	0.36
Butylated hydroxytoluene	33.848	7.01
Phenylethyl alcohol	33.998	0.40
Benzothiazole	35.063	0.55
2-methoxy-4-methylphenol	35.522	2.72
1,2,3-trimethoxy benzene	35.856	0.30

2,6-dimethyl naphthalene	36.010	0.21
p-mentha-1(7),8(10)-dien-9-ol	36.726	0.47
2,4-dimethylphenethyl alcohol	37.468	0.35
4-ethyl-2-methoxy phenol	37.892	0.17
1,6,7-trimethyl naphthalene	39.727	0.44
4-methyl phenol	39.949	4.51
2,6-dimethoxy phenol	40.097	0.06
Eugenol	42.145	0.20

The GC-MS analysis allowed to identify fifty seven constituents (Table 4), some of them like D-limonene have a repellency activity against insects including mealybugs, whiteflies, ticks, louses, acaruses and fleas (Hollingsworth, 2005). Another example is terpineol used with eugenol and cinnamic acid produce a neuro-insecticide mixture used against carpenter ants *Camponotus pennsylvanicus* De Geer and German cockroaches *B. germanica* (Enan, 2001).

It is possible that the major constituents detected in the essential oil from *E. cyclocarpum* heartwood, terpineol, eugenol, cinnamic acid and D-limonene act on *I. marginipennis* as repellent or as neuro insecticide as has been described.

Also, a small group of components that are not plant natural products and the tree intakes from the

environment by the following two mechanisms were detected: a) pollutants acquired by bioconcentration such as 2-butoxy ethanol, 1,2,3-trimethoxy benzene (used in paint industry), 2-ethyl-1-hexanol (a product of the biodegradation of the phthalate plasticizers and oil additives) and 2,5-dimethylhydroquinone (urease inhibitor) or, b) components acquired by microbial interaction such as octen-3-ol, 4-ethyl-2-methoxy phenol and 4-methyl phenol, all of them are fungal metabolites.

Finally, it is considered that dissuasive agents such as the anti-feedants and the repellents are a class of environmentally friendly drywood preservatives. These metabolites do not cause death in target and others organisms therefore it is better to use an agent with a dissuasive effect on drywood termite *I. marginipennis* than one with a lethal effect. The results described herein suggest that the essential oil from heartwood of *E. cyclocarpum* induced a dissuasive effect on termites.

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Cryopreservation of *Dendrobium* sonia-28 using an alternative method of PVS2 droplet freezing

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Abstract

Dendrobium sonia-28, an ornamental orchid in the Malaysian flower industry, is under risk of producing heterozygous progenies. Cryopreservation is a favoured long-term storage method for orchids with propagation problems. The droplet-freezing technique, utilising the principles of vitrification, has emerged as an important technique in the preservation of various plant species. This study was conducted to optimise the droplet-freezing technique for protocorm-like bodies (PLBs) of *Dendrobium* sonia-28, with viability tests conducted through the 2,3,5-triphenyltetrazolium chloride assay. The best results were obtained when 1-2 mm PLBs were precultured for 24 hours on half-strength Murashige and Skoog (MS) semi-solid medium supplemented with 0.5 M sucrose, placed in a loading solution for 10 minutes and dehydrated in PVS2 at 0°C for 20 minutes, prior to thawing, unloading in 1.2 M sucrose and recovery on semi-solid recovery medium composed of half-strength MS components and 2% sucrose for three weeks.

Key words: *Dendrobium* sonia-28, PVS2 droplet-freezing, Protocorm-like bodies

Introduction

Commercially valuable *Dendrobium* orchids such as *Dendrobium* sonia-28 are popular as cut flowers and ornamental plants due to their frequent flowering and high number of flowers for each inflorescence (Martin and Madassery, 2006). *Dendrobium* wholesale in Hawaii is valued at about USD 10 million (Sewake, 1999), while *Dendrobium* cut flowers contributed 80% of total cut flower production in Thailand (Bureau of Agricultural Economics Research, 2003; Samtinoranont and Wannakrairoj, 2010). The economic importance of *Dendrobium* orchids increases the urgency to conserve valuable orchid germplasm. The need of orchid conservation is also driven by various factors such as loss of orchid habitat in nature and illegal collection by hobbyist (Siregar, 2008).

Cryopreservation presently provides a long term conservation option, requiring only a minimum of space and maintenance (Sakai et al., 1991). For successful cryopreservation, intracellular freezing, which occurs during rapid cooling in liquid nitrogen (LN), must be avoided. One of the keys to successful cryopreservation by vitrification is the careful control of dehydration and prevention of injury by chemical toxicity. Specimens to be preserved have to be sufficiently dehydrated to avoid intracellular freezing and thus vitrify upon rapid cooling (Pandey et al., 2008).

The droplet-vitrification method, reported to be a promising technique in the plant cryopreservation field, is based on the droplet-freezing method that was established for potato (Schäfer-Menuhr et al., 1997) using 10% DMSO as the cryoprotectant. Droplet-vitrification is a cryopreservation method capitalising on the direct vitrification of small volumes of water containing cryoprotective additives placed on highly efficient, heat-conducting materials such as aluminium foil (Panis et al., 2005; Sakai and Engelmann, 2007; Galdiano Jr. et al, 2012). Successful attempts of this method have been reported for asparagus (Mix-Wagner et al., 2000), yam (Leunufna and Keller, 2003) and rose (Halmagyi and Pinker, 2006). Highly concentrated cryoprotectants such as plant vitrification solution 2 (PVS2) are used to protect the cells during freezing and to promote recovery of the plant tissue after storage in liquid nitrogen

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(Sakai, 1997). The droplet-vitrification method was the only method giving regeneration in three banana cultivars: Nakitengwa, Ingarama and Amou (Panis et al., 2004).

A post-cryopreservation viability test is defined as a test "that evaluates stress caused to plant tissue by cryopreservation and expresses the probability for survival or regrowth" (Verleysen at al., 2004). The best survival assessment of cryopreserved tissues is through regrowth of the target tissues into plantlets. However, regrowth is very slow in many cases, especially when a cryopreservation protocol is still in the optimisation stage. In this case, application of viability tests, based on the cellular absorption of dyes cannot be avoided, as most cryopreservation protocols require rapid assessments on whether the material survived the cryopreservation procedure (Leslie et al., 1995). The TTC assay, qualitative for large tissues and organs, is often used for embryos and axes, and is reduced into red-coloured formazan by respiration in mitochondria of the living cells. This method is based on the metabolic activity of living plant cells, as living cells or viable areas of the target plant sample absorbs the colourless TTC compound, which is then reduced by dehydrogenases, yielding triphenylformazan or reduced TTC (Van Waes and Debergh, 1986; Verleysen at al., 2004). Tsukazaki et al. (2000) reported that the TTC reduction assay and regrowth observations used in the assessment of survival of *Doritaenopsis* suspension culture cryopreserved by vitrification were correlated.

In this study, the plant vitrification solution 2, commonly known as PVS2 (Sakai et al., 1991) was used as a cryoprotectant to dehydrate and cryopreserve protocorm-like bodies (PLBs) of *Dendrobium* sonia-28 via the droplet-freezing method. The objectives of the present study are to ascertain the best PLBs size suitable for cryopreservation, and also to evaluate the effects of various sucrose concentrations, loading periods and PVS2 treatment periods on the viability of cryopreserved PLBs of *Dendrobium* sonia-28.

Materials and Methods

Plant material

In vitro cultures of protocorm-like bodies (PLBs) of *Dendrobium* sonia-28 were selected for this study. The PLBs were cultured on half-strength Murashige and Skoog (MS; Murashige and Skoog, 1962) semi-solid media supplemented with 1 mg/L benzylaminopurine (BAP), 20 g/L sucrose and 2.75 g/L Gelrite. The cultures were incubated at 25±2ºC under 16-hours photoperiod using white fluorescent lamps (Philips TLD, 36 W) set at 150 µmol m^{-2} s^{-1}. The PLBs were subcultured every four weeks.

Effect of PLB size

In order to determine the best PLB size in the application of the droplet-freezing method, four-week old 1-2 and 3-4 mm PLBs of *Dendrobium* sonia-28 were precultured on medium consisting of semi-solid half-strength MS components and 0.5 M sucrose. The PLBs were then incubated for 24 hours at 25±2ºC under 16 hours photoperiod using white fluorescent lamps (Philips TLD, 36 W) set at 150 µmol m^{-2} s^{-1}. The PLBs were then placed in sterile cryovials and immersed with a loading solution composed of liquid half-strength MS medium supplemented with 0.4 M sucrose and 2.0 M glycerol, for 20 minutes. For dehydration, the PLBs were immersed in PVS2 (30% [w/v] glycerol, 15% [w/v] ethylene glycol, 15% [w/v] Me_2SO and 0.4 M sucrose at 0ºC for 20 minutes (Sakai et al., 1991). The PVS2 was drained from the cryovials and the PLBs were then transferred onto 10 µl PVS2 droplets placed on a strip of sterile aluminium foil. The foils were carefully folded and submerged into LN for at least one hour. All solutions used were exchanged with fresh solutions after 10 minutes.

Effect of preculture

To determine the best sucrose concentration for preculture, PLBs with the optimal size (as selected from the previous treatment) were cultured on half-strength MS semi-solid medium supplemented with various sucrose concentration (0.0, 0.25, 0.5, 0.75, and 1.0 M). The osmoprotection and dehydration steps were conducted as in the determination of the best PLB size for cryopreservation.

Effect of loading and dehydration treatments

The best PLB size and preculture conditions were selected from the previous sections to proceed with the next steps of the treatment. In order to assess the effect of loading duration on the PLBs, the PLBs were immersed in loading solution for 10, 15, 20, 25, 30 and 35 minutes, with an exchange of fresh solution at half-time intervals. This was followed by immersing the PLBs in 1.5 ml PVS2 for 20 minutes at 0°C. The solution was exchanged after 10 minutes with fresh PVS2.

In order to assess the effect of dehydration on the PLBs, the PLBs were immersed in 1.5 ml loading solution (half-strength MS medium supplemented with 2 M glycerol and 0.4 M sucrose) at room temperature for 20 minutes. The loading solution was removed and replaced with 1.5 ml PVS2 at 0°C for 10, 20, 30 or 40 minutes.

Thawing, unloading and PLBs growth recovery

Non-cryopreserved and cryopreserved PLBs from all treatments were subjected to the recovery steps mentioned in this section. The foils were carefully removed from LN and rapidly thawed in 10 ml unloading solution (half-strength MS solution with 1.2 M sucrose) for 20 minutes at room temperature. After 20 minutes, the PLBs were transferred onto a piece of sterile filter paper to blot dry the unloading solution.

The PLBs were transferred onto two pieces of sterile filter papers placed on semi-solid recovery medium composed of half-strength MS components and 2% sucrose, and incubated in the dark for one day at 25±2ºC. The PLBs were then transferred onto fresh recovery medium without the filter paper and incubated in the dark at 25±2ºC for one week. The PLBs were slowly exposed to dim light by incubation under a layer of white cloth for one week. Finally, the PLBs were exposed to light and incubated for one more week at 25±2ºC under 16 hours photoperiod using white fluorescent lamps (Philips TLD, 36 W) set at 150 µmol m^{-2} s^{-1}. Non-cryopreserved PLBs were subjected to the same treatment as cryopreserved PLBs, except for storage in LN.

PLBs viability assessment

After 3 weeks of recovery, the viability of cryopreserved and non-cryopreserved PLBs were assessed using the 2,3,5-triphenyltetrazolium chloride (TTC) analysis, set at 490nm (Verleysen et al., 2004).

Statistical Analyses

The treatments consisted of six replicates, each containing 10 PLBs. Means in the determination of the best PLB size were analysed through independent sample's t-test. Means from other treatments were analysed with one-way ANOVA and differentiated with Tukey's test. The probability value was set at 0.05.

Results

Effect of PLB size

The results of this study showed that 1-2 mm cryopreserved PLBs produced higher absorbance values compared to 3-4 mm cryopreserved PLBs (Figure 1), hence their selection for the subsequent steps of the experiment. On the other hand, 3-4 mm PLBs produced higher absorbance values compared to 1-2 mm PLBs in the control treatments.

Effect of Sucrose Preculture

It was observed that the cellular viability of cryopreserved and non-cryopreserved PLBs was the highest when PLBs were precultured in 0.5 M sucrose (Figure 2). The viability of cryopreserved PLBs decreased when they were precultured in higher sucrose concentrations (>0.5 M) prior to storage in LN.

Effect of loading treatment

Results from the loading treatment showed that 10 minutes of loading produced the highest viability for cryopreserved PLBs of *D.* sonia-28 (Figure 3). The decrease in cellular viability after the 10th minute indicated that extended exposure to loading solution is harmful for PLBs of *D.* sonia-28.

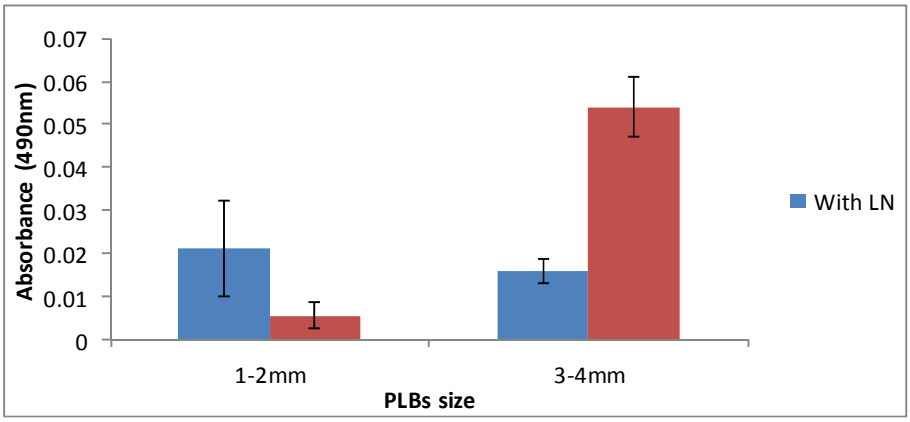

Figure 1. Effect of PLB size in the cryopreservation of *Dendrobium* sonia-28. The PLBs were precultured in 0.5 M sucrose prior to loading, dehydration and storage in LN. The PLB sizes tested were 1-2 mm and 3-4 mm. Error bars show corresponding standard deviation.

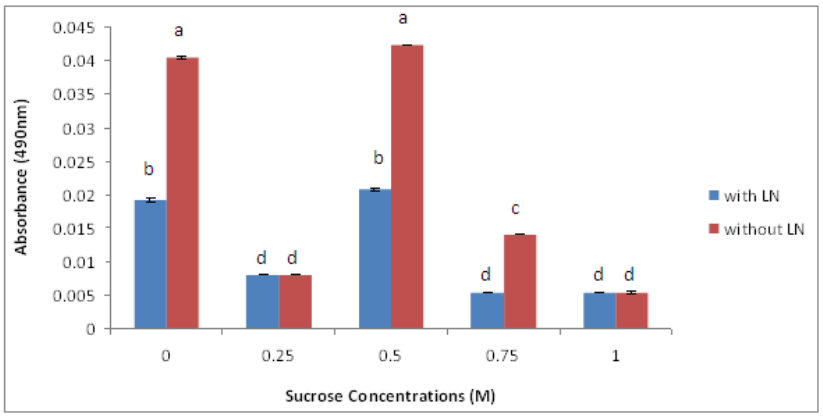

Figure 2. Effect of preculture on the viability of non-cryopreserved and cryopreserved PLBs of *Dendrobium* sonia-28. The tested sucrose concentrations were 0, 0.25, 0.50, 0.75 and 1.0 M. Error bars show corresponding standard deviation.

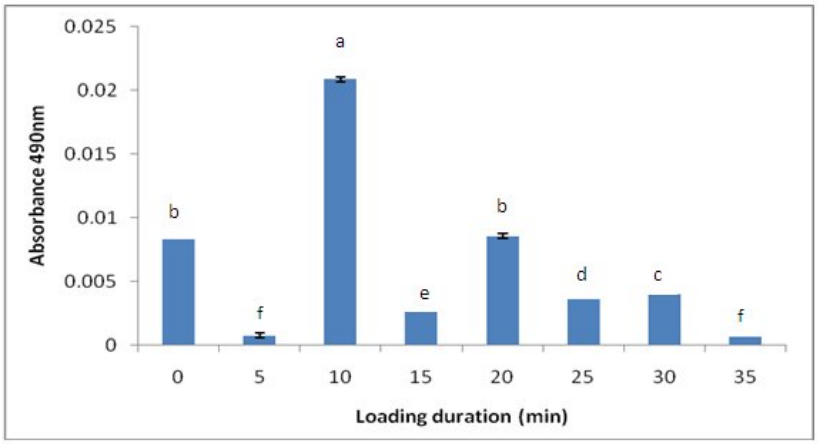

Figure 3. Effect of different loading durations on the viability of cryopreserved PLBs of *Dendrobium* sonia-28. The tested loading durations were 0, 5, 10, 15, 20, 30 and 35 minutes. Error bars show corresponding standard deviation.

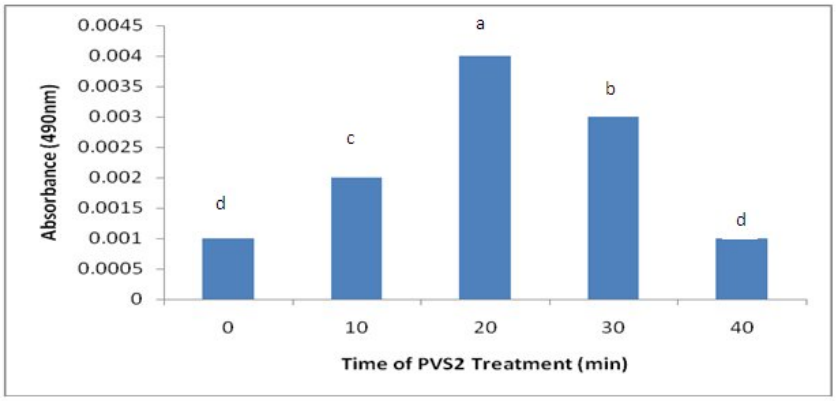

Figure 4. Effect of different dehydration durations on the viability of cryopreserved PLBs of *Dendrobium* sonia-28. The tested PVS2 durations were 0, 10, 20, 30, and 40 minutes. Error bars show corresponding standard deviation.

Effect of PVS2 treatment

Cryopreserved PLBs of *D*. sonia-28 showed increasing cellular viability with increased exposure to PVS2 (Figure 4). A significantly high cellular viability was recorded when PLBs were exposed for 20 minutes to PVS2, suggesting that the PLBs can be effectively dehydrated at the mentioned duration without causing harmful effect to the cells. The drastic decline of cellular viability in PLBs of *D*. sonia-28, with increasing exposure to PVS2 (30 and 40 minutes), could be due to the toxic effect of the solution.

Discussion

Effect of PLB size

The best results in the cryopreservation of PLBs of *Dendrobium* sonia-28 were obtained when 1-2 mm PLBs were used in the vitrification protocol, compared to 3-4 mm PLBs. This result corresponds to that obtained by Pouzi et al. (2011), who discovered that the best viability in the encapsulation-dehydration of *D.* sonia-28 was obtained when 1-2 mm PLBs were precultured in 1.0 M sucrose for 24 hours (Pouzi et al., 2011). In *Iris nigricans*, 2–4 mm somatic embryos produced higher survival rates compared to 1–2 mm or 4–6 mm embryos (Shibli, 2000; Lambardi et al., 2008). Lambardi et al. (2005) discovered that within the callus clumps of *Aesculus hippocastanum*, embryogenic masses at an advanced stage of somatic embryo maturation, for instance, the torpedo stage, produced optimum post-cryopreservation regrowth of healthy and proliferating embryogenic callus, performing better than callus clumps composed predominantly of globular, heart shaped and cotyledonary somatic embryos (Lambardi et al., 2008). Hence, the outcome of this cryopreservation treatment could have depended on the age or the growth stage of the PLBs prior to the cryopreservation process.

Effect of Sucrose Preculture

High sucrose concentrations applied in this study resulted in excessive cell dehydration, with consequences on the viability of explants (Halmagyi and Pinker, 2006). The cellular viability of the PLBs in this study displayed a similar pattern to that observed in cryopreserved *Lilium* (Chen et al., 2010). The best results in the encapsulation-vitrification of PLBs of *Dendrobium candidum* Wall. ex Lindl. were obtained when the PLBs were subjected to a five-day preculture in 0.75 M sucrose medium with BAP and 1-naphthaleneacetic acid (NAA) (Yin and Hong, 2009). Results of a study by Tan et al. (2010) showed that the best viability rate in the cryopreservation of *D.* sonia-28 was achieved when 3-4 mm PLBs were precultured in semi-solid half-strength MS media with 0.6 M sucrose and dehydrated in PVS2 at 0°C for 20 minutes. In the cryopreservation of *Dendrobium* Bobby Messina by vitrification, the best viability rate was obtained when 3-4 mm PLBs were precultured in either 0.6 M sucrose or 1.2 M sorbitol (Antony et al., 2010).

Preculture of plant materials in medium containing sucrose or sorbitol boosted the survival of cryopreserved shoot tips of white clover (Yamada et al., 1991), apple and pear (Niino et al., 1992). The beneficial effect of sucrose in cryopreservation could be due to two effects (Steponkus et al., 1992). First, sucrose, like other osmotically active substances, has an osmotic dehydration effect during treatment, leading to reduced water content in the tissue (Reinhoud et al., 1995, Tanaka et al., 2004). Furthermore, sucrose is able to penetrate the cells (Dumet et al., 1993), proven by histological observations of intracellular accumulation of starch during preculture (González-Arnao et al., 1998). The accumulation of sucrose within the tissue contributes to the cell viability by assisting in the removal of cellular water to the point of glassy state during vitrification (Steponkus et al., 1992). Sugars are also known to play a crucial role in the preservation of the membrane integrity (Crowe et al., 1988) and protein structure (Leslie et al., 1995) during dehydration.

Effect of loading treatment

The loading solution is credited in the reduction of injurious membrane changes resulting from severe dehydration (Ishikawa et al., 1997). In this study, the effect of the loading solution on the survival of PLBs of *Dendrobium* sonia-28 was neither readily apparent nor significant in either the control or freezing treatments. Wang et al. (2004) stated that the loading treatment, regardless of the immersion durations, did not influence the viability of grapevine embryogenic cell suspensions, with viability percentages recorded at about 85 and 76% for non-cryopreserved and cryopreserved cells respectively. Panis et al. (2005) also observed that loading treatment conducted for any duration did not significantly affect regeneration of control or cryopreserved *Musaceae* meristems. However, a study by Takagi et al. (1997) showed that a loading treatment for taro shoot tips was important in ensuring their survival post-cryopreservation, with

the best result obtained when the samples were osmoprotected for 20 minutes.

Effect of PVS2 treatment

Cryoprotectants such as PVS2 function to protect and to recover the plant material after storage in LN (Sakai, 1997). The optimal dehydration treatment of different plant species (with varying water content and membrane permeability) can differ considerably. Towill and Jarret (1992), and Sakai (1997) reported that long exposure of explants to highly concentrated vitrification solution is potentially injurious because of the phytotoxic effects of individual component or combined osmotic effect on cell viability.

Tiau et al. (2009) reported that 2-4 mm PLBs of *D.* sonia-28 gave the highest viability when PLBs were precultured in 0.25 M sucrose, followed by dehydration in PVS2 at 0°C for 20 minutes. This corresponds to the results obtained in this study. Cryopreserved PLBs of *Bratonia* had to be dehydrated for one hour in modified PVS2 supplemented with PEG instead of ethylene glycol to boost post-thawing recovery percentages up to 20.4% (Popova et al., 2010). Tsukazaki et al. (2000) discovered that the use of PVS2 was detrimental to the survival of *Doritaenopsis* suspension culture as the TTC stainability reduced to 80% when the cells were precultured in 0.056, 0.1, 0.2, 0.3 or 0.4M sucrose and immersed in PVS2, compared to cells that were simply precultured and not dehydrated (85%). However, cells that were not dehydrated did not survive the cryopreservation procedure at all. A three- to five-hour dehydration period was required for the embryos of *Bletilla striata* (Ishikawa et al., 1997). On the other hand, apical meristems of Japanese horseradish required only 10 minutes of dehydration in PVS2 prior to cryostorage (Matsumoto et al., 1994). The differences in the dehydration period for various plant species was attributed to the cell clump sizes, developmental stages and physiological condition of the explant (Tsukazaki et al., 2000).

Conclusion

In this study, it was found that the highest viability in the cryopreservation of PLBs of *Dendrobium* sonia-28 through the droplet-freezing method was achieved when 1-2 mm PLBs were precultured in half-strength MS medium supplemented with 0.5 M sucrose, placed in the loading solution for 10 minutes, and dehydrated in PVS2 for 20 minutes.

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PLANT SCIENCE

Pentacyclic triterpenoids with antimicrobial activity from the leaves of *Vernonanthura patens* (Asteraceae)

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Abstract

Vernonanthura patens (Kunth) H. Rob, is native to Mexico and South America and found in dry or wet forests or pine forests. They are reaching in height from 0 to 1865 meters above sea level (masl). They are distributed throughout South America, in Central America: Mexico, Belize, Costa Rica, El Salvador, Guatemala, Honduras, Nicaragua, Panama, and northern South America: Venezuela, western South America: Bolivia, Colombia, Ecuador and Peru. Three pentacyclic triterpenoids from the leaves were isolated by chromatographic (Colum Chromatography (CC) and Thin Layer Chromatography (TLC)) techniques. The structures of these isolated compounds were identified by 1H NMR and 13C NMR spectroscopic and MS analysis as lupeol, epilupeol and acetyl lupeol. Lupeol and epilupeol showed antifungal activity against *Fusarium oxysporum* and *Penicillium notatum*, whereas acetyl lupeol was inactive.

Key words: Pentaciclyc triterpenoids, Lupeol, Epilupeol, Acetyl lupeol, Antimicrobial, *Vernonanthura patens*

Introduction

Vernonanthura patens (Kunth) H. Rob (Asteraceae) is a wild plant broadly distributed throughout America. It grows from 0 to 2200 meters above sea level in the Ecuadorian region. Its leaves are cooked and used against malaria, for postpartum treatment and for healing infected wounds of animals as part of a plant mixture (Blair, 2005; Kvist, 2006). It is also used against headaches, treatment of leishmaniasis (Gacheta, 2010), preparation of antivenos (Tene, 2007), and to combat athlete's foot (Valadeau, 2009). Its usefulness for treating certain types of cancer has also been referred to by indigenous healers. However, there are scarce biological and chemical studies on this species. The only results published so far refer to the antimalarial activity against *Plasmodium falciparum*, Itg2 strain (Blair, 2005), anti-*Leishmania* activity (Valadeau, 2009) of the leaves and lack of antiprotozoal activity against different strains of Leishmania (Fournet, 1994). On the chemical composition of the species, there are reports of sesquiterpene lactones and sesquiterpenes present in the aerial parts (Mabry, 1975: Jakupovic, 1986). There are some references on *Vernonanthura* species that shows the presence of diterpene compounds (Portillo, 2005; Valadeau, 2009), flavonoids (Borkosky, 2009; Mendonça, 2009), triterpenes (Tolstikova, 2006; Gallo, 2009), saponins (Borkosky, 2009) and sesquiterpene lactones. In addition, different biological activities have been described assuming that certain chemical groups could be responsible for the therapeutic properties attributed to species of this genus (Pollora, 2003, 2004; Portillo, 2005; Bardón, 2007). In this work, we performed the extraction and fractionation of leaves of the species in the vegetative state using column chromatography increasingly polar solvent and thin layer

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chromatography. Through these procedures, three triterpenoids isolated, which were identified using Nuclear Magnetic Resonance Spectroscopy of ^{1}H and ^{13}C. For these compounds, antimicrobial activity was determined against *Fusarium oxysporum* and *Penicillium notatum*.

Materials and Methods

Plant material

Mature leaves of *Vernonanthura patens* (laritaco) were collected at the vegetative state in the Marcabelí town, province of El Oro, Ecuador, with the following coordinates S 3° 47' 06,14" W 79° 54' 32.66", to 316,58 m. Leaves were collected in the early morning in February 2011. A voucher specimen is on deposit in the National Herbarium of Ecuador (QCNE) and a duplicate sample (CIBE37) is kept as a herbal witness in the laboratory of the CIBE-ESPOL Bioproducts.

Extraction and isolation

Coarsely ground leaves (67 g) were extracted by maceration, with two liters of methanol (HPLC grade) in a closed container and in the absence of light and with agitation in a New Brunswick Scientific shaker model C-10. The extraction time was 8 days and was conducted until total depletion of plant material. The crude extract was evaporated to dryness on a rotary evaporator Heidolph brand Laborota 4001 model with reduced pressure at 50°C.

The crude methanol extract was subjected to CC over activated silica gel (60 - 200 mesh) using *n*-hexane, *n*-hexane: EtOAc (90:10, 80:20) and EtOAc, taking 150 mL fractions each time. Four fractions were obtained: Fr 1 *n*-hexane (79 mg), Fr 2 *n*-Hex/EtOAc (90:10) (1370 mg), Fr 3 *n*-Hex/EtOAc (80:20) (0.60 mg) and Fr 4 EtOAc (0.21 mg). From fractions three and four, on further purification by preparative layer chromatography, compounds 1 (57 mg), 2 (20 mg) and 3 (90 mg) were obtained, respectively.

Mass spectra were performed on an Agilent 7890A gas chromatograph, with Agilent 5975 detector (Avondale, PA.USA).

NMR analyses **[**^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz)] were recorded at room temperature with a Bruker DRX500 spectrometer with an inverse multinuclear 5 mm probe head equipped with a shielded gradient coil. The spectra were recorded in $CDCl_3$, and the solvent signals (7.26 and 77.0 ppm, respectively) were used as reference.

Bioassays

The diffusion method with potato dextrose agar (PDA) was used to determine the antifungal activity of three isolated pure compounds from *V. patens* leaves at 100 and 200 µg/mL (Avello, 2009). Dilutions were made with 10% dimethylsulfoxide (DMSO). Strains of *Fusarium oxysporum* and *Penicillium notatum* were isolated from infected *Pinus radiata* and *Citrus sinense* fruits and maintained in the Collection of Fungi at the University of Concepcion-Chile.

Holes of 5 mm diameter were made in the agar with a sterile cork borer and filled with 20 µL of each concentration of pure compound. DMSO 10% was used as a negative control in each plate. A disc (5 mm diameter) of already grown fungus was placed in the center of Petri dishes and incubated at 22°C. Radial growth assessment was made during 2 weeks.

Experimental design was completely randomized and each assay was performed in triplicate. Descriptive statistics of the experimental data were made in order to represent and point out its most important features.

Results and Discussion

Compound 1

Compound 1 was isolated as a white amorphous solid. The mass spectrum suggested formula $C_{30}H_{50}O$ (M m/z 426). Its $^{1}HNMR$ spectrum (Cl_3CD, 300 MHz), exhibited seven signals as singlet to δ 0.76 (3H, s, Me-28), 0.78 (3H, s, Me-25), 0.83 (3H, s, Me-24), 0.94 (3H, s, Me-23), 0.96 (3H, s, Me-27), 1.03 (3H, s, Me-26) and at lower fields other to δ 1.67 (3H, s, Me-30) assignable to a methyl group on double bond; a multiplet at δ 1.94 (2H, m, H-21); a double doublet to δ 2.4 (1H, dd, J=11.01 and 5.7 Hz, H-19) to be assigned to a methynic proton; a double doublet to δ 3.20 (1H, dd, J=10.9 y 5.3 Hz, H-3) assignable to a geminal proton of a secondary alcohol, two doublets for proton to an exocyclic methylene to δ 4.55 (2H, d, J=2.0 Hz, H-29) and 4.67 (2H, d, J=2.0 Hz, H-29). $^{13}CNMR$ (Cl_3CD, 125 MHz), showed 30 carbon signals; vinyl carbon signal occurs at 109.5 ppm, the signal corresponding to methylene - methylidene at 151.1 ppm and the carbon bound to the hydroxyl group appears at 79.1 ppm. By comparing the chemical shifts with those reported in the literature concluded that the compound is a pentacyclic triterpene lupeol (Jamal, 2008).

Compound 2

Compound 2 was isolated as a white amorphous solid. The mass spectral analysis has allowed establishing the molecular formula

$C_{32}H_{52}O_2$ from the molecular ion M m/z 468. The ^{1}HNMR spectrum (Cl_3CD, 300 MHz), shows the presence of eight tertiary methyl groups singlets at values of δ 0.77(3H, s, H-27), 0.79 (3H, s, H-26), 0.85 (3H, s, H-24), 0.87 (3H, s, H-23), 0.94 (3H, s, H-28), 1.05 (3H, s, H-25) and 1.65 (3H, s, H-30) and a methyl acetate to 2.07 (3h, s, H-2´) ppm. Singlets at the δ 4.62 (1H, s, H-29a) and 4.72 (1H, s, H-29b) indicate the presence of a exomethylene group. Moreover double doublet at δ 4.25 (1H, dd, J_{axax}=9.8, J_{axeq}=4.2, H-3) was caused by the proton bonded to carbon was connected to the functional group acetate. ^{13}CNMR (Cl_3CD, 125 MHz), shows a carbonyl group at δ 171.2 ppm and its connection to C-3 to δ 81.0 ppm. Are also located two olefinic carbons C-20 (151.2 ppm) and C-29 (109.5 ppm), connected to the single double bond exocyclic methylene group present in the structure. Spectrum signals have coincided with those reported for lupeol acetate (Bracho, 2009).

Compound 3

Compound 3 was isolated as a white amorphous solid. The mass spectrum suggested formula $C_{30}H_{50}O$ (M m/z 426). Its ^{1}HNMR spectrum (Cl_3CD, 300 MHz), exhibited too seven signals as singlet to δ 0.79 (3H, s, Me-28), 0.80 (3H, s, Me-25), 0.83 (3H, s, Me-24), 0.94 (3H, s, Me-23), 0.96 (3H, s, Me-27), 1.03 (3H, s, Me-26) and at lower fields other to δ 1.68 (3H, s, Me-30) assignable to a methyl group on double bond; a multiplet at δ 1.93 (2H, m, H-21); a double doublet to δ 2.4 (1H, dd, J=11.01 and 5.7 Hz, H-19) to be assigned to a methynic proton; a double doublet to δ 3.40 (1H, dd, J=10.9 y 5.3 Hz, H-3) assignable to a geminal proton of a secondary alcohol, this indicates that the hydroxyl group is in the alpha position, and that when the hydroxyl is in beta, this chemical shift occurs at 3.20 ppm. Two doublets for proton to an exocyclic methylene to δ 4.57 (2H, d, J=2.1 Hz, H-29) and 4.69 (2H, d, J=2.1 Hz, H-29). 13CNMR (Cl3CD, 125 MHz), showed 30 carbon signals; two signals are characteristic of a double bond in compounds having skeletons derived from lupane (109.6, 151.2ppm) which exhibit an isopropenyl group with specific chemical shifts those of 19.5, 151.2 and 109.6 ppm for the carbons C-30, C-20 and C-29, respectively. The spectrum showed a characteristic signal of carbon oxygen ported 76.4 ppm, assignable to hydroxyl group at position 3-α. By comparing the chemical shifts with those reported in the literature concluded that the compound is a pentacyclic triterpene epilupeol (Souza, 2001).

In Tables 1 and 2, the spectroscopic data for lupeol and epilupeol are presented for the identified compounds compared with those reported in the literature.

The most relevant antifungal activity was observed in the pure compounds 1 and 3 at both concentrations tested. The pure compounds showed selective inhibition properties and a certain concentration dependence on its antifungal activity. Compound 1 showed a rate of inhibition of 50 and 90% (100 and 200 µg/mL respectively) against *Penicillium notatum*, while compound 3 was capable to inhibit 80 and 100% of *Fusarium oxysporum* growth for each assayed concentrations. Compound 2 showed no antifungal activity against the microorganisms tested. Statistical differences ($P \leq 0.05$) with negative controls indicated that DMSO did not influence the results of biological evaluation (Figure 1).

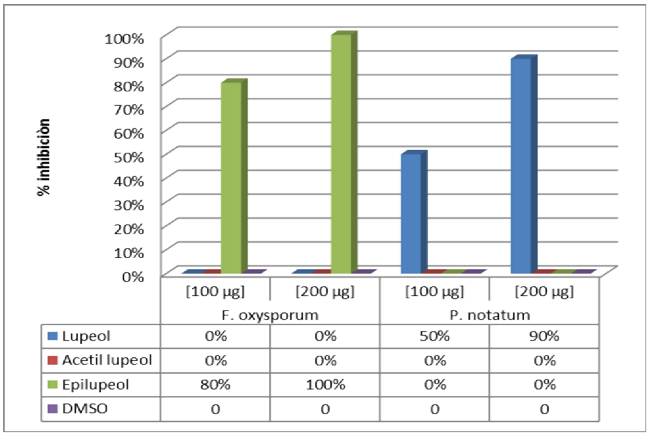

Figure 1. Percent Inhibition of isolated compounds *V. patents* against *F. oxysporum* and *P. notatum* at different concentrations.

Table 1. ^{1}HNMR shifts values of compounds 1 and 3 and reference compounds used as (δ_H).

H	1 $CDCl_3$	2 $CDCl_3$	Compound 1 $CDCl_3$	Compound 3 $CDCl_3$
3	3.20 m	3,40 m	-	-
19	2,28 m	2,38 m	-	-
21 a	-	-	-	-
21 b	-	-	-	-
23	-	-	0,94 s	0,96 s
24	-	-	0,83 s	0,78 s
25	-	-	0,78 s	0,85 s
26	1,03 s	1,03 s	1,03 s	1,02 s
27	-	-	0,96 s	0,93 s
28	0,79 s	0,79 s	0,76 s	0,82 s
29	4,57 s	4,57 s	4,65 m	4,67 m
	4.69 s	4,69 s	4,67 m	4,55 m
30	1,68 s	1,68 s	1,67 s	1,67 s

Legend:

1. Souza, 2001. 3β-hydroxy-lupeol
2. Souza , 2001 3α-hydroxy-lupeol

(-) unreported data

Tabla 2. ^{13}CNMR shifts values of compounds 1 and 3 and reference compounds used as (δ_C).

C	1 $CDCl_3$	2 $CDCl_3$	Compound 1 $CDCl_3$	Compound 2 $CDCl_3$
1	38,7	32,5	38,86	31,9
2	27,4	25,3	25,18	25,2
3	78,9	76,4	79,01	76,0
4	38,6	37,6	38,73	37,5
5	55,3	48,2	55,35	48,2
6	18,3	18,4	18,33	18,4
7	34,2	34,4	34,31	34,4
8	40,8	41,1	40,86	41,0
9	50,4	50,5	50,47	50,4
10	37,1	37,3	37,19	37,2
11	20,9	21,2	20,95	21,0
12	25,11	25,3	27,43	25,1
13	38,0	38,0	38,08	38,0
14	42,8	43,0	42,84	43,1
15	27,4	27,6	27,46	27,5
16	35,5	35,8	35,60	35,6
17	43,0	43,2	43,00	43,1
18	48,2	48,2	48,33	48,1
19	47,9	48,2	47,98	48,2
20	150,9	151,2	150.9	150,9
21	29,8	29,9	29,87	29,8
22	40,0	40,2	40,01	40,1
23	28,0	28,2	27,99	28,1
24	15,4	22,4	16,11	22,5
25	16,1	16,4	15,99	16,3
26	15,9	16,2	15,36	16,1
27	14,5	14,7	14,56	14,6
28	18,0	18,2	18,0	18,1
29	109,3	109,6	109,31	109,4
30	19,3	19,5	19,3	19,4

Legend:

1. Souza, 2001. 3β-hydroxy-lupeol
2. Souza , 2001 3α-hydroxy-lupeol

(-) unreported data

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ANIMAL SCIENCE

Morphometric studies on adult double humped camel of Ladakh, India

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Abstract

In India, double humped camel is living in Nobra valley of Ladakh region at an altitude of 18,300ft. The animal has been declared critically endangered by IUCN (1998), yet the animal has not been explored in detail. The study was conducted along the breeding tract of the Nobra valley. Thirty out 70 double humped camels having been shifted from their natural habitat, Nobra valley to Government farm chichoot, near the capital city, Leh were also included in the study. Biometric studies were done and the body measurements included measurement of Heart girth, Body Length, Lower jaw, Height up to wither, Tail length, Neck length, Distance between two eyes, Ear length, Face length, Hump 1, Hump 2, Distance between two humps, Length of fore leg, Length of hind leg, Fore foot pad, Hind foot pad and Lower jaw. The animal has relatively shorter legs compared to Mongolian Bactrian. The various biometric characteristics recorded are presented.

Key words: Double Humped Camel, Body measurements, Morphometry, Cold arid desert

Introduction

Dromedary and Bactrian camels are occupied different areas in the world, the dromedary living in hot desert (Gaili et al., 2000) and the Bactrian rather in cold desert (Konuspayeva and Faye, 2010). However, in some cases, they are cohabiting in the same country, even in the same farm for example in Kazakhstan (Faye et al., 2008). In India, the double humped camel is only distributed in Nobra valley, a cold arid region of Ladakh in Jammu and Kashmir State. The highest altitude breeding tract from sea level is 18600 ft (5670 m). Their genetic capability to survive at temperature ranging from -40 ºC to +40 ºC, their adaptability to scarce water and sparse feeding had made them the main mode of transport along the Silk Road. Therefore, ethnic importance of this species in the area is well appreciated. The area remains cut off from rest of the world for 6 months in winter during which feed is not adequate.

The double humped camel population in the Leh and Nobra Valley of Ladakh in the year 2005 was 150 heads which have now increased to 223 camels (Patil, 2011). However, there is an important and wide gap between the official number of camel heads and the camel population available with the nomads. With the launching of conservation drive and awareness after it was declared as critically endangered animal, there was an increase in number. The double-humped camel like the dromedary (Hjort af Ornas and Ali Hussein, 1993) is a multipurpose animal. It is a well-adapted animal to wide difference of temperature between summer and winter and to low oxygen pressure in high altitude.Due to its adaptability for cold arid high altitude area, it is used as mainly as an exclusive source of transportation of load.

A detailed report on phenotypic classification of Camelus *dromedarius* in Saudi Arabia has been recently undertaken (Abdallah and Faye, 2012) by their body measurements, but the morphometric characteristics in Ladakh double humped camel have not been studied so for and their differences on other related parameters to their general conformation were not clearly described. The present study was designed to characterize the Ladakh double humped camel (Figure 1).

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Figure 1. Side view of the Ladakh Bactrian camel.

Material and Methods

The Survey

The study was conducted in the Nobra area of the Ladakh. Four to six years animals belonging to the camel keepers inhabiting the studied area formed the material of the study. As the whole, 100 animals (88 males and 12 females) were submitted to measurement. The sample included 30 out of 70 double humped camels having been shifted from Nobra valley to Government farm chichoot, Leh in the capital city which is not actual habitat of the animal.

The measurements

The following distances were collected individually: Heart girth, Body Length, Lower jaw, Height up to wither, Tail length, Neck length, Distance between two eyes, Ear length, Face length, Hump 1, Hump 2, Distance between two humps, Length of fore leg, Length of hind leg, Fore foot pad, Hind foot pad and Lower jaw (Figure 2). Measurements of height, hump girth and thoracic girth were used to estimate the live weights by using the equation of Yagil (1994): W=50*HSH*THG*HG, where W=live weight in kg, HSH=the shoulder height using the measuring stick vertically from the ground to the top of scapula, THG=the thoracic girth using the meter ribbon around the body just behind the sternal pad, and HG=the hump girth using the measuring tape along the abdomen over the midpoint of the hump.

Figure 2. (a) Measuring Hump Length; (b) Measuring Heart girth; (c) Measuring Hump Length; (d) Measuring Length of foreleg.

The statistical analysis

For the data analysis, Microsoft Excel version 2007 computer package was employed. Differences between two means of quantified data were compared by Student's t-test. Comparisons between two qualitative data were performed using the chi-square test.

Results and Discussion

The measurements were achieved in both male and females animals and reported separately (Table 1). As usual, the male measurements were on average higher than for female (on average 6% more for the whole measurements). The most important difference occurred for neck circumference (40%). However, the measurements appeared higher in female than in male for some parameters as the heart girth and the distance between two eyes, the length of the hind leg and the hind food pad width (Table 1). In their study regarding dromedary camels in Saudi Arabia, Rahim and El-Nazier (1992) has found also sexual differences with on average, male measurements higher for all the parameters. The male head was 11% longer, the neck 12% longer, the neck circumference 13% higher, the thigh circumference 14%. The difference in height (4%) and girth circumference (6%) was less marked.

Table 1. Mean ±S.E of the different body measurements of adult Double Humped Camel of Ladakh (in cm) in male and female

Parameters	Male (n=88)	Female (n=12)
Trunk		
Body Length	152.4±3.1	129.5±2.0
Thorax girth	237.5±4.1	200.6±3.1
Heart girth	203.2±4.9	210.8±2.9
Appendages		
Tail length	50.8±3.1	48.3±0.6
Ear length	15.2±2.0	12.7±0.0
Distance between ears	15.2±2.0	12.7±0.1
Head and Neck		
Face length	44.4±3.03	40.6±0.82
Lower jaw	43.2±2.03	40.6±0.82
Neck length	94.0±4.02	91.4±4.07
Neck circumference	114.3±4.0	81.3±1.04
Distance between two eyes	21. ±2.2	22.9±0.88
Supra orbital foramina	8.9±1.7	7.6±0.32
Hump		
Hump circumference	78.7±3.1	116.2±2.7
Hump 1(Height)	45.7±2.7	38.1± 0.9
Hump 2(Height)	30.5±1.9	26.7±1.1
Distance between two humps	121.9±3.1	182.3±2.0
Total area under hump	121.9±3.1	99.1±2.0
Limbs		
Height upto wither	170.2±2.1	152.4±3.1
Shoulder height	248.6±4.0	212.5±3.8
Length of fore leg	147.3±4.1	152.4±3.05
Length of hind leg	152.4±3.1	175.3±2.3
Knee circumference	50.8±1.6	45.7±1.3
Fore foot pad	60.9±1.1	60.2±1.1
Hind foot pad	50.8±3.1	58.4±1.8

Table 2. Mean ± SE of linear measurements (in m) of camels in both males and females.

Parameter	Male		Female		Significance
	Mean ± SEM	Range	Mean ± SEM	Range	
Shoulder height	2.4±0.1	2.3-2.5	2.2±0.05	2.2-2.3	P <0.01
Hump girth*	2.5±0.1	2.3-2.6	2.4±0.1	2.4-2.5	P <0.01
Thoracic girth	2.3±0.2	2.1-2.4	2.2±0.02	2.2-2.3	P <0.01
Weight	672.5	550.1-811.8	587.0	568.5-657.3	

* (hump first +hump second)/2

Elsewhere in the world, the characterization of the camel has been done on dairy and meat performances (Faye and Bonnet, 2012) or racing performances (Shorepy, 2011). A first phenotypic characterization was achieved for most of the dromedary breeds of the world, based on general morphology but the double humped camel of Ladakh was never described, contrary to dromedary camel in Saudi Arabia (Hussain and Faye, 2012) or in Sudan (Ishag et al., 2011).

Regarding the sexual differences observed for the hump, the measurements in female appeared also higher than in male in our study. On average, the hump circumference was 32% higher in female and the distance between hump also (33%). At reverse, the height of the humps in male were higher, both for hump 1 (20%) and hump 2 (14%). The biometrics of humps revealed that the height of first hump was found greater than second hump both in male and female. Elsewhere, the hump stand erects during summer and was deviated to a side in winter. The shape of the humps of Mongolian Bactrian were small and Pyramid Shaped (Endo et al., 2000) while those of Ladakh Bactrian were distinctly large and irregular. However, the measurement of the hump is a quite debatable parameter to distinguish the phenotypes because the hump is the main fat storage form in camel representing on average 85% of the adipose tissue (Faye et al., 2001a) and its volume is linked to the body condition score which is not under genetic dependence, but linked to the feeding status of the animals (Kamili et al., 2006). In Bactrian camel, the first hump is on the withers and the second one is on the loin region.

Regarding the height up to wither, Endo et al. (2000) reported that the Mongolian Bactrian was higher at the level of hump (7 feet i.e 213.26 cm) than Ladakh Bactrian.

The face of Ladakh Bactrian was triangular and the split in the lip was smaller than that in Mongolian. Two broad toes on each foot have undivided soles and are able to spread widely as an adaptation to walking on hilly terrain and sand (Makhdoomi, 2006).

Conclusion

In camel belts of the world, the variation in morphometry is depending upon different breeds, farming systems or feeding systems. The current morphometric study description could be a first step for establishing standard of the double humped camel of Ladakh, a critically endangered animal. However, in addition of that, control of performances would be implemented for a better characterization of this Bactrian camel.

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ANIMAL SCIENCE

Effects of a phytogenic feed additive on growth performance, selected blood criteria and jejunal morphology in broiler chickens

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Abstract

The study was conducted to examine the effects of a phytogenic feed additive (PFA; BIOSTRONG® 510) on growth performance, selected blood parameters and jejunal morphology in broiler chickens. The PFA consists of a mixture of essential oils with thymol and anethole as leading active substances, as well as different herbs and spices. A total of 264 1-d-old Cobb male broilers were randomly allocated to 2 dietary treatments, with 6 replicates per treatment and 22 birds each replicate. The experiment lasted 42 days. The dietary treatments were a starter and grower diet without feed additives (control), or the diets supplemented with 150 mg/kg of the PFA. Body weight and feed intake were not significantly influenced by PFA feeding compared with the control during all experimental periods. During the grower phase (22 - 42 d of age) and during the whole period (1 - 42 d of age) the PFA significantly improved ($P<0.05$) feed conversion ratio. Serum total protein, albumin, total cholesterol and leucocytes were increased by PFA feeding. Furthermore, villus height to crypt depth ratio in the jejunum was increased by PFA feeding. In conclusion, the results of this study show that inclusion of PFA lead to morphological changes in the jejunum, which might influence nutrient absorption and thus, improved FCR.

Key words: Broiler chicken, Performance, Blood parameters, Jejunal morphology

Introduction

The ban of antibiotic growth promoters (AGP) in poultry feeds intensified the search for alternatives improving the health and productivity of broiler chickens (Barreto et al., 2008). Such alternatives are probiotics, prebiotics and organic acids, which are added to the feed (Huyghebaert et al., 2011). Also phytogenic feed additives (PFA) were shown to enhance performance in AGP-free livestock production (Alçiçek et al., 2003; Steiner, 2009). Phytogenic feed additives consist of a broad variety of substances, mainly extracts from plant materials, such as flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots (Burt, 2004). The active molecules include many different secondary plant metabolites, resulting in a broad range of physiological effects, like secretolytic and spasmolytic, or immune-stimulative effects (Lee et al., 2004a).

PFA are generally recognized as natural feed additives and safe to the animal. However, results of studies concerning the use of PFA in broiler nutrition are inconsistent (Windisch et al., 2008). Some authors stated significant improvement of broiler performance (Ertas et al., 2005; Cross et al., 2007), whereas others reported no effects on BW gain and feed intake (Lee et al., 2003; Jamroz et al., 2005; Nasir and Grashorn, 2010) or feed conversion ratio (Ocak et al., 2008). These discrepancies may be due to numerous factors such as type and parts of plants used, their physical properties, time of harvest, the preparation method of PFA and their compatibility with other feed components (Jang et al., 2007). Furthermore, the mode of action of these additives is not fully clarified yet and in vivo studies are limited. Plant extracts have been shown to influence digestion and secretion of digestive enzymes (Platel and Srinivasan, 2000; Williams and Losa, 2001) to increase absorption of micronutrients (Usha et al., 2010) and to exhibit antibacterial, antiviral and

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antioxidant activities (Brenes and Roura, 2010). However, only few studies investigated the effects of PFA on intestinal morphology in broiler chickens. Therefore, the aim of the present study was to examine the effect of a commercial PFA on performance, intestinal morphology and selected blood parameters in broiler chickens.

Materials and Methods

Two hundred sixty four 1-d-old male Cobb chickens were weighed and randomly allotted to 2 experimental treatments. Each treatment consisted of 6 replicates with 22 birds per replicate. The dietary treatments were a starter and grower basal diet without any feed additives used as a control or the basal diets supplemented with 150 mg/kg of a commercial PFA (BIOSTRONG® 510, Delacon, Steyregg, Austria) used as experimental diets. The PFA consisted of a mixture of essential oils, with thymol and anethole as leading active substances, as well as different herbs and spices. The compositions of the basal diets are shown in Table 1. Control and experimental diets were formulated to be iso-nutritive and to meet the requirements for broiler chickens during starter and grower phase. Feed and water were available *ad libitum*. Feed consumption and BW of the birds were recorded weekly and were used to calculate broiler performance (weight gain, feed intake, feed conversion ratio).

Blood samples were taken at 35 d of age from the brachial vein from 12 birds per treatment group (2 birds per pen). Blood samples were separated by centrifuge, and serum was analyzed for glucose, total cholesterol, triglycerides, albumin and total protein using commercial test kits. Blood cell counts were determined by commercial cell counter.

At 42 d of age, 9 birds per treatment were slaughtered and the jejunum was removed for assessment of tissue morphology. The tissue samples were taken from the middle part of the jejunum (from the entrance of bile duct to Meckel's diverticulum, pieces about 5 cm in length), carefully cleansed and then fixed in 4% buffered formalin for 2 days. The further processing consisted of serial dehydration in PBS and graded ethanol solutions, clearing with xylene and embedding in paraffin. Sections of 5 µm were prepared and placed on glass slides. Tissue samples were deparaffinised, rehydrated and stained with haematoxylin and eosin. Villus heights and crypt depths were examined with a photomicroscope (Photomikroskop III, Carl Zeiss, Oberkochern, Germany) fitted with a digital camera (MikroCam 3 MP, Bresser, Rhede, Germany) and images were analysed using image analysis software (Image software Bresser MikroCamLab Mikroskopie). A total of 16 intact well-oriented villus-crypt units were randomly selected at each tissue sample. Villus height was measured from the tip of the villus to the villus-crypt junction, and crypt depth was defined as the depth of the invagination between two villi.

Experimental data were analyzed by ANOVA using SPSS v. 17.0 (Statistical Packages for the Social Sciences, released August 23, 2008). Data was tested for homogeneity of variances, and comparison of means was performed by Tukey test. The significance level was set at $p < 0.05$.

Results

During the whole experiment, birds were healthy and the mortality was below 1%. Performance data for broiler chickens during the starter and grower phase and for the total experimental period are summarized in Table 2.

Table 1. Ingredients and nutrient composition of experimental diets.

Ingredients (%)	Starter 1 - 21d	Finisher 22 - 42 d
Soybean meal (48% CP)	34.50	31.80
Maize	28.46	29.47
Wheat	24.69	24.29
Soy oil	6.80	9.35
Limestone	1.80	1.51
Monocalcium-phosphate	1.40	1.35
Premix*	1.20	1.20
Chromium oxide	0.50	0.50
DL-Methionine	0.30	0.29
L-Lysine	0.25	0.14
Additives**	0.10	0.15
	100%	100%
Chemical composition (calculated)		
AMEn, (MJ/kg)	12.59	13.29
Crude protein(%)	22.89	21.50
Crude fibre (%)	2.39	2.32
Lysine (%)	1.43	1.26
Methionine (%)	0.64	0.61
Methionine + Cystein (%)	1.02	0.98
Calcium (%)	1.03	0.90
Total Phosphorus (%)	0.70	0.68

*Supplied per kg of diet: 4000 IU vitamin A (retinyl acetate); 400 IU cholecalciferol; 80 mg (α-tocopherole acetate); 3 mg vitamin K3 (menadione); 2.5 mg thiamin ; 2.5 mg riboflavin; 25 mg nicotinic acid; 4 mg pyridoxine; 0.02 mg cobalamin; 0.3 mg biotin; 10 mg calcium pantothenate acid; 1 mg folic acid; 800 mg choline chloride; 50 mg Zn (Zinc oxide); 20 mg Fe (Iron carbonate); 60 mg Mn (manganese oxide); 12 mg Cu (copper sulfate-pentahydrate); 0.45 mg J (calcium iodate; 0.30 mg Co (cobalt- (II)-sulfate-heptahydrate); 0.35 mg Se (sodium selenite); 1.3 g Na (sodium chloride); 0.55 g Mg (magnesium oxide) ; ** Additives: Control = without active substances (PFA); Treatment = Biostrong® 150 mg/kg feed.

For all experimental periods, there was no effect (P>0.05) of the PFA on BWG and feed intake. Feed conversion ratio was significantly improved (P<0.05) in the grower phase (22 - 42 d of age) as well as during the whole experimental period (1- 42 d of age) due to PFA supplementation.

Villus heights, crypt depths and villus height: crypt depth ratios of jejunal tissue samples are presented in Table 4. In broilers fed diets supplemented with PFA, there were no differences between the villus height and crypt depth could be observed compared to the control (Figure 1). Villus height: crypt depth ratio was significantly (P<0.05) increased in birds fed PFA compared to the control.

Effect of the phytogenic feed additive on some blood parameters in broilers were summarized in Table 3. Serum total protein, albumin, cholesterol and leucocytes were increased (P<0.05), while serum glucose, triglycerides, hemoglobin and erythrocytes were not affected by PFA feeding.

Table 2. Effect of the phytogenic feed additive on broiler performance during the starter and grower period (means ± SD).

Period	Treatments		*P-value*
	Control	PFA (150 mg/kg)	
Average body weight gain (g)			
1 - 21 d	797 ±67	813 ±28	0.62
22 - 42 d	2108 ±102	2144 ±150	0.64
1 - 42 d	2906 ±134	2956 ±173	0.58
Average feed intake (g)			
1 - 21 d	1124 ±108	1106 ±99	0.76
22 - 42 d	3332 ±130	3259 ±211	0.49
1 - 42 d	4456 ±202	4365 ±287	0.54
Feed conversion ratio (g/g)			
1 - 21 d	1.41 ±0.07	1.36 ±0.08	0.295
22 - 42 d	1.58^{a} ±0.04	1.52^{b} ±0.03	0.013
1 - 42 d	1.53^{a} ±0.04	1.48^{b} ±0.02	0.011

ab Values with different superscript within lines differ significantly (*P*<0.05)

Table 3. Effect of the phytogenic feed additive on some blood parameters in broilers (35^{th} d of age; means ± SD).

Parameters	Treatments		*P-value*
	Control	PFA (150 mg/kg)	
Serum total protein (g/l)	27.03^{a} ±3.65	30.64^{b} ±1.38	<0.004
Serum albumin (g/l)	11.67^{a} ±2.15	14.22^{b} ±0.87	0.001
Serum glucose (mmol/l)	14.22 ±1.49	14.99 ±0.60	0.11
Serum Cholesterol (mmol/l)	3.00^{a} ±0.65	3.84^{b} ±0.22	<0.001
Triglycerides (mmol/l)	1.33 ±1.23	1.86 ±0.71	0.21
Erythrocytes (Tpt/l)	2.27 ±0.10	2.34 ±0.27	0.31
Leucocytes (Tpt/l)	175.8^{a} ±2.4	183.5^{b} ±9.5	0.012
Haemoglobin (mmol/l)	4.94 ±0.25	5.22 ±0.48	0.09

ab Values with different superscript within lines differ significantly (*P*<0.05)

Table 4. Effect of the phytogenic feed additive on histomorphological parameters of the jejunum in broilers (means ± SD).

Parameters	Treatments		*P- value*
	Control	PFA (150 mg/kg)	
Villus height (µm)	1447±125.4	1569±120.9	0.068
Crypt depth (µm)	199±21.0	179±18.6	0.061
Villus height:crypt depth	7.3^{a}±0.8	8.8^{b}±0.9	0.004

ab Values with different superscript within lines differ significantly (*P*<0.05)

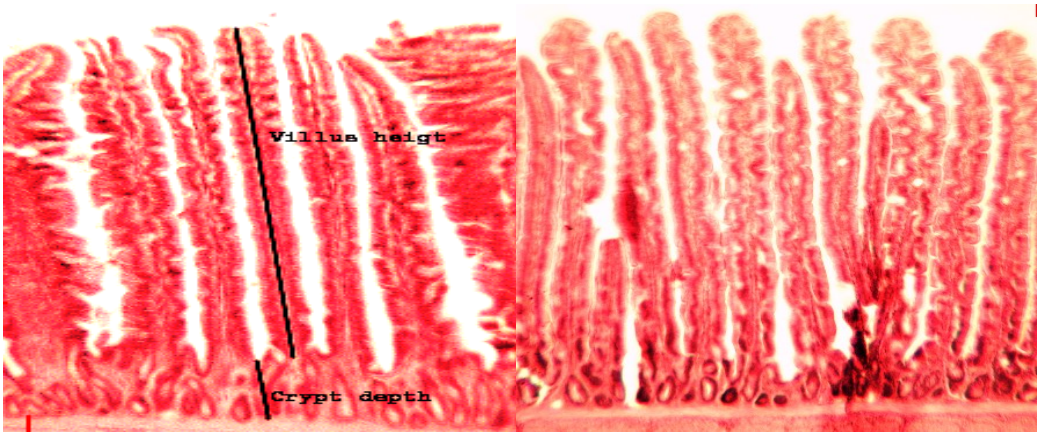

Figure 1. Villus height and crypt depth in the jejunum of broilers fed diets with or without the phytogenic feed additive at 42 days of age, left control, right phytogenic feed additive.

Discussion

In the present study, there was no effect of the tested PFA on BW and feed intake in broilers. These results are consistent with the studies of Jang et al. (2007) and Erdogan et al. (2010), who did not find effects of different phytogenic compounds on broiler growth performance. However, FCR was significantly improved in the present study. Consistently, Jamroz et al. (2005) reported improved FCR due to the addition of a plant extract, containing cinnamaldehyde, carvacrol and capsaicin, to a maize or wheat and barley based diet by 4.1 or 2.0%, respectively, whereas BW was not affected by treatment. Al-Kassie (2009) showed that the addition of 200 ppm oil extract derived from thyme and cinnamon to broiler diets significantly improved BW gain and FCR during a growing period of 6 weeks and Ocak et al. (2008) reported higher ($P< 0.05$) BW at 21 and 42 d of age as well as higher ($P< 0.05$) BW gain from 7 to 35 days of age in broilers fed peppermint and thyme compared to the controls. The above mentioned studies, reporting improved FCR, have in common, that the added substances mainly consist of essential oils. Essential oils exert antimicrobial activity in the digestive tract of animals (Lee et al., 2004; Al-Kassie, 2010). It is hypothesized that gut microflora reduces nutrients available to the animal by enforcing the intestinal cell turnover and thereby increasing the intestinal requirement for nutrients to maintain tissue integrity. Moreover, intestinal microflora and epithelial cells have to compete for nutrients (Dibner and Richards, 2005; Lan et al., 2005). By a reduction of intestinal microflora, essential oils may lead to moderate cell turnover, to decreased intestinal nutrient requirements and to less competition for available nutrients. As a consequence, FCR is improved because more nutrients are used for BW gain instead of tissue maintenance or microbial growth.

The increased nutrient supply for growth is reflected in enhanced nutrient transport in the blood. For example, Ghazalah and Ali (2008) observed higher levels of total protein, albumin and globulin in the blood serum of birds when fed 0.5% rosemary leaves. Similar results were obtained in the present study, where higher contents of total protein and albumin in the blood serum of PFA fed animals may also indicate enhanced nutrient supply and transport. Furthermore, Tekeli et al. (2006) reported increased blood glucose concentration by Zingiber officinale supplementation and increased triglyceride concentration by both Zingiber officinale and Syzygium Aromaticum supplementation. In the present study, there was no effect of PFA on blood glucose and triglyceride concentrations. Calislar et al. (2009) found effects neither on triglycerides nor on blood cholesterol levels by application of a PFA containing Origanum vulgare ssp. hirtum extract. In general, essential oils are more often associated with hypocholesterolemic properties (Lee et al., 2004). In contrast, in the present study chicken fed the PFA showed higher serum cholesterol concentrations compared to the control animals. This discrepancy may be due to the combination of essential oils and pungent substances which were also included in the used PFA. Pungent substances increase digestive secretions including enzymes and bile. The higher serum cholesterol content, which was observed in the PFA fed animals of the present study, may be the result of an increased lipid digestibility due to a higher secretion of bile and digestive enzymes. The improvement of FCR as

well as the increase of blood nutrient concentrations supports this assumption, although it remains speculative as nutrient digestibilities have not been investigated in the present study.

Regarding the intestinal morphology, heavier chickens are generally associated with longer villi, greater villus width and higher villus surface area as compared to lighter ones (Adibmoradi et al., 2006; Incharoen et al., 2010). In the present study there were insignificantly increased villus heights and decreased crypt depths in the jejunum of birds receiving 150 mg/kg PFA compared to the control. Accordingly, the villus height: crypt depth ratio was significantly higher in the birds fed the PFA compared to the control. These findings were consistent to Adibmoradi et al. (2006) who reported that jejunal villus height was increased whereas crypt depths were decreased, leading to increased villus height: crypt depth ratio in birds fed graded levels of garlic meal. It has been suggested that longer villi would result in an increased surface area and higher absorption of available nutrients (Caspary, 1992; Yasar and Forbes, 1999). A higher absorptive capacity of the intestine of PFA fed animals is also supported by higher blood nutrient concentrations of those animals, as observed in the present study. However, although Jamroz et al. (2006) reported similar improvement of FCR due to the supplementation with a plant extract containing carvacrol, cinnamaldehyde and capsicum oleoresin, the authors could not find an effect of the plant extract on intestinal morphology in broilers at 42 d of age. Thus, for PFA containing essential oils as well as pungent substances further, eventually synergistic, effects or other modes of action cannot be ruled out.

Conclusion

In the present study, the addition of the phytogenic feed additive BIOSTRONG® 510 to broiler diets significantly improved feed conversion ratio. The observed improvement of feed conversion ratio may be caused by a combination of different effects, including enhancement of digestive secretions, antimicrobial effects of essential oils as well as enlargement of intestinal absorptive surface. However, further studies are necessary to evaluate the effects of various substances present in phytogenic feed additives and to clarify their specific modes of action.

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AGRICULTURAL ECONOMICS

Farming and risk attitude

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Abstract

Data from a survey among Norwegian farmers (n=514), combined with tax register data and data on farming area and production, are used to explore various questions related to future farming, general attitudes to farming and risk attitude. To complement the raw data, several new variables were constructed in order to compare how variables such as investment, consumption and intensity of farm production in the farmers' opinion would be affected by sudden, unexpected monetary losses and gains. These new variables say something about how various types of behavior are asymmetrically exhibited when facing unexpected gains and losses. In general, and as expected, farmers claim that a sudden gain would affect their behavior less than a sudden loss. The main exception is farm investment, which would be more affected by a sudden gain than a sudden loss would affect divestment. This is interesting, as it captures some important aspects of farming lifestyle: A sudden gain is likely to be invested in the farm, but a sudden loss would, if possible, be financed without farm divestment, as this often would lead to giving up farming, or at least some important aspects of the farming lifestyle. Overall, these results are not surprising. Nevertheless, it is argued that the results could have some interesting policy implications, both with regards to design of hedging schemes, general agricultural support schemes, and rural policy.

Key words: Farming lifestyle, Investment, Risk attitude

Introduction

The purpose of this paper is to contribute to the literature on risk attitude among farmers with insights from data on Norwegian farmers and their plans, behavior, and risk attitude. Risk attitude among farmers (for instance related to investment and operation plans) is interesting for several reasons. First, farmers are dealing with real investments, with potentially important and long-term consequences. Second, for many farmers, important decisions also have an emotional impact because the decisions will affect both future lifestyle and personal identity as a farmer – for the current and possibly future generations. Examples of such decisions include decisions to quit farming, leave the farm, or change to a different farming system. On the other hand, large investments may be under consideration which would be likely to "lock in" the farmer and his family for a number of years.

A fundamental starting point is the relation between farm/farmer characteristics, perceptions and decisions/behavior. Several similar studies (Flaten et al., 2005; Borges and Machado, 2012) use van Raaij's (1981) model as a building block. Figure 1 below illustrates the core of this model:

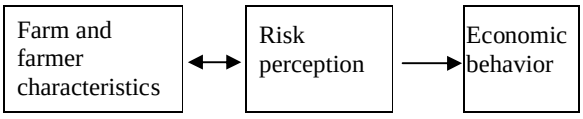

Figure 1. Van Raaij's model of a firm's decision making environment.

This model implies that different farmers will have – and state – different risk perceptions, which in turn will lead them to different decisions and different economic behavior. Hence, understanding risk perceptions in relation to differences in farm characteristics is useful with regards to understanding decisions and economic behavior.

Whereas expected utility theory traditionally has been the most commonly used model for

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decision-making under risk, a number of studies point out that this approach often fails to explain observed behavior (some classic references include Kahneman and Tversky, 1979 and Rabin and Thaler, 2001). Hence, it is even more important to understand the decision maker's frame of reference and perceptions of risk, as these often affect decisions and behavior more than "objective" considerations about expected utility (March and Shapira, 1987).

A large body of literature exists on risk and risk management in agriculture. Useful starting points are Moschini and Hennessy (2001) and Hardaker et al. (2004). In particular, Flaten et al. (2005) conducted a survey among Norwegian farmers about their risk perceptions. This literature provides a fairly good overview of the relevant risk sources and risk attitudes, both among farmers in general and Norwegian farmers in particular. Our objective in this study is more modest – to assess whether Norwegian farmers are equally risk averse in all areas, and what consequences this might have. Flaten et al. (2005) touch upon this by asking farmers to rate their willingness to take risks *compared to other farmers* in three areas: production, marketing, and finance and investment. The differences between the three areas seem small, with a slightly larger comparative risk aversion in the marketing area. However, this does not necessarily translate to a higher risk aversion in that area. On the other hand, similar comparative risk aversions in these three areas does not exclude the possibility that farmers as a group have very different risk attitudes in different situations, as long as each farmer perceives his own attitude compared to his peers to be similar in all three areas.

In this study, we look at (perceived) risk attitudes in several areas, and instead of comparative risk attitudes, we are concerned with how farmers would spend a large and sudden gain versus how they would finance a large and sudden loss. Our dataset only allows a proxy measure of risk aversion, but this measure fits well with earlier measurements of risk aversion, and allows us to look at differences in risk attitude in various areas, and potential consequences for policy and risk management schemes.

Materials and methods

In this paper, we utilize data from a 2008 survey among Norwegian farmers (n=514), combined with tax register data and data on farming area and production, to explore various questions related to future farming, general attitudes to farming, risk aversion, and how different factors affect the utility curve of farmers.

For the development of the survey used in this study, we in part used a structure that had been used for another survey (Flaten et al., 2005; Lien et al., 2008). We also looked at questionnaires from other countries as a source of reference (e.g., Pennings and Garcia, 2001). Before the survey was conducted among the farmers, a draft questionnaire was tested, and the final questionnaire was the result of several rounds of testing.

Most questions were closed, i.e., each respondent was asked to tick one/several of a number of pre-defined alternatives. Attitudes towards listed statements were mostly measured by 7-points Likert scales, where the respondent was asked to rate his or her degree of agreement/disagreement on a scale from 1 to 7. The final question was open, and respondents were asked to give comments in their own words. The response quality was very good, indicating that the questions were understandable and not too numerous.

The questionnaire was sent by mail to a stratified sample (with regard to age, region, and size of farms) from the Norwegian Agricultural Authority's register of farmers receiving production support. Virtually all farmers in Norway are on this register. In total, 1001 questionnaires were mailed out. Those who had not responded were sent a reminder postcard approximately four weeks later. In total, 551 responses were received. This constitutes a response rate of 56%, which is satisfactory for a mail response survey. As mentioned above, the general quality of responses was very good. Nevertheless, 37 forms were incomplete and had to be rejected, thus leaving us with a total sample size of 514.

We were able to merge the survey data on attitudes etc. with financial data obtained from the Norwegian Tax Authority. These records include both farm and off-farm income for both the farmer and partner, typically specified with regard to income source (income from farming, income from other farm-related activities, off-farm salary, and capital income/capital gain). The financial data also contained information on taxable wealth, debt etc; thus giving a reasonably good overview of the farm household's financial situation.

Finally, we also merged the two datasets from the survey and the Tax Authority with a third dataset; the Norwegian Agricultural Authority's register of farming area and production. In short, this register contains information about farmland used for different purposes, and livestock numbers.

To study risk attitudes in different areas more carefully, some new variables were constructed in order to compare how variables such as investment, consumption and intensity of farm production in the farmers' opinion would be affected by sudden, unexpected monetary losses and gains.

To construct the new variables, we used responses to two different questions. In one question, farmers were asked to consider a situation where they won MNOK 1 (approx. $160 000), for instance through a lottery. They were presented various opportunities to spend/invest this amount, and were asked to rate each opportunity on a scale from 1 (nothing) to 7 (everything) with regard to how much of the prize they would allocate to each opportunity. The opportunities were farm investments, investment in farm-related activities, off-farm investments, running the farm less intensively (thus probably reducing the income), work less off the farm, increase private consumption, gifts/inheritance to children, gifts to charity, pay off debt and/or bank savings, and buy shares/equity. In a related question, they were asked how they would finance a sudden loss of MNOK 1. Again, they were asked to consider various opportunities on a scale from 1 (would definitely not use) to 7 (would definitely use). The opportunities here were sale of the farm (or parts of it), increased forest harvesting, running the farm more intensively, work more off the farm, sell shares/equity, reduce private consumption, and reduce bank savings/increase loans.

A reviewer introduces a highly relevant point – that risk perceptions could depend on the source of the sudden gain or loss. A sudden drop in market prices leading to a loss of 1MNOK does not necessarily trigger the same response as a sudden cut in subsidies leading to the same loss. However, such differences are beyond the scope of this paper to explore. In this study, the farmers were asked to consider the gain as a result of winning the lottery, and the loss as a result of losing some unspecified, unexpected legal case. Hence, both the gain and loss were framed as unexpected, unrelated to the day-to-day business of farming, and without any direct implications for future farming.

Responses to the two questions were paired, and reordered in an increasing/ordinal order from zero to six. In other words, spend/invest alternatives in case of gain were scaled from 0 (nothing) to 6 (everything), while spend/save alternatives in case of loss got a scale between 0 (would definitely not use) and 6 (would definitely use). The constructed measures of risk attitudes are illustrated in Table 1 below, and simply measure the difference between the responses to the two questions. If the value of the new variable “invest” equals 0, this means that the average effect on investment from a sudden gain is reported to be as strong as the effect on divestment (sale of farm) from a sudden loss. If a farmer claims that a gain he would use “everything” from a gain to invest in the farm (score 6), and would “definitely not” finance a loss by sale of the farm, this would give him a score of 6–0 = 6 for the new variable “invest”. In short, a positive value indicates that the effect on divestment from a loss is stronger, whereas a negative value indicates that the effect on investment from a gain is stronger.

Table 1. Constructed measures of risk attitude.

New variable	Variable used, gain	Variable used, loss	Interpretation, high score (>0)
Invest	Investment in farm	Sale of farm/parts of farm	A gain means less for farm investment than a loss means for giving up farming
Intense	Extensify farm production	Intensify farm production	A gain means more for “extensification” than a loss means for “intensification”
Off_farm	Less off-farm work	More off-farm work	A gain means more extra off-farm work than a loss means reduced off-farm work
Consume	More private consumption	Less private consumption	A gain means more extra private consumption than a loss means reduced private consumption
Loan	Increase savings/reduce loans	Increase loans/use savings	A gain means more extra bank savings/reduced loans than a loss means increased loans/reduced savings
Equity	Buy shares/equity	Sell shares/equity	A gain means more extra investments in shares/equity than a loss means sales of shares/equity
Overall	Average of all new variables		A gain means more change in behavior than a loss

At this stage, it is important to emphasize how these new variables should – and shouldn't – be interpreted.

First, we know from standard expected utility (EU) theory that risk aversion implies that a monetary gain increases utility less than a loss of the same size reduces the utility. Our new measures are related to this concept, but it is not quite the standard risk aversion we are measuring. First of all, we are not concerned with the (perceived) effect on utility, but rather with the effect on behavior. Although a conceptual deviation from the standard EU framework, this is beneficial, as it is behavior we are interested in. It is, however, worth pointing out that it is known from the behavioral economics literature that a loss – or something framed as a loss – has larger consequences for behavior than a gain (see e.g. Bertrand et al. *(*2006) and references therein). Hence, we would expect the same tendency in our material, that (potential) losses have larger behavioral consequences than gains.

One additional weakness should be pointed out. As the questionnaire was designed for several purposes, the wording in the two questions (about gains and losses) is slightly different. Farmers were asked how large a share of a gain they would spend/invest in various areas; while they were asked how likely they were to use various sources to finance a loss. One could argue that whereas the sum of scores in the gain question should add up to 100% (or, 7 on our scale), no such relation exists in the loss question – there is nothing wrong with definitely using all the listed sources to cover the loss, and hence getting a 7 as response to all the sources.

This problem is important to be aware of, yet we will argue that it does not destroy the study. First of all, we are uncertain to what extent the respondents in fact has grasped the difference in wording. We assume that many respondents have treated the two questions in the same way, and simply ignored the difference in wording. Finally, it is worth noting that any such difference – by definition – should be identical for all the new variables. This means that no matter how much faith one has in the overall result, the relative size of each new variable (compared to the other new variables) should still be reliable.

Results and Discussion

Some general, descriptive statistical results from the survey are presented in Bergfjord et al. (2011). Some of the main results from the raw data are as follows: We observe that the farmers exhibit a clear, but not extreme risk aversion. The differences between different subsets of the sample are generally small, with the exception that full-time farmers with high household incomes are less risk averse than others. This is reasonable. These farmers probably are better equipped to take on some risks in order to achieve future gains. Also, some of the difference is probably caused by underlying differences in marital status. High-income households usually have two incomes, often including one off-farm income, whereas the group with low income to a larger extent consists of one-income households. This means that the high-income group has a more diversified income base, and thus better opportunities to pursue more risky business strategies.

The focus of this paper is the constructed measures of risk attitude, the information they provide about behavior, and their possible implications. The size, standard errors and 99% confidence intervals of these new variables are presented in the table below. All variables are significantly different from 0, and all risk attitude measures are estimated to have absolute values above 0.5.

Table 2. Descriptive statistic for the risk attitude measures. N = 514.

Variable (n=514)	Size	Std error	99% confidence interval	
Invest	1.34	0.11	1.05	1.64
Intense	-0.74	0.10	-0.99	-0.49
Off_farm	-1.22	0.12	-1.52	-0.92
Consume	-1.29	0.11	-1.58	-1.02
Loan	-0.58	0.11	-0.87	-0.29
Equity	-0.65	0.09	-0.89	-0.42
Overall	-0.88	0.06	-1.03	-0.73

All the new variables are negative, except from the first one. This can be interpreted as follows:

1. A gain means more for farm investment than a loss means for farm divestment
2. A gain means less for “extensification” than a loss means for “intensification”
3. A gain means less extra off-farm work than a loss means reduced off-farm work
4. A gain means less extra private consumption than a loss means reduced private consumption
5. A gain means less extra bank savings/reduced loans than a loss means increased loans/reduced savings
6. A gain means less extra investments in shares/equity than a loss means sales of shares/equity
7. A gain generally means less change in behavior than a loss

In general, and as expected, farmers claim that a sudden gain would affect their behavior less than a sudden loss. Although the measure used is not strictly comparable to risk aversion, this is in line with both the risk aversion derived from the raw data in Bergfjord et al. *(*2011) as well as results from behavioral economics that losses affect behavior more than gains.

We have not found important differences between different subgroups of farmers, for instance based on on- and off-farm income or production types. This is interesting, and indicates that the attitudes found are common for most types of farmers. However, there is a strong correlation between the various constructed variables for each farmer. This means that if a farmer's equity holding is more affected by a loss than a gain, it is likely that also for instance his consumption behavior will be more affected by a loss than a gain. This is also to be expected. Overall, our results are not surprising, in the sense that both moderate risk aversion, larger effect of losses than gains, and generally small differences between different types of farmers in this sample could expected based on theory and earlier work.

Although expected, some further comments regarding the different variables could be useful. For the second variable, about intensification, part of the reason might be the structure of Norwegian agriculture. Many farms are already run rather extensively, and many farmers have other, more important sources of income, almost reducing farming to a hobby. Thus, further “extensification” is difficult and/or pointless, whereas intensification is an option if financial reasons make it necessary.

For our third variable, about off-farm work, there are several potential explanations, in addition to the standard risk aversion component. A related reason is that for less wealthy farmers, increased off-farm income could be a necessity to compensate for a large loss. There are hence good reasons to expect farmers to increase off-farm work in face of a loss, but why do they not reduce off-farm work as much after a gain? One potential reason is the structure of the labor market: If you have an off-farm job, it is often not easy to, say, reduce this from a 100% to a 50% position – often the alternative would be to quit the job altogether, which might not be an attractive option. Finally, this can be viewed as a sign that many farmers find their off-farm work rewarding also beyond the financial aspects, and hence would like to work approximately as much as they currently do, even if a financial gain allowed them to work less.

For our fourth variable, about private consumption, we see the same pattern. A potential extra reason for this is that many farmers already have a “sufficiently” high level of private consumption, and expect their consumption to stay more or less the same even if they could afford to spend more.

For our fifth variable, about debt and savings, one additional reason for the negative value could be that farmers have good bank connections and are relatively comfortable with their current debt level, and thus see no urgent need to reduce this. Another reason might be that other options are more attractive than debt payments or bank savings after a sudden gain – for instance farm investments, as indicated by variable 1.

For our sixth variable, about shares and equity, the variable is again – as expected – negative. An alternative interpretation of this could be low interest in the stock market – alternatively, low expectations about future returns in the stock market.

The one important exception in this picture is farm investment, where a gain apparently means more for investment than a loss means for divestment. Although unexpected based on general theory, we will argue that the result makes sense in this particular setting. Many farming families have off-farm income which is enough to cover most daily expenses. The farm thus is not only a source of profit, but also a home and an instrument for saving. Whatever is left after all the bills are paid is invested in the farm, maybe to generate larger profits from the farm in the future, but also to make

it a better home, and because it is considered a good investment alternative. This explains one side of the equation – why a sudden gain is perceived to have large impacts on farm investment. The other side of the equation – why a sudden loss would have relatively little impact on farm divestment – is also relatively easy to understand. The extreme effect of farm divestment would be to give up farming. A loss of MNOK 1 is large enough for this to be a real threat for many farmers. If quitting is not necessary, a less drastic farm divestment is likely to be both less convenient and more emotionally painful than, say, a sale of off-farm assets. In a sense, the lifestyle importance of farming is supported by the results from this study: A sudden gain could well be invested in the farm, but to finance a sudden loss, most other options would be preferred rather than farm divestment and a possible new lifestyle as a non-farmer.

Implications

The results are overall not surprising. The general risk attitude corresponds well to previous studies, and the negative sign of the new «invest» variable is also easy to explain. Nevertheless, we think the results could have some interesting policy implications.

1. Risk management schemes
   Farmers are very reluctant to farm divestment and the treat of quitting farming. A reasonable interpretation of this is that the average farmer is able and willing to cope with risk and sudden losses – as long as he is able to handle them without divesting. Hence, it could be proposed that both private (i.e., insurance) and public risk management schemes should be adjusted to account for this. General crop insurance or any similar scheme is likely to be imperfect for most farmers. For relatively wealthy farmers, a bad crop will not force them to divest – they will be able to handle the loss by other means. Hence, insurance could be useful, but it will not be crucial for survival, and self-insurance might be as beneficial in the long run. For some farmers, however, the situation will be the opposite. Standard crop insurance might be useful, but it will typically not eliminate all extra costs associated with a bad crop. For farmers with few off-farm assets, even the (relatively small) losses they have to carry will make it necessary to divest and possibly quit farming. Hence, the insurance scheme will, in some sense, be of little use to them, because a bad crop will force them to give up farming anyway.
2. Policy and general support programs
   Most developed countries spend large amounts on agricultural subsidies – often through different schemes with different target groups and objectives. Lobley and Potter (2004) recommend a more integrated agricultural policy to take into account the diversity of farmers, including «lifestyle oriented» policies, directed at improving rural living in general, instead of supporting certain types of farm production in particular. Our study supports this recommendation. A reasonable interpretation of the reluctance to divest is that this is based on a strong will to stay on the farm and maintain a farming lifestyle – even if it becomes necessary to cut into other savings or increase the off-farm workload. If more of the support schemes are directed at improving rural living conditions, this would make it easier for many to stay on the farm. Even if their agricultural production is different or smaller than before, and they for instance have to work more off-farm, this is likely to be a good alternative for many farmers.

Conclusions

Farmers claim that a sudden gain would affect their behavior less than a sudden loss. The main exception is farm investment, which would be more affected by a sudden gain than a sudden loss would affect divestment. This is interesting, as it captures some important aspects of farming lifestyle: A sudden gain is likely to be invested in the farm, but a sudden loss would, if possible, be financed without farm divestment, as this often would lead to giving up farming, or at least some important aspects of the farming lifestyle.

These results are not surprising, as they are in line with both theory and earlier studies. However, the results could have implications for both insurance schemes and general support programs. As most farmers are very reluctant to farm divestment, this treat could be considered more specifically when designing insurance schemes. Also, as maintaining farming lifestyle is considered so important, general support programs could aim at improving rural living in general, rather than supporting specific types of production.

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