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Survival of probiotic *Lactobacillus casei* and *Enterococcus fecium* in Domiati cheese of high conjugated linoleic acid content

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Abstract

Domiati cheese was made from reconstituted milk (20% total solids) containing normal (C) and high level (T) of conjugated linoleic acid (CLA) content respectively using mixed probiotic *Lactobacillus casei* and *Enterococcus fecium* (1:1) as starter. The cheeses were stored in brine at 25±5°C for 60 d storage. The added probiotics grow equally well in C and T cheeses. The total viable and *Lb casie* counts increased to a maximum after 15 d of storage and then decreased while the counts of *Enterococcus fecium* and total lactic acid continued to increase until the end of the storage period. The cheeses retained high counts of these probiotics (>10⁷ cfu g⁻¹) throughout the storage period. The acidity, soluble nitrogen, free amino acid, and conjugated dienes increased significantly in Domiati cheese during storage with small differences between treatments except for the significantly higher conjugated dienes in T than C treatment.

Key words: Domiati cheese, Conjugated linoleic acid, Probiotics, Cheese microflora, *Lactobacillus casei*, *Enterococcus fecium*

Introduction

Functional foods are defined as foods that affect specific functions or systems in the human body, providing health benefits beyond energy and nutrients. The market of functional dairy products has developed markedly during the last decade (Granto et al., 2010). At the current time, the largest markets for functional foods and supplements are the United States, Europe, and Japan, accounting for 33.6%, 28.2% and 20.9% of sales in 2003, respectively with a yearly growth potential of 10% (Granto et al., 2010). This growth is mainly due to the segment of probiotic fermented milks. Analysis of the North American probiotics markets for human nutrition found that the probiotics sector earned revenues of US\$ 698 million in 2006 and expected to reach US\$ 1.70 billion in 2013, with an annual growth rate of 13.7%. Meanwhile, in Europe, consumption of probiotics is equally strong: between 2002 and 2007, consumption in Western Europe grew by an annual growth rate of 13% and by 18% in and consumption in Eastern Europe (Granto et al., 2010).

However, the major challenge facing producers of probiotic fermented milk is to maintain the viability of probiotics from processing until it reaches the consumer. Probiotics have been reported to suffer from low viability in fermented dairy products due to several factors (Granto et al., 2010) such as inhibition by acid, peroxide and other metabolites.

Cheese has been used as an alternative form of dairy products for the delivery of probiotics. The high solids and fat content of cheese offer better protection for probiotics in simulated human gastrointestinal tract (Mäkeläinen et al., 2009). Also, the continuous proteolysis of cheese during ripening provides probiotics with needed nitrogen source for their growth and activity. Consumption of probiotic cheese creates a buffer against the high acidic environment in the gastrointestinal tract, and thus creates a more favourable environment for probiotic survival throughout the gastric transit, due to higher pH. Moreover, the dense matrix and relatively high fat content of cheese may offer additional protection to probiotic bacteria in the stomach (Gomes daCruz et al., 2009). Similar to other functional probiotic foods, maintaining the viability of probiotics during cheese processing and storage is a challenge.

Several technological hurdles have been described in the development of probiotic cheeses including the salting step and storage conditions (Gomes daCruz et al., 2009). Domiati cheese is

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unique in that salt is added to milk before renneting, which would affect the survival of added probiotics. Domiati cheese is also stored in high salt brine (>8%) at ambient temperatures which would affect the viability of added probiotics during storage. Kasmó et al. (2004) found that *Lactobacillus acidophilus* survived to numbers $> 10^7$ cfu g⁻¹ in Turkish white cheese particularly when stored in vacuum without brine. Sarantinopoulos et al. (2002) found that the number of enterococci in Greek Feta cheese made with the use of *Enterococcus faecium* as an adjunct starter to increase until day 15 of ripening and then remained constant. El-Soda and Abd El-Salam (2002) reported that *Enterococcus faecium* and a combination of mesophilic and thermophilic lactobacilli developed the characteristic flavour of Domiati cheese made from pasteurized milk. Pirauzian et al. (2010) found that the inclusion of *Enterococcus faecium* improved the quality of ultrafiltered (UF) white cheese.

Conjugated linoleic acid (CLA) is a generic name that refers to a mixture of linoleic acid isomers in which the double bonds are conjugated. These isomers have received much attention recently for their beneficial health properties as anticarcinogens reducing potential cardiovascular diseases, immune modulation and body fat reduction (German and Dillard, 2006). Research (Whitlock et al., 2006) has demonstrated that alteration of dairy cow diets by inclusion of sources of linoleic and linolenic acid (precursors for bacterial CLA) will increase yield of CLA and trans fatty acids in milk fat.

During the course of developing probiotic Domiati cheese with high level of conjugated linoleic acid (CLA) it was important to study the

behaviour of the added probiotic under prevailing conditions in its manufacture and storage.

The aim of the present study was to investigate the survival of a combination *Lactobacillus casei* and *Enterococcus faecium* of potential probiotic characteristics in Domiati cheese made from milk of normal and high conjugated linoleic acid respectively. Additionally, changes in its composition during manufacture were followed.

Materials and Methods

Production of milk of high level in CLA content

The production of milk rich in CLA was carried out in the Experimental farm of the Dairy Science Department, South Dakota University, Brookings, USA as described by Whitlock et al. (2006). Two groups of multiparous Holstein cows were used. The first group received control diet (C) with 0% supplemental fat and second group received the C diet with 1% fish oil (FO) and 1% of extruded soy beans (ESB). In the fat supplemented diet FO replaced a portion of the cracked corn and the ESB replaced a portion of the cracked corn and a portion of the soybean in the control diet. Total mixed diets were formulated to be isonitrogenous at 18% crude protein and contain more than adequate amounts of major elements (NRC, 1989). Supplemental vitamin E was included in the diet at 53 IU/Kg of DM to help prevent oxidation of the milk because increased concentration of unsaturated fatty acids in milk fat may be more susceptible to oxidation. Cows were milked twice daily and the milk from each group was combined and spray dried using a pilot spray drier (Niro Atomizer, APV, Denmark). The milk powders had the following composition (Table 1).

Table 1. Chemical composition and properties of spray dried whole milk containing normal (Control) and high CLA contents.

Type of Milk	Moisture %	Fat %	Protein %	pH %	Acidity %	Solubility index %	Peroxide meq/kg Fat	Acid value ml 0.1 N KOH/100g fat
Control	3.37	28.64	24.21	6.62	0.25	2.25	4.3	1.90
High CLA	3.58	23.98	26.13	6.11	0.30	2.02	27.9	2.85

Milk fat was extracted from the powder and analyzed for fatty acids and results have been reported before (Abd El-Salam et al., 2011). The most obvious differences in the fatty acid composition of milk fat from animals receiving control and experimental diets were found in the CLA isomer *cis9, trans11* of the two milks being 0.5 and 1.25 g 100 g⁻¹ fat respectively. Also, the

milk fat with high level of CLA contained slightly higher contents of the trans isomers of the fatty acids; C_{15:1}, C_{16:1}, C_{17:1} and C_{18:1} than the one with normal CLA content.

Source of cultures

Lactobacillus casei was obtained from Chr. Hansen's Lab., Denmark. Probiotic *Enterococcus faecium* isolated from traditional dairy products

was obtained from Dairy Microbiology Lab, National Research Center, Dokki, Cairo, Egypt (El-Shafei et al., 2002). The strains were maintained and transferred weekly in sterilized reconstituted skim milk and stored at 4°C. Cultures were propagated in sterilized reconstituted skim milk (10% TS) at 35°C for 12-16 hrs before its use in the manufacture of Domiati cheese.

Cheese manufacture

The manufacture and analysis of Domiati cheese was carried out in the Dairy Department, National Research Centre, Cairo, Egypt. Whole milk powders with normal and high levels of CLA content were reconstituted in pre-boiled water to give 20% total solids in the reconstituted milks. The milks were heated to 60°C for 30 min, homogenized using two stage laboratory homogenizer (Lab-20, Rannie, APV, Copenhagen) at 20 and 5 MPa for the 1st and 2nd stages respectively. The milk was cooled to 40°C, 8% NaCl were added and stirred until the salt was completely dissolved. Rennet (calf rennet powder, Ch. Hansen, Copenhagen, Denmark) was added at the ratio of 0.5 g kg⁻¹ reconstituted milk and left to complete coagulation. The curd was ladled in rectangular frames (20 x 20 x 20 cm) lined with cheese cloth and the drained whey was collected to be used a brine for cheese storage and maturation. The fresh cheese was cut into cubes (~0.5 kg each), stored in salted whey in tightly closed containers at room temperature (20±5°C). Samples were taken from the fresh cheese and cheese stored for 15, 30 and 60d respectively for chemical and microbiological analysis. The whole experiment was repeated twice.

Methods of analysis

Sampling

Samples were taken aseptically by cutting a section from the cheese cubes with a sterilized sharp knife. About 1 cm of the surfaces of the obtained section, and the remaining part was divided into two portions. The first was kept for microbiological analysis and the second was kept frozen for chemical analysis.

Methods of chemical analysis and pH

The pH value of the cheese was determined at room temperature (20± 2°C) using digital pH meter (Hanna, Germany) equipped with a combined electrode. Cheeses were analyzed for total solids and fat contents and acidity according to A.O.A.C (1995) methods.

Determination of proteolysis

Cheeses were analyzed for soluble nitrogen (SN) content by Kjeldahl method (IDF, 1993) and total free amino acids (Folkertsma and Fox, 1992).

Determination of conjugated dienes

Analysis of conjugated dienes was carried as an index for changes in CLA content of cheese during storage. Five grams of the cheese sample were extracted with 6 ml chloroform:methanol (2:1). Two milliliters of chloroform were added to the cheese sample, mixed well, followed by 2 ml methanol and then 2 ml chloroform. The mixture was then centrifuged at 3000 x g for 15 min. The chloroform layer was withdrawn, dried with anhydrous sodium sulphate and then evaporated to dryness. The residue transferred to a capped tube, flushed with nitrogen and stored frozen until analyzed. The AOAC (1995) official method 957.13 was used for the determination of conjugated dienes in the extracted cheese lipids.

Microbiological analysis

The cheese samples were analyzed for total viable count (APHA, 1993), lactic acid bacteria (Elliker et al., 1956), *Lb. casei* on modified clbc medium (Nighswonger et al., 1996) and for *Enterococcus faecium* on kanamycin aesculin azide agar (Oxoid, 1998).

Statistical analysis

Two way independent factorial analysis of variance was carried out using Vassarstats computing website (Lowry (2009) (<http://faculty.vassar.edu/lowry/anova2corr.html>)). Significance of differences between treatments (type of milk and starter), storage periods and their interactions were determined using Tukey test at significance level of 0.05.

Results and Discussion

Physicochemical characteristics

Domiati cheese from milk of high CLA content had lower total solids (TS) content than that from normal milk throughout the storage period. This can be attributed to the softer texture of cheese from milk containing high level of CLA content which results in retaining more moisture than cheese from normal milk. Jones et al. (2005) reported that cheeses of soft texture have been obtained from high CLA milk which agrees with the present finding.

Analysis of Domiati cheese (Table 2) revealed significantly lower fat content in cheese made from milk with high level of CLA content as compared to cheese from milk of normal CLA content. This

can be explained by the differences in the fat content of the two milks and increased losses of fat during the cheese manufacture from high CLA milk. Feeding animals rations supplemented with fish oil reduced the milk fat content (Whitlock et al., 2006) which would explain the lower fat in milk powder of high CLA level prepared in the present study. Also, milk of high CLA was reported to produce cheese of less firm texture than normal milk (Jones et al., 2005) which may explain the higher fat losses during the manufacture of Domiati cheese from milk of high CLA content.

The rate of acidity development in Domiati cheese from milk of high CLA content was slightly less than that from normal milk (Table 2) but the differences were not significant (Table 2). On the other hand, the pH of cheese from milk of high CLA was slightly higher, but not significant, than that from normal milk throughout the storage. This

can be due to the differences in the developed acidity, and the higher buffer capacity of Domiati cheese from high CLA milk due to its high protein content.

The free amino acids (FAA) increased gradually in Domiati cheese from normal and high CLA milks during storage (Table 2). The development of FAA in the two cheeses was not significantly different (Table 3) indicating comparable proteolysis in the two cheeses.

The conjugated dienes content increased gradually in cheese from both treatments reaching 0.84 and 1.93 g 100 g⁻¹ cheese fat after 60 d. This increase may be attributed to the growth and activity of the added probiotic starter which is capable of synthesizing CLA (Abd El-Salam et al., 2010). The differences in the conjugated dienes of Domiati cheese due to storage period and type of milk used were found significant (Table 2).

Table 2. Changes in the composition of Domiati cheese from milk with normal (C) and high (T) conjugated linoleic acid during storage (Average of two replicates).

Treatment	Storage Period			
	Fresh	15	30	60
Total Solids (g 100g ⁻¹)				
C	35.75 ^{aA}	37.95 ^{bA}	39.07 ^{cA}	40.84 ^{dA}
T	35.37 ^{aA}	36.35 ^{bB}	38.11 ^{cB}	39.29 ^{dB}
Fat (g 100g ⁻¹)				
C	17.50 ^{aA}	18.00 ^{aA}	19.00 ^{bA}	21.00 ^{cA}
T	13.00 ^{aB}	13.00 ^{aB}	14.00 ^{bB}	15.00 ^{cC}
Acidity (%)				
C	0.28 ^a	0.74 ^b	0.79 ^b	1.21 ^c
T	0.26 ^a	0.70 ^b	0.85 ^c	1.20 ^d
pH				
C	5.90 ^a	4.95 ^b	4.71 ^{bc}	4.04 ^d
T	5.97 ^a	5.17 ^b	4.74 ^c	4.25 ^d
Soluble N (g 100g ⁻¹)				
C	0.34 ^a	0.34 ^a	0.36 ^a	0.36 ^a
T	0.36 ^a	0.36 ^a	0.39 ^a	0.39 ^a
Free amino acids (mg 100g ⁻¹)				
C	24 ^{aA}	30 ^{bA}	54 ^{cA}	64 ^{dA}
T	30 ^{aB}	35 ^{bB}	63 ^{cB}	85 ^{dB}
Conjugated dienes (g 100g ⁻¹ fat)				
C	0.39 ^{aA}	0.55 ^{bA}	0.69 ^{bcA}	0.84 ^{cdA}
T	0.84 ^{aB}	1.50 ^{bB}	1.64 ^{bcB}	1.93 ^{cdB}

* Small letters (a,b,c) different subscripts in the same row indicate significance

* Capital letters(A,B) different subscripts in the same column indicate significance

Effect of added probiotics on total viable and lactic acid bacteria counts

Figures 1 and 2 shows that the changes in the total viable count (TVC) and lactic acid bacteria (LAB) count from different treatments. The TVC increased to a maximum after 15 days of storage and then decreased slightly thereafter and statistical analysis showed that the effect of storage on TVC was highly significant as shown in Table 3. The changes in TVC during storage followed its normal pattern of changes during storage where TVC increased during the first two weeks of storage and then decreased after that (Abd El-Salam and Benkerroum, 2006). Slight but not significant differences were found in the TVC of Domiati cheese from milk of high and normal levels of CLA content (Figure 1 and Table 3). This indicates that the changes in CLA content of milk had no probable effect on the TVC of Domiati cheese. The count of LAB in Domiati cheese increased slightly throughout storage and these increases were highly significant (Table 3).

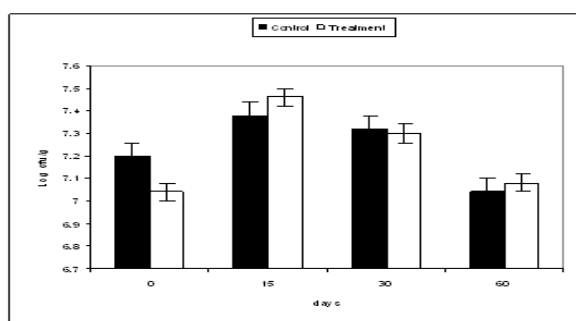


Figure 1. Changes in the total bacterial count (mean Log cfu g⁻¹ ± Standard deviation) of Domiati cheese from milk containing normal and high CLA content.

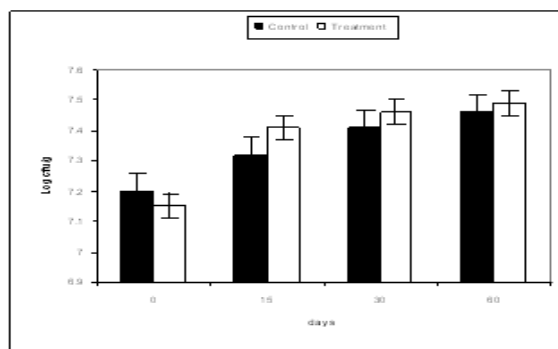


Figure 2. Changes in the total LBA count (mean Log cfu g⁻¹ ± Standard deviation) of Domiati cheese from milk containing normal and high CLA content

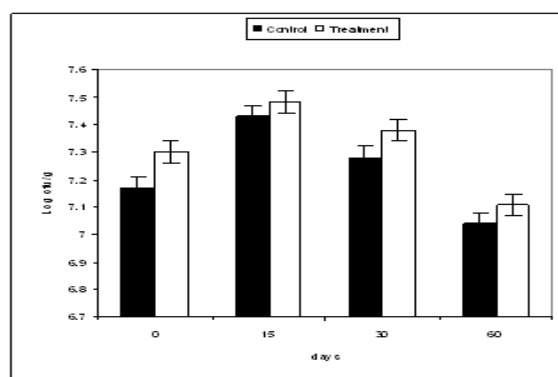


Figure 3. Changes in the total *Lb casei* count (mean Log cfu g⁻¹ ± Standard deviation) of Domiati cheese from milk containing normal and high CLA content.

Previous studies showed that changes in LAB followed a similar pattern as TVC which was not found in the present study.

Table 3. Analysis of variance of chemical composition and microbiological quality of Domiati cheese as affected by storage and type of milk (treatment) used.

	Effect of Storage		Effect of treatment		Interaction (Storage x Treatment)	
	Probability	Tukey HSD test (0.05)	Probability	Tukey HSD test (0.05)	Probability	Tukey HSD test (0.05)
Total	<0.0001	0.84	0.0002	0.43	—*	—
Solids	<0.0001	0.57	<0.0001	0.29	0.016	0.99
Fat	<0.0001	0.11	—*	—	—*	—
Acidity	<0.001	0.31	—*	—	—*	—
pH	—*	—	—*	—	—*	—
Soluble N	<0.0001	1.6	<0.0001	0.82	<0001	2.8
Free AA**	<0.0001	0.16	<0.0001	0.08	0.008	0.28
Dienes	<0.0001	0.08	—*	—	0.008	0.14
TVC**	<0.0001	0.07	—*	—	—*	—
LAB**	<0.0001	0.08	0.006	0.04	0.02	0.14
<i>Lb. casei</i>	—*	—	—*	—	—*	—
<i>E. faecium</i>						

* not significant at P<0.05, Tukey HSD 0.05, absolute difference between two means required for significance at P 0.05.

** Free AA, free amino acids; TVC, total viable count LAB, lactic acid bacteria

The changes in the LAB also followed a similar pattern to changes in *Enterococcus faecium* (Figure 4) which show that the count of LAB was affected by the added adjunct starter. On the other hand, the type of milk used had no significant effect on the count of LAB of Domiati cheese. Coakley et al. (2007) reported that elevating the CLA level in cheese did not affect the growth of starter bacteria or nonstarter lactic acid bacteria in cheese during ripening.

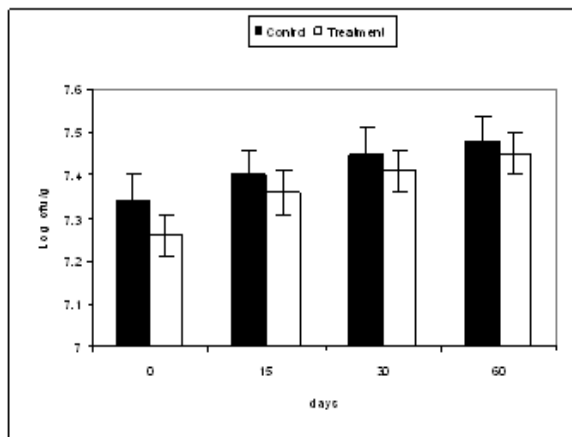


Figure 4. Changes in the total *Enterococcus faecium* count (mean Log cfu g⁻¹ ± Standard deviation) of Domiati cheese from milk containing normal and high CLA content.

Survival of probiotics in Domiati cheese

After a storage period of 15 days, the viable count of *Lb casei* increased by 0.26 and 0.18 log cycles cfu g⁻¹ in Domiati cheese of normal and high level of CLA content respectively (Figure 2). However, the *Lb casei* count of normal and high level of CLA content decreased by 0.15 and 0.19 log cycles cfu g⁻¹ respectively in comparison with the initial count after 2 mo of storage. The count of *Ent faecium* increased by 0.06 and 0.14 and 0.1 and 0.19 log cycles cfu g⁻¹ after 15 d and 2 mo for Domiati cheese of normal and high levels of CLA respectively. The high viability of added probiotics during storage can be attributed to the high TS of Domiati cheese. High TS in milk was reported to offer protection for the growing microorganisms (Gün and İstkli, 2007; Mahdian and Tahrani, 2007) which agree with the present finding. Also, *Ent. faecium* was reported as being able to tolerate the high salt in brined cheeses (Sarantinopoulos et al., 2002) which may explain the continuous increase of this probiotic in Domiati cheese during storage in brine. Donkor et al. (2007) reported that storage time play an important role in the extent of overall proteolytic activity, and consequent increase in the

amount of liberated amino acids may cause a higher growth rate of probiotic bacteria even in an acidic environment.

Conclusions

Domiati cheese retained high counts of probiotics *Lb casei* and *Ent faecium* (> 10⁷ cfu g⁻¹) throughout the storage of 2 months indicating the high survival of the used probiotics under the storage conditions. Also, the level of CLA in Domiati cheese had no probable effect of the survival of the used probiotics. Therefore, the obtained Domiati cheese can be considered as a functional probiotic cheese with normal or high levels of CLA content.

References

- Abd El-Salam, M. H. and N. Benkerroum. 2006. North African cheeses. In "Brined Cheeses" (Ed.A.Y. Tamime) pp 139-187, Blackwell Pub. Oxford, UK.
- Abd El-Salam, M. H., K. El-Shafei, O. M. Sharaf, B. A. Effat, F. M. Assem and M. El-Aassar. 2010. Screening of some potentially probiotic lactic acid bacteria for their ability to synthesis conjugated linoleic acid. Inter. J. Dairy Technol. 63:62-69.
- Abd El-Salam, M. H., A. R. Hippen, K. El-Shafie, F. M. Assem, H. Abbas, M. Abd El-Aziz, O. M. Sharaf and M. El-Aassar. 2011. Preparation and properties of probiotic concentrated yoghurt (labneh) fortified with conjugated linoleic acid. Inter. J. Food Sci. Technol. 46:2103-2110.
- AOAC. 1995. Official Methods of Analysis, vol. 1: Agricultural Chemicals, Contaminates, Drugs. 16th ed. Washington D.C.
- APHA. 1993. Standard Methods for Examination of Dairy Products, 16th ed. American Public Health Association, Washington D C, USA.
- Coakley, M., E. Barrett, J. J. Murphy, R. P. Ross, R. Devery and C. Stanton. 2007. Cheese manufacture with milk with elevated conjugated linoleic acid levels caused by dietary manipulation. J. Dairy. Sci. 90:2929-2927.
- Donkor, O. N., S. L. I. Nilmini, P. Stolic, T. Vasiljevic and N.P. Shah. 2007. Survival and activity of selected probiotic organisms in set-type yoghurt during cold storage. Int. Dairy J. 17:657-665.

- Elliker, P. R., A. W. Anderson and G. Hannesson 1956. An agar medium for lactic acid streptococci and lactobacilli. J. Dairy Sci. 3:1611-1618.
- El-Shafei, K., G. A. Ibrahim and N. F. Tawfik 2002. Beneficial uses of locally isolated lactic acid bacteria. Egypt J. Dairy Sci. 30:15-25.
- El-Soda, M. and M.H. Abd El-Salam 2002. Cheese matured in brine. In: H. Roginski, F. W. Fruquay and P. F. Fox (Eds), pp.406-4011. "Encyclopedia of Dairy Science Elsevier Science, London.
- Folkertsma, B. and P. F. Fox. 1992. Use of the Cd-ninhydrin reagent to assess proteolysis in cheese during ripening. J. Dairy Res. 59:217-224.
- German, J. B. and C. J. Dillard. 2006. Composition, structure and absorption of milk lipids: A source of energy, fat-soluble nutrients and bioactive molecules. Crit. Rev. Food Sci. Nutr. 46:57-82.
- Gomes daCruz, A., F. C. A. Buriti, C. H. Matista de Souza, J. A. F. Faria and S. M. I. Saad. 2009. Probiotic cheese: Health benefits, technological and stability aspects. Trends Food Sci. Technol. 20:344-354.
- Gün, Ö. and N. D. İştikli. 2007. Effect of fat and non-fat dry matter of milk, starter type, on the rheological properties of set during the coagulation process. Int. J. Food Sci. Technol. 42:352-358.
- Granato, D., G. F. Branco, A. G. Cruz, J. A. F. Faria and N. P. Shah. 2010. Probiotic dairy products as functional foods. Comprehensive Rev. Food Sci. Food Safety 9:455-470.
- IDF. 1993. Milk. Determination of nitrogen content by Kjeldahl method. Standard B20. International Dairy Federation, Brussel, Belgium.
- Jones, E. L., K. J. Shingfield, C. Kohen, A. K. Jones, B. Lupoli, A. S. Grandison, D. E. Beever, C. M. Williams, P. C. Calder and P. Yaquob. 2005. Chemical, physical and sensory properties of dairy products enriched with conjugated linoleic acid. J. Dairy Sci. 88:2923-2937.
- Lowery 2009. Vassarstats (<http://faculty.vassar.edu/lowry/anova2corr.html>).
- Kasmo, A., M. Göncüo and S. Akgün. 2004. Probiotic white cheese with *Lactobacillus acidophilus*. Int. Dairy J. 14:1067-1073.
- Mäkeläinen, H., S. Forssten, K. Olli, L. Granlund, N. Rautonen and A. C. Ouwehand. 2009. Probiotic lactobacilli in a semi-soft cheese survive in the simulated human gastrointestinal tract. Int. Dairy J. 19:675-683.
- Mahdian, E. and M. M. Tehrani. 2007. Evaluation the effect of milk total solids on the relationship between growth and activity of starter cultures and quality of concentrated yoghurt. Am-Eurasian J. Agric. Environ. Sci. 2:587-592.
- Nighsowonger, B. D., M. M. Brashears and S. E. Gilland. 1996. Viability of *Lactobacillus acidophilus* and *Lactobacillus casei* in fermented milk products during refrigerated storage. J. Dairy Sci. 79:212.
- NRC. 1989. Nutritional Requirements of Dairy Cattle. 6th ed. National Academy of Science, Washington DC.
- Oxoid Manual. 1998. Eighth Edition. Compiled by E. Y. Bridson. Oxoid Ltd, Basigtoke, UK.
- Pireuzian, H.R., J. Hesari, S. Farajnia, M. Moshaddam, S. Ghiassifor and M. Manafi. 2010. Inclusion of *Enterococcus faecalis* and *Enterococcus faecium* to UF white cheese. World Acad. Sci. Eng. Technol. 66:850-853.
- Sarantinopoulos, P., G. Kalantzopoulos and E. Tsakalidou. 2002. Effect of *Enterococcus faecium* on microbiological, physicochemical and sensory characteristics of Greek Feta cheese. Int. J. Food Microbiol. 76: 93-105.
- Whitlock, L. A., D. L. Schingoethe, A. A. AbuGhazaleh, A. R. Hippen and K. F. Kalscheur. 2006. Milk production and composition from cows fed small amounts of fish oil and extruded soybeans. J. Dairy Sci. 89:3972-3980.

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The microbial quality of processed date fruits collected from a factory in Al-Hofuf City, Kingdom of Saudi Arabia

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Abstract

The microbial contamination of samples of processed date fruits collected from a factory in Al-Hofuf City, Kingdom of Saudi Arabia, was measured immediately after processing and then after refrigerated storage at 4-7°C for 2, 4 and 6 months. Freshly processed samples were found contaminated with potential spoilage microorganisms including mesophilic aerobic bacteria, molds, and yeasts. The amount of contamination in general decreased steadily with storage time. The main contaminants of freshly processed fruits were mesophilic aerobic bacteria and the mold *Aspergillus niger*. The yeasts *Zygosaccharomyces mellis*, *Debaryomyces hansenii*, *Candida lipolytica*, and *Torulaspora delbrueckii* were detected in stored samples up to 6 months. Coliforms, fecal coliforms and nonpathogenic *Escherichia coli* strains were detected in freshly processed samples and in samples stored for 2 months. The potential pathogens *Staphylococcus aureus* and *Aspergillus flavus/parasiticus* were detected in the freshly processed samples only.

Key words: Date fruits, Microbial contamination, Cold storage, Spoilage, Pathogens

Introduction

Dates (*Phoenix dactylifera* L.) are mainly grown in Middle East and North African countries, with a worldwide annual production of about 6 million tones. Saudi Arabia produces yearly about 900 thousand tones and ranks as the third largest producer in the world (FAO, 2008). About 50% of the produce in Saudi Arabia is consumed locally as human food, only about 4% is exported, while the rest is mainly used as animal feed (Al Eid, 2010). Microbial contamination, especially with molds, is a major obstacle facing international marketing of Saudi dates (Al Eid, 2010). Dates are fairly dry fruits, with water and sugar contents of 10-15% and 60-88% (on dry basis), respectively (Barreveld, 1993), hence they are generally regarded as stable to microbial spoilage. However some contaminants, especially osmotolerant yeasts and molds, may survive for longer times or even grow on the fruits. Microbial contaminants isolated from date fruits include yeasts, molds, lactic acid bacteria and some potential pathogens like *Staphylococcus aureus*,

E. coli, and *A. flavus/parasiticus* (Bolin et al., 1972; Abu-Zinada and Ali, 1982; El-Sherbeeney et al., 1985; Nussinovitch et al., 1989; Abdulsalam et al., 1991; Aido et al., 1996; Kader 2007; Hamad, 2008).

This study was undertaken to investigate the microbial contamination of processed date fruits collected from a factory in Al-Hofuf City, Kingdom of Saudi Arabia. Tests were performed on samples immediately after processing, and on samples stored at 4-7°C for 2, 4, and 6 months.

Materials and Methods

Samples

Samples were collected from a date processing factory in Al-Hofuf City, Saudi Arabia. The processing line consists of a conveyer belt on which the dates are first rinsed with water to prevent clumping, and then rinsed with chlorinated water, then with water again, before drying with hot air, and finally packaging under partial vacuum in sealed high density polyethylene containers as unpitted pressed fruits. A total of 40 samples (each sample 1 kg package) were collected, 10 samples from lots processed on the same day (samples 1-10), and then each time other 10 samples taken after 2, 4, and 6 months of storage at 4-7°C in cold stores (samples 11-20, 21-30 and 31-40, respectively), which is the storage practice followed in this factory. The samples were taken to the laboratory and analyzed on the same day.

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Microbiological analysis

Five replicates were tested from each sample as required by the Saudi Standard Specifications for Foods, so that 200 replicates were tested from the 40 date fruit samples collected. The samples were aseptically destoned using sterile forceps and microbial loads calculated for the flesh. To test for aerobic mesophilic bacteria and coliforms, the samples (10g) were weighed into sterile stomacher bags, 90 ml sterile peptone water (Oxoid, CM0009) added, homogenized in a stomacher (Lab-Blender 400, Seward Medical, England) for 45 seconds and then serial dilutions prepared. Using the pour plate method (1 ml inoculum size), aerobic mesophilic bacteria were counted on Plate Count Agar dishes (PCA Oxoid, CM0325) incubated at 30°C for 2 to 3 days. Enumeration of *E. coli* and coliform bacteria was done using the most probable number (MPN) method. Lauryl Tryptose Broth (LST, Oxoid, CM0451) incubated at 35°C for 24-48 h was used for the presumptive test for coliforms, fecal coliforms, and *E. coli*. Brilliant Green Bile 2% Broth (BGLB, Oxoid, CM0263) incubated at 35°C for 48 h for the confirmed test of coliforms, EC broth (Oxoid, CM0853) incubated at 45.5°C for 24-48 h for the confirmed test of fecal coliforms and *E. coli*, and Levine's Eosin-Methylene Blue Agar (L-EMB Agar, Oxoid, CM0069) incubated at 35°C for 18-24 h for the completed test of *E. coli*. Five suspicious colonies from each L-EMB plate were transferred to PCA slants, incubated for 18-24 h at 35°C and used for further identification. The further identification of *E. coli* was performed using the api 20 E method, and the tested isolates identified using the api 20 E analytical profile index (bioMérieux sa, France). The pathogenicity of *E. coli* isolates was tested serologically (DENKA SEIKEN CO. LTD, REF. 295347). To enumerate yeasts, moulds, *A. flavus/parasiticus* and *Staphylococcus aureus*, 20 ml sterile peptone water were added to 10g samples (1:3 dilution), to account for low loads (further dilutions performed when necessary). Yeasts and molds were counted on Potato Dextrose Agar medium (PDA Oxoid, CM0139) to which 100 mg/l chloramphenicol (SR0078, Oxoid) were added to suppress bacterial growth. In both tests the spread plate method was used and 0.5 ml sample aliquots were added to the dishes. The dishes were left in the upright position for about 15 minutes until inoculum was absorbed by agar. Yeasts were incubated at 30°C for 3 days, and molds at 20-30°C for 3 to 7 days. Representative isolates (10 isolates from each colony form) were made from yeasts found in counts around 10^2 cfu/g in samples stored at 4-7°C for 2, 4 and 6 months (potential spoilage

organisms). The isolates were purified by repeated streaking on PDA plates and then stored in slopes in the refrigerator for identification. Lactic acid bacteria (LAB) were enumerated using De Man, Rogosa, and Sharpe for lactobacilli (MRS Agar, Oxoid, CM0361) and M₁₇ Agar media for streptococci and lactococci (Oxoid, CM0785). The plates were incubated in anaerobic jars with anaerobic gas generating kits (Oxoid, BR0038) for 2 to 3 days at 30°C. *Staphylococcus aureus* was enumerated on *Staphylococcus* medium No. 110 (CM0145, Oxoid) incubated at 35-37°C for 24-48 hours and identified using the Staphylase Test (DR0595, Oxoid). *Aspergillus flavus/parasiticus* was detected and enumerated on *Aspergillus flavus-parasiticus* agar (AFPA, CM0731, Oxoid) incubated at 30°C for 48 hours.

Moisture content was determined by difference after heating about five gram samples overnight in an oven at 105°C.

Identification of yeasts

Identification of yeasts was done according to the methods described by Barnett et al. (2000). First, microscopical examination of the appearance of non-filamentous vegetative cells grown in shake flasks in malt extract broth (CM0057) for 2 days at 25°C, and microscopical examination for filamentous growth using the slide culture technique was performed. The isolates were then examined for glucose fermentation in Durham tubes and for sporulation on; a. malt-yeast-glucose-peptone (YM agar), b. Gorodkova agar, c. McClary acetate agar, d. Malt extract agar, the dishes incubated at 25°C and examined after 3 days for up to 6 weeks. After that, the appropriate subsequent tests according to the identification keys 1, 2, 3, and 4 were performed. Using these results, the isolates were then identified after the above mentioned identification keys.

Identification of molds

Molds were identified from the macroscopic morphology of the colony and the microscopic morphology of the hyphae, conidia and conidiophores of cultures grown on Czapek Dox Aar (Oxoid, CM0097) dishes incubated at 20-30°C for 3-7 days (Larone, 1995).

Results and Discussion

Contamination of the fruits with potential spoilage microorganisms

Contamination of freshly processed samples

The moisture content of all samples, freshly processed and stored, was in the range 13-15% (results not shown). As can be seen in Table 1, all

of the 10 samples analyzed on the same day of processing were contaminated with mesophilic aerobic bacteria and molds; while yeasts contamination was detected in 5 samples (loads for each sample were averages of 5 replicates). The loads of mesophilic aerobic bacteria in three samples were in the order 10^5 cfu/g, in other three samples in the order 10^4 cfu/g and in four samples in the order 10^3 cfu/g. This relatively high amount of contamination is similar to values we detected in raw date fruits (results not shown), indicating that the washing process practiced in this factory didn't result in a significant reduction in the microbial load.

Contamination with molds was in the order 10^3 cfu/g in three samples, 10^2 cfu/g in five, and less than 10^2 cfu/g in two samples. This is also a high level of contamination. The limits for mold contamination in date fruits according to the Saudi Standard Specifications are (these specifications include limits for molds, yeasts and *E. coli* only): in 2 out of 5 replicates tested from a sample the targeted limit is 10^2 cfu/g and no replicate should reach a load of 10^3 cfu/g (SASO 1999). The three samples with loads 10^3 cfu/g (samples 4, 6, and 7 in Table 1) were therefore out of specification. In addition, samples 1, 5, and 9 were also out of specification because 3 to 4 replicates of each sample contained more than 10^2 cfu/g (results not

shown). Hence only four samples met the requirements for limits of mold contamination. About 95% of the mold contaminants were identified as *Aspergillus niger*. The high level of mold contamination can be attributed to the fact that dates are harvested in the dry windy months of July – September. Airborne mold spores can easily contaminate the fruits of the tall palm trees.

Five out of the 10 samples were found contaminated with yeasts. One sample contained 1.4×10^2 cfu/g, and the other 4 samples 19-76 cfu/g (Table 1). The limits for yeast contamination in date fruits according to the Saudi Standard Specifications are: in 2 out of 5 replicates tested from a sample the targeted limit is 10 cfu/g and no replicate should reach a load of 10^2 cfu/g (SASO 1999). Accordingly, sample 9 was out of specification, and also sample 6 was out of specification because 3 out of its 5 replicates tested contained more than 10 cfu/g (results not shown). Contamination with lactic acid bacteria was minimal. It was detected in only two samples at low concentrations (Table 1).

From the above results it appears that only four out of the 10 samples tested met the Saudi requirements for microbiological limits in date fruits, and that mold contamination was the main problem.

Table 1. Contamination of date fruit samples with yeasts, molds, and mesophilic aerobic bacteria, analyzed on the same day of processing (packaging). Results of each sample are averages of 5 replicates.

Sample No.	Yeasts (cfu/g)	Molds (cfu/g)	Mesophilic aerobic bacteria (cfu/g)	LAB (cfu/g)
1	38	9.0×10^2	1.4×10^3	n.d.
2	n.d.	52	1.3×10^5	n.d.
3	24	2.1×10^2	6.0×10^5	73
4	n.d.	1.0×10^3	1.5×10^5	62
5	n.d.	8.8×10^2	6.4×10^4	n.d.
6	76	5.2×10^3	3.4×10^4	n.d.
7	19	4.2×10^3	2.0×10^3	n.d.
8	n.d.	1.5×10^2	9.9×10^3	n.d.
9	1.4×10^2	7.1×10^2	1.1×10^4	n.d.
10	n.d.	87	4.2×10^3	n.d.

n.d. = not detected, LAB = lactic acid bacteria

Contamination of samples stored for 2 months after processing

The microbial loads of the samples stored at 4-7°C for 2 months (samples 11-20) were much less than those of the samples analyzed immediately after processing. Four samples were found contaminated with mesophilic aerobic bacteria at loads in the order 10^2 cfu/g, other four samples contained less than 10^2 cfu/g, and two samples were

free of detectable contamination with these types of bacteria. This amount of contamination is less than that of the freshly processed samples by about 2-3 log cycles (Tables 1 and 2). It is apparent that the mesophilic aerobic bacterial contaminants were quite sensitive to the conditions of low temperature storage, low water activity and high sugar content in the fruits and the low level of oxygen in the

packages so that the majority of them couldn't survive.

All samples were found contaminated with molds, but the amounts of contamination were also generally less than those of the freshly processed ones. One sample contained mold contamination in the order 10^3 cfu/g, four samples in the order 10^2 cfu/g, and five samples less than 10^2 cfu/g. Sample 18 (Table 2), which contained 1.4×10^3 cfu/g, was out of Saudi specification, and also samples 17 and 20 were out of specification because more than two replicates from each sample contained more than 10^2 cfu/g mold contamination (results not shown). Molds, which are generally more tolerant to low water activity levels than bacteria, seem to be more persistent in the conditions prevailing in the package. Again about 90% of the molds were identified as *A. niger*.

Yeast contamination was detected in 5 samples. Sample 19 (Table 2) was out of specification because one of its replicates tested contained 1.2×10^2 cfu/g, and also sample 17 was out of specification because 3 of its replicates contained more than 10^2 cfu/g (results not shown). The yeast strain detected in sample 19 was identified as *Zygosaccharomyces mellis* (Table 6). This is an osmotolerant yeast showing growth on 50 and 60% glucose, hence it seems that it was able to survive and grow on the date fruits under the prevailing storage conditions. *Z. mellis* is also psychrophilic yeast with maximum growth temperature for some strains of about 30°C (Barnett et al., 2000). This yeast was reported as a spoilage organism of date fruits at the rutab stage (Hamad 2008). Lactic acid bacteria were detected in only three samples at low concentration.

Table 2. Contamination of processed date fruit samples stored at $4-7^\circ\text{C}$ for 2 months with yeasts, molds, and mesophilic aerobic bacteria. Results of each sample are averages of 5 replicates.

Sample No.	Yeasts (cfu/g)	Molds (cfu/g)	Mesophilic aerobic bacteria (cfu/g)	LAB (cfu/g)
11	n.d.	60	n.d.	n.d.
12	n.d.	94	4.9×10^2	n.d.
13	n.d.	2.0×10^2	95	n.d.
14	33	79	1.3×10^2	n.d.
15	25	1.1×10^2	89	58
16	n.d.	83	2.6×10^2	n.d.
17	79	7.4×10^2	86	83
18	17	1.4×10^3	n.d.	n.d.
19	1.2×10^2	58	2.5×10^2	n.d.
20	n.d.	6.8×10^2	76	61

n.d. = not detected, LAB = lactic acid bacteria

Contamination of samples stored for 4 months after processing

The amounts of contamination with mesophilic aerobic bacteria and molds in the samples stored for 4 months at $4-7^\circ\text{C}$ (samples 21-30) were still lower than those of the samples stored for 2 months (Table 3). Only two samples contained loads of mesophilic aerobic bacteria in the order 10^2 cfu/g, 5 samples contained less than 10^2 cfu/g, and 3 samples were free of detectable contamination. With respect to contamination with molds, only one sample was out of specification because three of its replicates tested contained more than 10^2 cfu/g (results not shown). Six samples contained less than 10^2 cfu/g and 3 were free of detectable mould

contamination. In case of yeasts it seems that some osmophilic and psychrotrophic strains were able to grow in the packages. Samples 24 and 28 (Table 3) contained 2.1×10^2 and 1.6×10^2 cfu/g yeasts, respectively and hence were out of specification. Two samples contained 16 and 21 cfu/g while 6 samples were free of detectable contamination with yeasts. The yeast found in sample 24 was *Z. mellis*, and that in sample 28 was *Candida lipolytica* (the asexual state of *Yarrowia lipolytica* (Barnett et al., 2000). The later yeast is osmotolerant showing growth at 50% glucose (Table 6) and is also a psychrotrophic organism (Deak, 2008). No lactic acid bacteria were detected in any sample.

Table 3. Contamination of processed date fruit samples stored at 4-7°C for 4 months with yeasts, molds, and mesophilic aerobic bacteria. Results of each sample are averages of 5 replicates.

Sample No.	Yeasts (cfu/g)	Molds (cfu/g)	Mesophilic aerobic bacteria (cfu/g)	LAB (cfu/g)
21	n.d.	n.d.	78	n.d.
22	n.d.	59	n.d.	n.d.
23	n.d.	64	1.1x10 ²	n.d.
24	2.1x10 ²	n.d.	53	n.d.
25	21	94	85	n.d.
26	n.d.	n.d.	2.7x10 ²	n.d.
27	16	82	93	n.d.
28	1.6x10 ²	75	n.d.	n.d.
29	n.d.	1.4x10 ²	n.d.	n.d.
30	n.d.	88	69	n.d.

n.d. = not detected, LAB = lactic acid bacteria

Contamination of samples stored for 6 months after processing

The 10 samples stored at 4-7°C for 6 months (samples 31-40) contained still lower microbial contamination, except for one sample (Table 4). Only one sample contained mesophilic aerobic bacteria in the order 10² cfu/g, 5 samples contained less than 10² cfu/g and 4 were free of detectable contamination. In case of molds, 5 samples contained less than 10² cfu/g, 5 were free of detectable contamination and none of them was out of Saudi specification. The only one sample, namely sample 40, which was out of specification was contaminated with yeasts at 4.1x10³ cfu/g. The contamination was a mixed population of *Debaryomyces hansenii* and *Torulaspora delbrueckii* at about 2:1 ratio. The two strains were osmotolerant at 50% and 60% glucose (Table 6)

and are also known to be psychrotrophic (Deak, 2008). All 5 replicates of this sample contained more than 10² cfu/g yeasts, which indicate that the yeasts were growing in the package. No contamination with lactic acid bacteria was detected.

These results indicate that most microbial contaminants of dates die with time if the fruits are packaged and stored at refrigeration temperature. Date fruits are also known to contain some antimicrobial components. For example, some varieties contain up to 2.5% tannins (Al-Hooti et al., 1997; Myhara et al., 2000), which have been reported to cause growth inhibition to many species of fungi and bacteria (Nelson et al., 1997; Ishida et al., 2006). Only some osmotolerant yeasts seem to be able to survive or grow in packaged date fruits stored under refrigeration conditions.

Table 4. Contamination of processed date fruit samples stored at 4-7°C for 6 months with yeasts, molds, and mesophilic aerobic bacteria. Results of each sample are averages of 5 replicates.

Sample No.	Yeasts (cfu/g)	Molds (cfu/g)	Mesophilic aerobic bacteria (cfu/g)	LAB (cfu/g)
31	n.d.	n.d.	69	n.d.
32	n.d.	77	n.d.	n.d.
33	23	n.d.	n.d.	n.d.
34	n.d.	68	78	n.d.
35	n.d.	n.d.	n.d.	n.d.
36	n.d.	n.d.	86	n.d.
37	n.d.	79	75	n.d.
38	n.d.	n.d.	94	n.d.
39	n.d.	83	1.7x10 ²	n.d.
40	4.1x10 ³	64	n.d.	n.d.

n.d. = not detected, LAB = lactic acid bacteria

Contamination with potential pathogenic microorganisms

As can be seen in Table 5, potential pathogens were detected in some freshly processed samples (samples 1-10) and in some samples stored for 2

months at 4-7°C (samples 11-20), but none in the samples stored for 4 and 6 months (samples 21-40). Coliforms were found in 3 out of the 10 freshly processed samples and in 2 out of the 10 samples stored for 2 months (Table 5). Sample 8 showed the

highest level of contamination with coliforms, where 3 of the 5 replicates contained up to 150 MPN/g. Two of the 5 replicates in samples 1 and 6 also contained coliforms, and in samples 18 and 20 only one replicate contained coliform. Fecal coliforms were detected in 2 replicates of samples 1 and 8 and in one replicate of samples 6 and 18. *E. coli* was detected in 2 replicates of sample 8 and in one replicate in each of samples 1, 6, and 18. Thirteen out of 20 isolates (isolates 1-5, 10, 11, 13 and 16-20) made from the contaminated samples were identified as *E. coli* 1 and 7 isolates (isolates 6-9, 12, 14 and 15) as *E. coli* 2 (Table 7). Isolates 1 to 5 (from sample 1) gave identical numerical profile of 5144552 and the quality of identification as *E. coli* 1 was good with %id = 97.7 and T value = 1.0. Isolates 6-9 (from sample 6) gave identical numerical profile of 4044102 and the quality of identification as *E. coli* 2 was good with %id = 99.8 and T value = 0.98. Isolates 10, 11, 13 and 17 (from samples 6, 8 and 18), gave identical numerical profile of 1044552 and the quality of identification as *E. coli* 1 was good with %id = 69.3 and T value = 0.86. Isolates 12, 14 and 15 (from sample 8) gave identical numerical profile of 0044112.

Discrimination from *Shigella* spp. was low, and the %id and T value for *E. coli* 2 were 70.1 and 0.95, respectively. Isolates 16 and 18-20 (from sample 18) gave identical numerical profile of 5044572 and the quality of identification as *E. coli* 1 was very good with %id = 99.2 and T value = 0.91. The pathogenicity tests showed that none of these isolates belonged to the 43 pathogenic strains described in the test kit. Still, sample 8 will be regarded out of Saudi specification which requires that the load of a maximum of 2 out of 5 replicates of a sample of date fruits should not exceed 10 cfu/g *E. coli* (SASO, 1999).

Samples 4, 7, and 9 were found contaminated with *S. aureus*. Contamination was relatively high in sample 4 where 3 of the 5 replicates contained loads up to 2.2×10^2 cfu/g. Two of the replicates of sample 7 were contaminated with *S. aureus*, while only one replicate of sample 9 was contaminated with the bacterium. The potential aflatoxin producer *A. flavus/parasiticus* was detected in only one replicate of samples 3 and 10. These results indicate that pathogenic microorganisms don't grow or survive for long times in dates stored at refrigeration temperature.

Table 5. Contamination of date fruit samples with potential pathogenic microorganisms. Five replicates were tested for each sample, and the results shown are loads of individual replicates. Samples 1-10 analyzed immediately after processing and samples 18 and 20 analyzed after 2 months storage at 4-7°C.

Sample No.	Coliforms (MPN/g)	Fecal coliforms (MPN/g)	<i>E. coli</i> (MPN/g)	<i>Staph. aureus</i> (cfu/g)	<i>A. fl./para.</i> (cfu/g)
1	43/93*	9/21	21	n.d.	n.d.
3	n.d.	n.d.	n.d.	n.d.	65
4	n.d.	n.d.	n.d.	97/130/220	n.d.
5	n.d.	n.d.	n.d.	n.d.	n.d.
6	21/43	15	15	n.d.	n.d.
7	n.d.	n.d.	n.d.	135/165	n.d.
8	43/93/150	75/75	39/43	n.d.	n.d.
9	n.d.	n.d.	n.d.	79	n.d.
10	n.d.	n.d.	n.d.	n.d.	73
18	15	15	15	n.d.	n.d.
20	21	n.d.	n.d.	n.d.	n.d.

*Each digit represents the microbial load of one replicate of a sample, results of replicates containing no detectable loads are not shown. n.d. = not detected

Table 6. Profiles of aerobic assimilation of different carbon sources and ability to grow at 50% and 60% glucose concentration for yeasts isolated from date fruit samples.

Carbon source	Isolates identified as			
	<i>Z. mellis</i>	<i>D. hansenii</i>	<i>C. lipolytica</i>	<i>T. delbrueckii</i>
D-galactose	-	+	±	±
D-glucosamine	-	±	-	-
D-xylose	-	+	-	-
Sucrose	±	+	-	±
Maltose	-	+	-	±
A, α-Trehalose	-	+	-	+
Me α-D-glucoside	-	-	-	-
Cellobiose	-	±	-	-

Salicin	-	-	-	-
Arbutin	-	-	-	-
Raffinose	-	+	-	-
Melezitose	-	±	-	-
Glycerol	+	+	+	-
Ribitol	-	+	-	-
Xylitol	±	+	-	-
D-glucitol	+	+	+	+
D-mannitol	+	+	+	-
D-glucono-1,5-lactone	-	-	+	-
2-keto-D-gluconate	-	+	-	-
D-gluconate	+	±	±	+
DL-lactate	-	-	-	-
Succinate	-	+	+	-
Citrate	-	±	+	-
Ethanol	-	-	+	+
Propane 1,2 diol	-	-	-	-
Butane 2,3 diol	-	-	-	-
D-galactonate	-	-	-	-
50% D-Glucose	+	+	+	+
60% D-Glucose	+	+	-	+

Tests conducted according to Barnett *et al.*5; -, no growth; +, growth; ±, some isolates gave growth others didn't.

Table 7. Profiles of the api 20 E biochemical tests for *E. coli* strains isolated from date fruit samples. Isolates 1-5, 10, 11, 13, and 16-20 (numerical profiles 5144552, 5044572 and 1044552) identified as *E. coli* 1 and isolates 6-9, 12, 14 and 15 (numerical profiles 0044112 and 4044102) as *E. coli* 2.

Test name	Isolates																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
B-galactosidase	+	+	+	+	+	-	-	-	-	+	+	-	+	-	-	+	+	+	+	+
Arginine dehydrogenase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lysine decarboxylase	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	+	-	+	+	+
Ornithine decarboxylase	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Citrate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H ₂ S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tryptophan deaminase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Indole	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Voges Proskauer	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sorbitol	+	+	+	+	+	-	-	-	-	+	+	-	+	-	-	+	+	+	+	+
Rhamnose	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
Sucrose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+
Melibiose	+	+	+	+	+	-	-	-	-	+	+	-	+	-	-	+	+	+	+	+
Amygdaline	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Conclusion

All date fruit samples tested immediately after processing were found contaminated with mesophilic aerobic bacteria and molds. Some samples were also found contaminated with yeasts, coliforms, and some potential pathogens like *E. coli*, *Staph. aureus*, and *A. flavus/parasiticus*. The

amount of contamination decreased with time in samples stored at refrigeration temperature, indicating that most contaminants were not able to survive the conditions of low temperature, low water activity and high sugar concentration prevailing in the fruit packages. The contaminants may also have been affected by antimicrobial

compounds such as tannins which are probably present in the date fruits. The yeasts *Zygosaccharomyces mellis*, *Candida lipolytica*, *Debaryomyces hansenii* and *Torulaspora delbrueckii* were detected in some samples stored for 2-6 months, indicating that they were able to survive and/or grow in the samples. In general, it can be said that when date fruits are packaged and stored at refrigeration temperature, they will be safe of most spoilage and pathogenic microorganisms.

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References

- Abdulsalam Khaled S., Ahmed A. Musa and Ahmed Abdulmohsin. 1991. The effect of three fungi and their combinations on the chemical constituents of two cultivars of date palm fruits. *Emir. J. Agric. Sci.* 3:81-95
- Abu-Zinada, A. H., and M. I. Ali. 1982. Fungi associated with dates in Saudi Arabia. *J. Food Prot.* 45:842-844.
- Aidoo, K. E., R. F. Tester, J. E. Morrison and D. MacFarlane. 1996. The composition and microbial quality of pre-packed dates purchased in Greater Glasgow. *Int. J. Food Sci. Technol.* 31:433-438.
- Al Eid, S. M. 2010. Date Palm Research Center, King Faisal University, Saudi Arabia. Personal communication, unpublished data.
- Al-Hooti, S., J. S. Sidhu and H. Qabazard. 1997. Physicochemical characteristics of five date fruit cultivars grown in the United Arab Emirates. *Plant Foods Hum. Nutr.* 50:101-113.
- Barnett, J. A., R. W. Payne and D. Yarrow. 2000. *Yeasts: characteristics and identification*, 3rd ed. Cambridge University Press, Cambridge.
- Barreveld, W. H. 1993. Date palm production. *FAO Agricultural Services bulletin* 101. Food and Agriculture Organization, Rome.
- Bolin, H. R., A. D. King, W. L. Stanely and L. Jurd. 1972. Antimicrobial protection of moisturized Deglet Noor dates. *Appl. Microbiol.* 4:799-802.
- Deák, Tibor. 2008. *Handbook of food spoilage yeasts*, 2nd edition, CRC Press. Boca Raton, London, New York.
- El-Sherbeeny, M. R., M. F. Saddik and F. L. Bryan. 1985. Microbial profiles of foods served by street vendors in Egypt. *Int. J. Food Microbiol.* 2:355-364.
- FAO (UN Food and Agriculture Organization) 2008. "Date Palm." <http://faostate.fao.org/site/340/default>.
- Hamad, S. H. 2008. Microbial spoilage of date Rutab collected from the markets of Al-Hofuf City in the Kingdom of Saudi Arabia. *Journal of Food Protection.* 71(7):1406-1411.
- Ishida, K., J. C. P. de Mello, D. A. G. Cortez, B. P. D. Filho, T. Ueda-Nakamura and C. V. Nakamura. 2006. Influence of tannins from *Stryphnodendron adstringens* on growth and virulence factors of *Candida albicans*. *J. Antimicrob. Chemother.* 58:942-949.
- Kader, A. A. 2007. Recommendations for maintaining postharvest quality. Department of Plant Science, University of California, Davis. Available at: <http://postharvest.ucdavis.edu/ProduceFacts/Fruit/Dates.shtml>.
- Larone, D. H. 1995. *Medically important fungi, a guide to identification*, 3rd ed. ASM Press, Washington, D.C.
- Myhara, M. R., A. Al-Alawi, J. Karkalas, and M. S. Taylor. 2000. Sensory and textural changes in maturing Omani dates. *J. Sci. Food Agric.* 80:2181-2185.
- Nelson, K. E., A. N. Pell, P. H. Doane, B. I. Giner-Chavez, and P. Schofield. 1997. Chemical and biological assays to evaluate bacterial inhibition by tannins. *J. Chem. Ecol.* 23:1175-1194.
- Nussinovitch, A., B. Rosen, H. Salik, and I. J. Kopelman. 1989. Effect of heating media on the microbiology and shelf life of heat pasteurized soft dates. *Lebensm. Wiss. Technol.* 22:245-247.
- Saudi Standards, Metrology and Quality Organization (SASO). 1999. Microbiological limits for food materials, No. 1556, Kingdom of Saudi Arabia.

NUTRITION AND FOOD SCIENCE

Evaluation of nutrients, trace metals and antioxidant activity in *Volvariella volvacea* (Bull. Ex. Fr.) Sing

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Abstract

Volvariella volvacea (Bull. Ex. Fr.) Sing, were collected from mushroom farms at Namakkal district, Tamil Nadu, South India. The different organic and inorganic additives were used in mushroom culture. Methanolic extracts were prepared from these mushrooms and their antioxidant properties, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, reducing power, and nutrient analysis were studied. Biomass productions, the additives Calcium carbonate and Sawdust were recorded better results. Calcium carbonate recorded maximum levels of enzymatic antioxidants that are catalase, superoxide dismutase, glutathione peroxidase, peroxidase, glutathione-s-transferase and Glutathione reductase, DPPH scavenging activities ($31.71 \pm 0.05 - 92.05 \pm 0.06$ mg/ ml), reducing power ($0.29 \pm 0.05 - 0.45 \pm 0.04$ mg/ ml), nutrients and trace metals were also significantly high in Calcium carbonate. Similar trends were recorded in nutrient analysis and mushroom yield potential (1356.98 ± 1.1 g/ bed). The ongoing research will lead to a new generation of nutritional food because *V. volvacea* may have potential as natural antioxidants and the calcium carbonate could be used as a suitable additive to improve the production of antioxidative substances, nutritive value and yield in the mushroom food industry.

Key words: Antioxidant activity, Calcium carbonate, DPPH, Nutrients, Trace metals, *Volvariella volvacea*

Introduction

The genus *Volvariella* (paddy straw mushroom) comprises a group of several species, which can be found growing on a variety of substrates in tropical and subtropical regions. *V. volvacea* (Bull. Ex Fr.) Sing., is probably the best known species, as it has been traditionally cultivated in Southeast Asia since the eighteenth century (Chang, 1977). Currently, *V. volvacea* is the third most important cultivated mushroom reaching total world production of 287 metric tones (Chang and Miles, 1993). In India, Su and Seth (1940) first cultivated straw mushroom; however, scientific cultivation using spawn was successfully demonstrated by Thomas et al. (1943). Mushrooms are considered to be a good source of antioxidants, such as variegatic acid and diboviquinone, which have been found in

mushrooms (Kasuga et al., 1995). Methanol and water extracts from *Volvariella volvacea* and *Lentinus edodes* were found to rich antioxidative activities (Cheung et al., 2003). In recent years, the use of some synthetic antioxidants has been restricted because of their possible toxic and carcinogenic effects (Gazzani et al., 1998). Foods rich in antioxidants have been shown to play an essential role in the prevention of cardiovascular diseases (Dragsted et al., 1993), neurodegenerative diseases, Parkinson's and Alzheimer's diseases (Joseph et al., 1999) inflammation, and problems caused by cell and cutaneous aging (Ames et al., 1993). Organism cells are prepared with many more protection systems against free radical damage, including oxidative enzymes, namely catalase and superoxide dismutase (Niki et al., 1994). Exogenous chemical and endogenous metabolic processes in the human body or in the food system might produce highly reactive free radicals, especially oxygen derived radicals, which are capable of resulting in cell death and tissue damage. However, antioxidant supplements containing foods might be used to help human body cells to reduce oxidative scratch (Halliwell and Gutteridge, 2003). Thus the natural antioxidants

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present in foods and other biological materials have attracted considerable interest because of their presumed safety and potential nutritional and therapeutic effects (Ames et al., 1993). In this study was discussed about, antioxidant properties, DPPH radical scavenging activity, reducing power, and nutrient analysis and trace metals were studied.

Materials and Methods

Isolation

Mushroom samples of *V. volvacea* (paddy straw mushroom) were collected from local mushroom form at Namakkal District, Tamil Nadu and South India. After collected mushrooms were immediately transferred to the laboratory. The sporophores of individual mushrooms were first swabbed with 80% of ethyl alcohol to remove external microbial contaminations. At the junction of pileus and strip, tissue bits were taken separately and using a sterile forceps, the tissue bits were surface disinfected with 10% of sodium hypochloride (commercial) for 5 min. and rinsed with three changes of sterile distilled water. The surface-sterilized tissue bits were placed on Potato Dextrose Agar (PDA) in petri dishes and incubated at laboratory temperature ($30 \pm 2^\circ\text{C}$) for 7 to 10 days. Six isolates were used for the present investigation.

Biomass production

For biomass production, 2% calcium carbonate was added separately in potato-dextrose broth. The 2% calcium carbonate was enhanced the mycelia growth, compared to other concentration (Kalaiselvan, 2007). Amended media (50 ml) were dispensed with additives in 100 ml Erlenmeyer flask and sterilized at 15 lbs pressure for 1 h. After cooling, the flask was inoculated with a disc (9 mm dia) taken from the peripheral growth of 7-day-old PDA culture of *V. volvacea*. For each treatment ten replications were maintained. The broth without amendment served as control. They were incubated at laboratory conditions ($25 \pm 2^\circ\text{C}$) for complete coverage of mycelia. After extracting the supernatant from the individual treatment flask, the mycelial growth retained in weighed Whatman No. 1 filter paper was recorded. The mycelial was taken from individual flasks and oven dried separately, at 80°C for 6 h and the mycelial dry weight (g/ lit) was determined.

Preparation of mushroom bed

The mushroom spawn and bed was prepared according to the method of Sudha et al. (2008) with some modifications. In mushroom bed preparation, organic and inorganic additives were used. Their

six different additives were consisting of six beds; each bed was three replications were maintained. The harvested mushrooms were used for further study.

Nutrients and trace metals

Standard procedures (Jackson, 1958) were used to determine the proximate composition of dried samples of paddy straw mushrooms. Nutrients and trace metals such as Nitrogen (N), Phosphorus (P), Potassium (K), Calcium (Ca), Magnesium (Mg), Copper (Cu), Zinc (Zn), Iron (Fe), and Manganese (Mn) were analyzed.

Samples Preparation and estimation

The different harvested mushroom samples were dried in an oven at 60°C for 30 min. The samples were powdered using pestle and mortar for the estimation of nutrients and trace metals. Oven-dried sample (0.5g) was digested over a hot plate with 50 ml of triple acid mixture (nitric acid: sulphuric acid: perchloric acids in the ratio of 9: 2: 1 v/ v). The silica that settled down was filtered through Whatman No. 42 filter paper ($2.5 \mu\text{m}$). Total nitrogen content of sample was estimated by Kjeldhal method (Peper, 1966). Phosphorus content was analyzed by Vandomolybdate method (Jackson, 1958). Flame photometer (Jackson, 1958) was used for estimation of potassium. The calcium and magnesium was determined by colorimetric method (Versenate method). The content of manganese, zinc, iron, and copper were determined in an Atomic Absorbance Spectrometer (AAS) using appropriate Cathode tubes and wave lengths (Cu = $3247.5 \text{ \AA}$, Zn = $2319 \text{ \AA}$, Fe = $2483.3 \text{ \AA}$, Mn = $2794 \text{ \AA}$).

Enzymatic antioxidant activity

Sample preparation

A fine dried mycelial mat (biomass filtrate paper) and harvested mushroom powder (20 meshes) samples (5g) were continuously extracted with methanol in a Soxhlet apparatus for 24h. The methanolic extract was evaporated to dryness at 40°C and redissolved in methanol at a concentration of 5 mg/ ml, and stored at 4°C prior to further use (Barros et al., 2007).

Catalase (CAT)

The assay mixture contained 0.5 ml of H_2O_2 , 1.0 ml of 0.01 M Phosphate buffer (pH 7.0) and 0.4 ml water. To initiate the reaction, 0.2 ml of the enzyme was added. Dichromate/acetic acid reagent (2 ml) was added after 0, 30, 60, and 90 seconds of incubation. To the control tube, the enzyme was added after the addition of acid reagent. The tubes were then heated for 10 min and the colour developed

was read at 610 nm (Sinha 1972). The activity of catalase is expressed as μM of H_2O_2 decomposed /min/mg protein.

Superoxide dismutase (SOD)

An aliquot of 1.4 ml reaction mixture was pipetted out in a test tube. Enzyme extract (100 μl) was added followed by a pre-incubation at 37°C for 5 min. Riboflavin (80 μl) was added and the tubes were exposed for 10 min to 200 W Philips fluorescent lamps. The control tube contained equal amount of buffer instead of sample. The sample and its respective control were run together. At the end of the exposure time, 1.0 ml of Greiss reagent was added to each tube and the absorbance of the colour formed was measured at 543 nm (Chao, 2001). The enzyme activity is expressed as inhibition of 50 per cent nitrite formation/min/mg protein.

Glutathione peroxidase (GPX)

An aliquot of 0.4 ml of 0.4 M sodium phosphate buffer (PH 7.0) was pipetted out along with 0.1 ml of sodium azide, 0.2 ml of reduced glutathione, 0.1 ml of H_2O_2 , 0.2 ml of enzyme extract and 1.0 ml of water to a final incubation volume of 2.0 ml. The tubes were incubated for 0, 30, 60, and 90 seconds. The reaction was then terminated by the addition of 0.5 ml trichloroacetic acid. To determine the glutathione content, 2.0 ml of the supernatant removed by centrifugation was added to 3.0 ml disodium hydrogen phosphate solution and 1.0 ml of DTNB (2, 2, Dithiobis 5 nitro pyridine) reagent. The colour developed was read at 412 nm. Standards in the range of 200–1000 μg were taken and treated in the similar manner (Rotruck et al., 1973). The activity was expressed in terms of μg of glutathione utilized/min/mg protein.

Peroxidase (POX)

A reaction mixture was prepared in a test tube by adding 3.0 ml of 0.05 M pyrogallol solution and 0.1 ml of enzyme extract. The spectrometer was adjusted from 0–430 nm. Then 0.5 ml of 1 per cent H_2O_2 was added in the test cuvette mixed and the change was recorded in absorbance for every 30 sec up to 3 min. The difference in OD change per minute with and without enzyme addition was a measure of peroxidase activity (Sadasivam and Manickam, 1992). The activity is expressed in terms of μ moles of pyrogallol oxidized/min/mg protein.

Glutathione-S-transferase (GST)

A reaction mixture was prepared in a test tube by adding 1.0 ml of 0.5 M Phosphate buffer, 0.1 ml of enzyme extract, 1.7 ml of water and 0.1 ml of CDNB (1-chloro 2,4 dinitrobenzene) was incubated

at 37°C for 5 min. After incubation, 0.1 ml of reduced glutathione was added. The increase in optical density of the enzyme was measured against the blank at 340 nm (Lin, 1999). The enzyme activity was calculated in terms of μM of CDNB conjugate formed/min/mg protein.

Glutathione reductase (GTR)

A mixture consisting 0.2 ml of enzyme extract, 1.5 ml of 0.3 M Phosphate buffer, and 0.5 ml of EDTA (Ethelene diamine tetra acidic acid) 0.2 ml of GSSG (Oxidised glutathione) and 0.1 ml NADPH (Nicotinamide adedine nucleotide disphosphate) were prepared in a test tube. The decrease in optical density of the enzyme was measured against the blank at 340 nm (Lin, 1999). The enzyme activity was calculated in terms of μM of glutathione utilized/min/mg protein.

DPPH radical scavenging activity

The scavenging activity of the free and bound extracts on 1, 1-diphenyl 2-picrylhydrazyl (DPPH) radical was measured according to the method of Cheung et al. (2003) with some modifications. Aliquots (various concentration of methanol extract) of 0.8 ml of 0.2 mM DPPH methanolic solution was mixed with 0.2 ml of the extracts. The mixture was vigorously shaken and left to stand for 10 min. under subdued light. The absorbance was measured at 520 nm. The DPPH radical scavenging activity (%) was calculated by the following equation:

$$\text{Radical scavenging activity (\%)} = (1 - A_{\text{sample}} / A_{\text{control}}) \times 100$$

Where A_{sample} is the absorbance in the presence of sample and A_{control} is the absorbance in the absence of sample, respectively. All extracts were analyzed in triplicate.

Reducing power

The reducing power was determined according to the method of Oyaizu (1986) with some modifications. Various concentrations of methanolic extracts from mushrooms (2.5 ml) were mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6, Wako Pure Chemical Co., Osaka, Japan) and 2.5 ml of 1% potassium ferricyanide (Sigma), and the mixture was incubated at 50°C for 20 min. After 2.5 ml of 10% trichloroacetic acid (w/v, Wako) were added, the mixture was centrifuged at 650 rpm for 10 min. The upper layer (5 ml) was mixed with 5 ml of deionised water and 1 ml of 0.1% ferric chloride (Wako), and the absorbance was measured at 700 nm in a 2020 Double Beem Spectrophotometer. A higher absorbance indicates a higher reducing power.

Statistical Analysis

Statistical analysis was performed on the data by Duncan's Multiple Range Test (DMRT) with means followed by a common letter are not significantly different at the 5% level by DMRT. For graphical displays, Box plots and whisker plots were used for treating the results by ORIGIN PRO 6.0.

Results and Discussion

The successful commercial cultivation of paddy straw mushroom depends to a large extent on quality and purity of spawn (Kalaiselvan, 2007). In this *in vitro* study, it was seen that calcium carbonate significantly enhanced the biomass production up to 2.95 ± 0.05 g/l for the additives Calcium carbonate followed by Sawdust (Figure 1). The beneficial effect of calcium carbonate on fungal growth might be due to the creation of neutral pH in the medium that enhanced the fungal growth, chlamydospore formation and density, and biomass production (Kalaiselvan, 2007). Sangeetha (2004) reported that the media amended with gram powders enhanced the fungal growth and chlamydospores density of *V. volvacea* due to increase in protein content of the substrate as stated by Kattam et al. (1991). The great potentiality of *V. volvacea* by virtue of its fast mycelial growth and greater biomass production due to calcium carbonate was evident from this study.

Yield evaluations for selecting a better performing strain have also been performed earlier by several researchers (Ahlawat et al., 2008). However, some of them have correlated morphological and biochemical characteristics of a strain with yield potential. They considered these parameters for the selection of promising strains for commercial cultivation. This study showed that isolate Calcium carbonate recorded highest yield of 1356.98 ± 1.1 g/ bed and was followed by Sawdust (1305 ± 0.9 g/ bed). The other additives were recorded fewer yields compared to Calcium carbonate and Sawdust (Figure 2). A variation in nutrients and trace metal levels of 26 strains of *V. volvacea* was also reported by Ahlawat et al. (2008). In this study, fabulous yielding isolates Calcium carbonate

and Sawdust recorded high levels of macro and micronutrients, this results; N: 1.47 ± 0.22 ; $1.45 \pm 0.37\%$, P: 1.22 ± 0.14 ; $0.92 \pm 0.06\%$, K: 879.77 ± 9.50 ; 841.41 ± 9.2 ppm/g, Ca: 11.45 ± 0.96 ; 10.73 ± 1.09 ppm/g; Mg: 2.13 ± 0.03 ; 1.95 ± 0.05 ppm/g, Cu: 0.33 ± 0.06 – 0.29 ± 0.02 ppm/g, Zn 1.38 ± 0.98 – 1.12 ± 0.02 ppm/g, Fe: 0.41 ± 0.1 – 0.35 ± 0.05 ppm/g, Mn: 7.86 ± 0.04 ; 3.45 ± 0.05 ppm/g (Table 1).

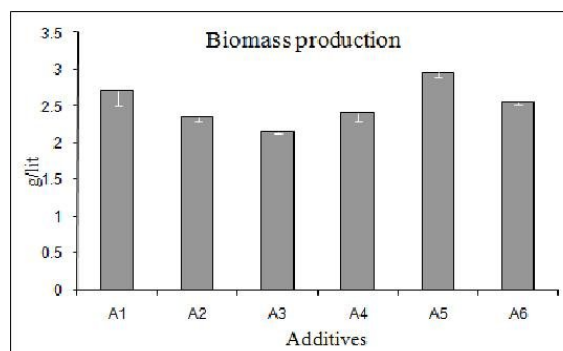


Figure 1. Biomass production of *Volvariella volvacea*.

A1- Sawdust, A2- Gypsum, A3- Calcium carbonate + gypsum, A4- Sawdust + rice bran, A5- Calcium carbonate, A6- Rice bran.

Each value is expressed as mean $\pm$ standard deviation.

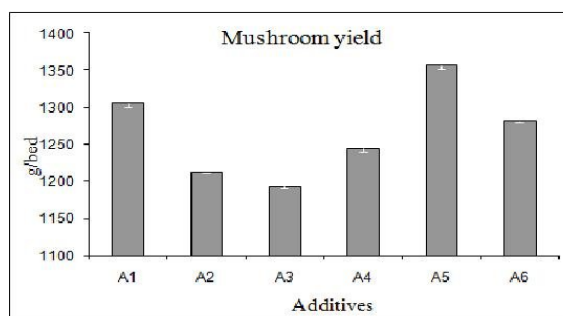


Figure 2. Yield performances in *Volvariella volvacea*.

A1- Sawdust, A2- Gypsum, A3- Calcium carbonate + gypsum, A4- Sawdust + rice bran, A5- Calcium carbonate, A6- Rice bran.

Each value is expressed as mean $\pm$ standard deviation

Table 1. Nutrient and trace metal levels in *Volvariella volvacea*.

Additive	N	P	K	Ca	Mg	Cu	Zn	Fe	Mn
A1	1.45 ± 0.37^a	0.92 ± 0.06^b	841.41 ± 9.2^b	10.73 ± 1.09^b	1.95 ± 0.05^b	0.29 ± 0.02^b	1.12 ± 0.02^b	0.35 ± 0.05^b	3.45 ± 0.05^b
A2	0.97 ± 0.17^c	0.72 ± 0.07^c	666.60 ± 4.07^d	7.66 ± 0.90^c	0.79 ± 0.09^d	0.16 ± 0.04^d	0.93 ± 0.21^c	0.27 ± 0.07^c	2.93 ± 0.03^d
A3	0.44 ± 0.13^d	0.53 ± 0.06^d	439.11 ± 2.83^e	6.64 ± 0.94^d	0.43 ± 0.03^e	0.11 ± 0.06^e	0.77 ± 0.22^d	0.19 ± 0.09^d	2.11 ± 0.1^e
A4	0.92 ± 0.09^c	0.72 ± 0.07^c	680.74 ± 9.51^c	7.68 ± 1.00^c	0.89 ± 0.04^c	0.18 ± 0.03^c	0.93 ± 0.05^c	0.31 ± 0.05^b	3.03 ± 0.03^d
A5	1.47 ± 0.22^a	1.22 ± 0.14^a	879.77 ± 9.50^a	11.45 ± 0.96^a	2.13 ± 0.03^a	0.33 ± 0.06^a	1.38 ± 0.98^a	0.41 ± 0.1^a	7.86 ± 0.04^a
A6	1.14 ± 0.20^b	1.01 ± 0.12^b	841.41 ± 5.61^b	10.63 ± 1.16^b	0.94 ± 0.05^c	0.19 ± 0.05^c	0.96 ± 0.04^c	0.33 ± 0.02^b	3.25 ± 0.05^c

A1- Sawdust, A2- Gypsum, A3- Calcium carbonate + gypsum, A4- Sawdust + rice bran, A5- Calcium carbonate, A6- Rice bran. Mean of three replicated. Means followed by a common letter are not significantly different at the 5% level by DMRT. Units: Nitrogen, phosphorus- %, Potassium, calcium, magnesium, copper, zinc, iron, and manganese- ppm/ g.

Food rich in antioxidative substances have been shown to play an essential role in the prevention of cardiovascular disease and cancers (Dragsted et al., 1993), neurogenerative diseases and inflammation (Joseph et al., 1999). The use of synthetic antioxidants has been restricted because of their possible toxic and carcinogenic effects (Gazzani et al., 1998). The present study clearly showed that the isolate Calcium carbonate (mycelial mat; Dried mushroom) recorded highest levels of antioxidants; catalase (90.15 ± 0.15 ; 37.37 ± 0.06 μmol), superoxide dismutase (22.69 ± 0.09 ; 29.21 ± 0.04 μmol), glutathione peroxidase (17.88 ± 0.03 ; 48.88 ± 0.07 μmol), peroxidase (5.11 ± 0.03 ; 6.35 ± 0.05 μmol),

glutathione-S-transferase (30.52 ± 0.06 ; 12.93 ± 0.07 μmol), and glutathione reductase (8.15 ± 0.05 ; 27.44 ± 0.03 μmol), in mycelial mats and dried mushroom (Table 2, 3). This was followed by Sawdust and Sawdust + rice bran. Recently, Kalaiselvan (2007) reported high levels of antioxidative substances due to calcium carbonate activity in *P. euos* and *V. volvacea*. Murcia et al. (2002) reported that all truffles (*Terfezia* and *Piscea* spp.) and five mushrooms (*Leptista nuda*, *Lentinus edodes*, *Agrocybe cylindracea*, *Cantharellus lutescens*, and *Hydrum sepundum*) exhibited higher percentage of oxidative inhibition based on lipid peroxidation, deoxyribose, and peroxidase.

Table 2. Antioxidant activity of the methanol extracts in mushroom mycelial mat.

Additives	CAT	SOD	GPX	POX	GST	GTR
Sawdust	88.21 ± 0.20^b	20.11 ± 0.04^b	16.74 ± 0.06^b	4.23 ± 0.09^b	29.85 ± 0.06^b	8.03 ± 0.06^b
Gypsum	80.65 ± 0.05^e	18.13 ± 0.1^e	14.15 ± 0.06^e	3.48 ± 0.07^e	27.95 ± 0.05^e	7.59 ± 0.05^d
Calcium carbonate + gypsum	79.39 ± 0.09^f	15.19 ± 0.09^f	13.15 ± 0.04^f	3.04 ± 0.06^f	27.05 ± 0.06^f	7.04 ± 0.07^e
Sawdust + rice bran	84.43 ± 0.10^d	18.63 ± 0.03^d	14.39 ± 0.1^d	3.62 ± 0.02^d	28.16 ± 0.05^d	7.76 ± 0.06^e
Calcium carbonate	90.15 ± 0.15^a	22.69 ± 0.09^a	17.88 ± 0.03^a	5.11 ± 0.03^a	30.52 ± 0.06^a	8.15 ± 0.05^a
Rice bran	86.03 ± 0.63^c	18.82 ± 0.08^c	16.63 ± 1.72^c	3.88 ± 0.07^c	28.73 ± 0.04^c	7.84 ± 0.08^c

Inhibition of 50 per cent nitrite formation / min / mg protein, GPX- 1 μmole of glutathione utilized / min / mg protein, POX- 1 μmole of pyrogallol oxidized / min / mg protein, GST - 1 μmole of CDNB conjugate formed / min / mg protein, GTR - 1 μmole of glutathione utilized / min / mg protein.

Table 3. Antioxidant activity of the methanol extracts in dried mushroom.

Isolate	CAT	SOD	GPX	POX	GST	GTR
Sawdust	35.05 ± 0.06^b	28.13 ± 0.06^b	47.29 ± 0.06^b	5.9 ± 0.4^b	12.05 ± 0.05^b	26.48 ± 0.24^b
Gypsum	32.42 ± 0.04^e	25.15 ± 0.05^e	45.28 ± 0.08^d	4.95 ± 0.06^e	10.75 ± 0.06^d	23.69 ± 0.11^e
Calcium carbonate + gypsum	29.05 ± 0.08^f	23.92 ± 0.07^f	41.12 ± 0.03^e	4.09 ± 0.03^f	10.18 ± 0.36^e	22.18 ± 0.05^f
Sawdust + rice bran	32.95 ± 0.05^d	26.01 ± 0.08^d	46.19 ± 0.04^c	5.01 ± 0.04^d	10.71 ± 0.09^d	23.96 ± 0.06^d
Calcium carbonate	37.37 ± 0.06^a	29.21 ± 0.04^a	48.88 ± 0.07^a	6.35 ± 0.05^a	12.93 ± 0.07^a	27.44 ± 0.03^a
Rice bran	33.93 ± 0.61^c	27.16 ± 0.06^c	47.02 ± 0.08^b	5.16 ± 0.03^c	11.09 ± 0.07^c	24.14 ± 0.06^c

Mean of three replications Means followed by a common letter are not significantly different at the 5% level by DMRT. Units: CAT - 1 μmole of H_2O_2 decomposed / min / mg protein, SOD - μmole of Inhibition of 50 per cent nitrite formation / min / mg protein, GPX- 1 μmole of glutathione utilized / min / mg protein, POX- 1 μmole of pyrogallol oxidized / min / mg protein, GST - 1 μmole of CDNB conjugate formed / min / mg protein, GTR - 1 μmole of glutathione utilized / min / mg protein.

Methanol and water crude extracts from shiitake and paddy straw mushroom were investigated for their antioxidant capacity in three different assays, the β -carotene and linoleic acid system, DPPH, radical scavenging activity, and inhibition of haemolysis of rat erythrocyte induced by peroxyl radicals (Cheung et al., 2003). In this study, DPPH scavenging activities (31.71 ± 0.05 – 92.05 ± 0.06 mg/ ml) (Figure 3) and reducing power (0.29 ± 0.05 – 0.45 ± 0.04 mg/ ml) (Figure 4) in dried mushroom were also higher in calcium carbonate isolate; this was followed by the isolate sawdust. Methanol extract of *P. florida* have potent hydroxyl radical scavenging and lipid peroxidation inhibition (antioxidant) activities. The antioxidant activities in

Ganoderma lucidum, *P. florida*, and *P. sajor-caju*, as scavenging activity were demonstrated by Lakshmi et al. (2004). Cheung et al. (2003) reported that the four mushroom extracts from *L. edodes* showed the most potent radical scavenging activity in each assay, showing 75.9% (at 20 mg/ ml) in the β -carotene bleaching method, 55.4% in the DPPH radical scavenging method (at 6 mg/ ml), and 94.0% of inhibition of erythrocyte hemolysis (at 5 mg/ ml). Total phenolics in the water extracts were higher antioxidant activity compared to methanol extracts. The occurrence and distribution of different macro and micro elements in fruiting bodies of certain mushrooms is not only an important problem for physiology and ecology

of fungi, but also has practical environmental and toxicological aspects. As evident in this and several other studies (Maria Rudawska et al., 2005).

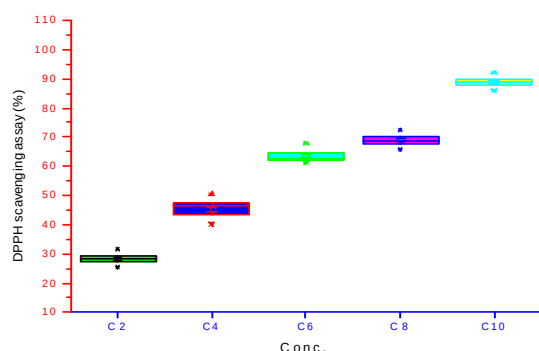


Figure 3. Scavenging effect of methanolic extracts from *Volvariella volvacea* on 1, 1-diphenyl-2-picrylhydrazyl radical.

Concentration: C2- 2mg/ ml, C4- 4mg/ ml, C6- 6 mg/ ml, C8- 8 mg/ ml and C10- 10 mg/ ml
Each value is expressed as mean $\pm$ standard deviation.

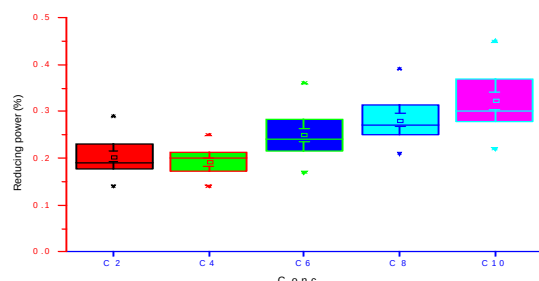


Figure 4. Reducing power of different concentrations of methanol extract of dried mushrooms.

Concentration: C2- 2mg/ ml, C4- 4mg/ ml, C6- 6 mg/ ml, C8- 8 mg/ ml and C10- 10 mg/ ml
Values are expressed as mean $\pm$ standard deviation.

Conclusion

According to the results of this study, it is clearly indicated that the methanolic extract of mushroom species has significant antioxidant activity against various antioxidant systems. From the above assays, the possible mechanisms of the antioxidant activity of the mushroom extracts include scavenging of free radicals also get good responsible. Moreover, the mushroom species can be used as an easily accessible source of natural antioxidants and as a possible food supplement or in pharmaceutical industry.

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References

- Ahlawat, O. P., P. Gupta, K. Shwet and B. L. Dhar. 2008. Development of molecular and biochemical markers for selecting a potential high yielding strain of paddy straw mushroom, (*Volvariella volvacea*). J. Plant Biochem. Biotech. 17(1):57–63.
- Ames, B. N., M. K. Shigena and T. M. Hagen. 1993. Oxidants, antioxidants, and the degenerative diseases of ageing. Proceedings of the National Academy of Sciences, USA, 90:7915–7922.
- Barros, L., M. J. Ferreira, B. Queiros, C. F. R. Isabel Ferreira and P. Baptista. 2007. Total phenols, ascorbic acid, β - carotene and lycopene in Portuguese wild edible mushrooms and their antioxidant activities. Food Chem. 103(2):413–419.
- Chang, S. T. 1977. Biological and commercial aspects of straw mushroom (*Volvariella volvacea*) cultivation. Mushroom Sci. 9:157-165.
- Chang, S. T. and G. P. Miles. 1993. The nutritional attributes and medicinal value of edible mushrooms. In: Edible Mushrooms and Their Cultivation. First Indian Reprint. pp. 27-39.
- Chao, G. R. 2001. Antioxidant properties and polysaccharide composition analysis of ear mushrooms. National Chung-Hsing Univ., Taiwan pp.54-58.
- Cheung, L. M., P. C. K. Cheung, V. E. C. Ooi. 2003. Antioxidant activity and total phenolics of edible mushroom extracts. Food Chem. 81(2):249-255.
- Dragsted, L. O., M. Strube and J. C. Larsen. 1993. Cancer protective factors in fruits and vegetables: biochemical and biological background. Pharma. Toxi. 72:116-135.
- Gazzani, G., A. Pepetti, G. Massolini and M. Daglia. 1998. Anti- and prooxidant activity of water soluble components of some common diet vegetables and the effect of thermal treatment. J. Agri. Food Chem. 46(10):4118–4122.
- Halliwell, B. and J. M. C. Gutteridge. 2003. Free Radicals in Biology and Medicine. Oxford University Press, 3rd edition, Oxford, UK.

- Jackson, M. L. 1958. Soil Chemical Analysis. Prentice hall of India. Pvt. Ltd., New Delhi, India. pp.498.
- Joseph, J. A., B. Shukit- Hale and N. A. Denisova. 1999. Reversal of Age-Related declines in neuronal signal transduction, cognitive and motor behavioral deficits with blue berry, spinach or strawberry dietary supplementation. J. Neurosci. 19(18):8114-8121.
- Kalaiselvan, B. 2007. Studies on modern Techniques for cultivation of paddy straw mushroom (*Volvariella volvacea* (Bull. ex Fr.)) Sing., on commercial scale. M.Sc. (Agriculture) Thesis, Tamilnadu Agricultural University, Coimbatore. pp.89-95.
- Kasuga, A., Y. Aoyagi and T. Sugahara. 1995. Antioxidant activity of fungus *Suillus bovinus* (L:Fr.) O. Kuntze. J. of Food Sci. 60:1113-1115.
- Kattam, M. H., I. A. Helmy, M. H. Mahmmud and K. A. Kawi. 1991. Effect of additives on oyster mushroom production. Mushroom Sci. Tech. 11:67-74.
- Lakshmi, B., J. C. Tilak, S. Adhikari, T. P. A. Devasagayan and K. K. Janardhanan. 2004. Evaluation of antioxidant activity of selected Indian mushrooms. Int. J. Pharma. Bio. 42:179-185.
- Lin, H. C. 1999. Evaluation of taste quality and antioxidant properties of edible and medicinal mushrooms. Master's thesis, National Chung-Hsing University, Taichung, Taiwan. pp.245-250.
- Maria Rudawska. and Tomasz Leski. 2005. Macro- and microelement contents in fruiting bodies of wild mushrooms from the Notecka forest in west-central Poland. Food Chem. 92:499-506.
- Murcia, A. M., M. Martinez- Tome, A. M. Jimenez, A. M. Vera, M. Honrubid and P. Parras. 2002. Antioxidant activity of edible fungi (Truffles and mushrooms): losses during industrial processing. J. Food. Prot. 65:1614-1622.
- Niki, E., H. Shimaski and M. Mino. 1994. Antioxidantism- Free Radical and Biological Defence. Gakkai Syuppan Center, Tokyo. pp.3-16.
- Oyaizu, M. 1986. Studies on products of browning reactions: antioxidative activities of products of browning reaction prepared from glucosamine. Jap. J. Nutr. 44:307-315.
- Peper, C. S. 1966. Soil and plant analysis. Hanos publishers, Bombay. pp.368.
- Rotruck, J. T., A. L. Pope, H. E. Ganther and A. B. Swason. 1973. Selenium: Biochemical role as a component of glutathione peroxidase. Science 179:588-590.
- Sadasivam, P. and S. Manickam. 1992. Biochemical methods. Second Edition. pp.187.
- Sangeetha, G. 2004. Exploring the possibilities of increasing the yield potential of paddy straw mushroom, *Volvariella volvacea* (Bull. ex Fr.) Sing. M.Sc. (Agriculture) Thesis, Tamilnadu Agricultural University, Coimbatore, India. pp.23-38.
- Sinha, A. K. 1972. Colorimetric assay of catalase. Anal. Biochem. 47:389-94.
- Su, U. T. and L. N. Seth. 1940. Cultivation of Straw Mushroom. Indian Farm. 1:332-333.
- Sudha, A., P. Lakshmanan and B Kalaiselvan. 2008. Antioxidant properties of paddy straw mushroom (*Volvariella volvacea* (Bull. ex Fr.)) Sing. Int. J. Appl. Agri. Res. 3(1):9-16.
- Thomas, K. M., T. S. Ramkrishna and I. L. Narasimhan. 1943. Paddy straw mushroom. Madras Agric. J. 31:57-59.

PLANT SCIENCE

Effect of some medicinal plants extracts and cypermethrin against Khapra Beetle (*Trogoderma granarium* Everts)

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Abstract

Effects of ethanol extracts of *Datura stramonium*, *Solanum nigrum*, *Quercus infectoria* and *Xanthium strumarium* on the mortality, egg hatching, reduction in F1 progeny and repellents on the red flour beetle were investigated. The results showed that cypermethrin had the best effect on the mortality of adults (100%) on all concentrations, whereas the fruits of *Datura stramonium* and *Xanthium strumarium* had a mortality rate of 100% in 2% and 4% concentrations. Egg hatching was also delayed, and began after four days of treatment, in *Quercus infectoria* and *Xanthium strumarium*. Ethanol plant extracts of *Datura stramonium* caused 100% mortality of larvae at a concentration of 1% after eight days of treatment, followed by the alcohol extract of *Solanum nigrum* with 97.43% mortality in 1% concentration. Mix of ethanol plant extract with food reduced F1 progeny. Cypermethrin and *Datura stramonium* were given the best effect that reaches 17.06% and 18.11% respectively. Repellents percentage showed that ethanol extracts of *Datura stramonium* and *Solanum nigrum* showed repellents of 91.87% and 91.45% in 4% concentration after 24 hours of treatment.

Key words: *Trogoderma granarium*, Plant extracts, Repellent, Toxicity

Introduction

The Khapra beetle, *Trogoderma granarium* (Coleoptera, Dermestidae) is considered to be one of the most serious pests of stored grain products, various leguminous crops, rice, oat, barley, and rye throughout the world (Lowe et al., 2000). It is originally occurred in India, and spread to Africa, Europe, South America and East Asia (Harris, 2009). The Khapra beetle occurs in very low numbers and can survive for a long period as an inactive state (Dwivedi and Shekhawat, 2004).

According to FAO estimate, 10 to 25% of the world harvested food is destroyed annually due to insects and rodent pests (Anonymous, 1980). Losses caused by *Trogoderma granarium* have been reported to range from 0.2 to 2.9% over a period of 1 to 10.5 months (Irshad et al., 1988).

Chemical insecticides such as malathion, cypermethrin, bifenthrin are used for rapid control, but are expensive, not readily available and may be

poisonous to humans and environment (Tsumura et al., 1994). Moreover, malathion and cypermethrin have gone ineffective due to development of resistance in insect pests of stored grain, particularly in *Trogoderma granarium* (Saxena and Sinha, 1995). The larval period of development of Khapra beetle had been prolonged after treatment with DDT (Shantaram, 1958).

Local alternatives such as the natural products are cheaper, easily available way for controlling pests, which are safe for humans and environment. Most pesticide plants also have medicinal values, and some are consumed by humans as spices (Okonkwo and Okoye, 1996). There is a growing interest in entomological research to identify and evaluate plant species with insecticide properties for control of various insect pests, including *S. zeamais* (Hassan et al., 1990; Owusu, 2001; Akob and Ewete, 2007; Odeyemi et al., 2008).

Plant insecticides are often alternatives effective as organophosphates or other neurotoxins for pest control due to multiple modes of action. It included toxicity, anti-feedant and anti-oviposition effects (Sutherland et al., 2002). Natural products contain secondary plant compounds such as terpenes (monoterpenes, sesquiterpenes and triterpenes), steroid, alkaloids, phenol and cardiac glycosides (Duke, 1990; Baser et al., 1998).

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Identifications of plant extracts exhibited the above described deleterious effects on pest insect physiology, behavior and represents a potential alternative strategy for development of directional controls that could replace synthetic neurotoxins (Duke, 1990).

Khalif and Al-Farhani (2008) studied the effects of leave's powders of *Nicotiana tabacum*, *Nerium oleander*, *Ziziphus spinachristi*, *Vitex agnus-castus*, *Lantana camara*, *Myrtus communis*, *Clerodendron inerum* and *Eucalyptus globules* on the mortality reduction in F1 progeny and repellents. Leaves powder on the red flour beetle showed that the leaves powders of *N. tabacum*, *C. inerum* and *Z. Christi* had the best effect on the mortality of adults after seven days (100%).

Nadi (2001) studied the toxicity of aqueous, methanol and acetone extracts of three plants *Rhazya stricta*, *Azadirachta indica*, and *Heliotropium bacciferum* on Khapra beetle (*Trogoderma granarium* Everts) larvae.

A number of plant species like *Azadirachta indica* A. Juss, *Melia azedarach* L., *Lantana camara* L., *Cannabis sativa* L., *Nerium indicum* Mill., *Eucalyptus* sp., *Ricinus communis* L. as well as *Solanum nigrum* L. are known to possess insecticidal properties, although only a few of these have been exploited commercially. The compounds from these plants have a number of useful activities like toxicity, repellence, feeding and oviposition deterrence and insect growth regulate activity (Mordue, 2004).

Recently some indigenous plants have been reported to possess repellent property against Khapra beetle. Dwivedi and Bajaj (2000) assessed of the *Cassia* leaf extract on the repellent activity on Khapra beetle, while Dwivedi and Sharma (2002) investigated repellents of five plant extracts against Khapra beetle.

Present trends in the world directed to the use plants that have insecticides properties. Some plant extracts are highly effective and safe for human beings and environment, convenience and

inexpensive for protection of stored grains. The toxicity of plant extracts had been checked against a number of stored product insects (Hasan et al., 2006). We need in this time, to research programmers to determine the response of *Trogoderma granarium* to different doses of plant and synthetic insecticide.

Materials and Methods

Rearing of test insect

Mixed population of *Trogoderma granarium* (Everts) was collected from infected stored wheat for rearing in the laboratory. The insects were collected and kept in wide jars covered with muslin cloth. The insects were kept in the laboratory for two months for rearing. The adults of *T. granarium* were sieved and placed in breeding containers in the medium of uninfected wheat grains. The samples were placed in an incubator at $30 \pm 2^\circ\text{C}$ and $65 \pm 5\%$ relative humidity for three days (72 hours). Then, the insects were transferred from the jars to new containers.

The media (wheat grains) in which the adults of *T. granarium* were initially retained for 72 hours contained enough eggs laid by females. In these jars, the first larvae appeared after four days and the highest number of larvae appeared after seven days. Same age and size progeny were removed from these stocks. It was further kept for another period of five days before testing (Hasan et al., 2006).

Collection of plant materials

Samples were collected from plants included fruit of *Datura stramonium* L. and *Quercus infectoria* DL. As well as leaves of *Solanum nigrum* L. and *Xanthium strumarium* L. from different places Sulaimanya, Arbile and Basrah in 2008-2009 (Table 1). All the identified plant materials were dried by using air for 6-7 days, while others were dried by using the oven at a temperature of 30°C for a period of 24 hours. The dried materials were macerated and powdered in blender machine type (Moulinex, 241, France).

Table 1. Plants and insecticide used against *Trogoderma granarium*.

Family name	Plants	Tissue used
Solanaceae	<i>Datura stramonium</i> L.	Fruit
Fagaceae	<i>Quercus infectoria</i> DL.	Leaves
Solanaceae	<i>Solanum nigrum</i> L.	Leaves
Compositae	<i>Xanthium strumarium</i> L.	Fruit
Cypermethrin	-	-

Preparation of ethanol plant extracts and cypermethrin solutions

Extracts of leaves and fruits were prepared by mixing 50g from sample in 500 mL of ethanol in a flask. The flask was closed with a piece of cotton and aluminum foils and placed in rotary shaker at 30°C for 24 hours or soxhlet for extraction. The extract was filtered to obtain the stock solution. From the stock solution, 1, 0.5, 2 and 4% concentrations were prepared.

10% master stock solution of cypermethrin 25% EC was prepared by dissolving technical grade of cypermethrin in primary non-volatile solvents (olive oil). Further concentrations such as 0.5, 1, 2 and 4% were prepared from 10% stock solution for non-volatile solvents.

Seventy two Petri dishes were taken, and filter papers were cut according to the size of Petri dishes for both test solutions. Plant extract and cypermethrin were spread uniformly on the filter papers (Whatman No. 1.7 cm diameter). Then, 0.5 millimetres of the extract and cypermethrin were placed on the filter paper by the syringe. Insect mortality was recorded after seven days of treatment. Three replicates were calculated for each concentration.

Abbott's formula (Abbott, 1925) was used to correct the mortality. Corrected mortality (PT) was calculated using the following formula:

$$PT = [(Po - Pc) / (100 - Pc)] \times 100$$

Where Po = observed mortality and Pc = controlled mortality.

Toxicity on F1 progeny

The effects of treated wheat grains on the F1 progeny of *Trogoderma granarium* were studied using cypermethrin and ethanol plant extracts of the four plant species diluted with ethanol (95%) to five different concentrations, 0 (control), 0.5, 1, 2, and 4%. Twenty-five grams of wheat grains were placed in five jars and treated with 0.5 mL to each of the five different concentrations of plant extracts and cypermethrin. The jars were well shaken manually for optimum coverage of grain surface. Five pairs (five male and five female) of Khapra beetle were put together into each jar, and then covered with mesh. Replicates of the treatments (four plant species extract as well as cypermethrin and four concentrations) and then the samples were analyzed in a randomized complete block (RCB) design.

After seven days all adult of Khapra beetles were removed, and the jars with grains were left undisturbed for 30 days. This was observed daily and F1 progeny emergence were recorded.

Repellent effect

The repellent effect of the cypermethrin and four ethanol plant extracts against *Trogoderma granarium* were evaluated using the method as described by McDonald et al. (1970). The treated and untreated grains were placed adjacent to each other in the Petri dish (11 cm diameter). Ten adult *T. granarium* of three – five days old were introduced into the middle of the Petri dishes. Each treatment was replicated four times and the number of insects present in control (Nc) and treated (Nt) grains were recorded after one hour and up to six hours. A percent repellent (PR) was computed as:

$$PR = [(Nc - Nt) / (Nc + Nt)] \times 100$$

And the data were analyzed using the ANOVA test after converted into arcsine values. All negative PR values were treated as zero (Obeng-Ofori et al., 1997).

The five concentrations of the extracts and the five replications were arranged in a completely randomized design (CRD). The numbers of insects present on the control (untreated) and treated were recorded after 30 minute exposure.

Statistical analysis

Statistical analysis of variance was carried out with the Statistical Package for Social Science (SPSS), using a factorial completely randomized design (CRD). Treatment means showing significant difference ($P < 0.01$) were separated by using Least Significant Difference.

Results

Toxicity of cypermethrin and ethanol plant extracts on adult insects

Ethanol extracts of fruits of *Datura stramonium* and *Xanthium strumarium*; as well as leaves of *Quercus infectoria*, *Solanum nigrum* and cypermethrin, using various concentrations had significant ($P < 0.01$) contact toxicity on *Trogoderma granarium*.

The data in a table (2) showed mean percent mortality of *T. granarium* after eight days under the various treatments of the ethanol plant extract and cypermethrin. Cypermethrin gave maximum mortality 100% in all concentrations. In the concentration of 2% and 4% of *Datura stramonium*, *Solanum nigrum* extracts gave maximum mortality 100%, while 0.5% concentration yielded the minimum mortality.

Table 2. Toxicity of cypermethrin and ethanol plant extracts on *Trogoderma granarium* after eight day of treatment.

Plant extracts	Concentration %				Mean extract plant
	0.5%	1%	2%	4%	
<i>Datura stramonium</i>	11.34	65.34	100	100	69.17
<i>Quercus injector</i>	10.43	33.64	46.43	46.43	34.23
<i>Solanum nigrum</i>	13.23	52.08	100	100	66.32
<i>Xanthium strumarium</i>	15.67	55.78	80.91	85.87	59.55
cypermethrin	100	100	100	100	100
Control	0	0	0	0	
Mean concentration	12.13	61.36	85.46	86.46	
L.S.D. (0.01) of plant extract	9.3				
L.S.D. (0.01) Interaction between plant extract and concentration	15.3				

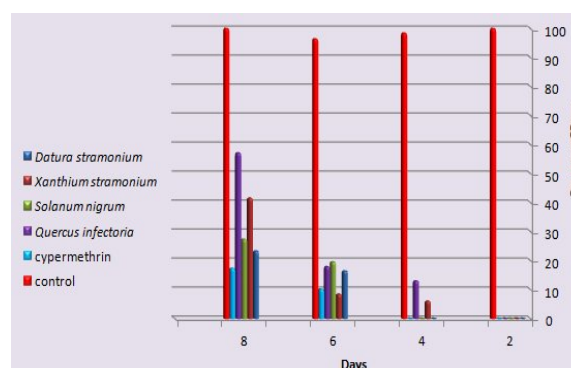
Toxicity on egg hatching

Ethanol extracts of all the selected plants, delayed the hatching of eggs of *Trogoderma granarium* (Figure 1). The effect of different concentration levels and time exposure on hatching of eggs varied with material and exposure time, we also noticed that the insects did not lay the eggs in 2% and 4% concentration, and that the female insect did not lay the eggs until the fourth day after the treatment in 1% concentration. In addition, hatching started just after four days in *Quercus infectoria* and *Xanthium strumarium*, as for the other treatments, no hatching was observed until six days. Inhibition in egg hatching was significantly higher in treatments than control. The percentage of hatching eggs were 17.34% and 23.31% in cypermethrin and *Datura stramonium* respectively, while the percentages of hatching in the control was 100%.

Toxicity on larvae

The results showed that cypermethrin larval mortality reached 100% after eight days of exposure in all concentrations (Table 3). Ethanol

plant extracts of *Datura stramonium* showed 100% larval mortality in 1%, 2% and 4% concentration, while *Quercus infectoria* and *Xanthium strumarium* extracts showed 76.54% and 92.43% mortality of larvae at a concentration in 4% after eight days of exposure.



L.S.D. of plant extracts = 0.9.

L.S.D. of time = 4.5

Figure 1. Toxicity of cypermethrin and plant extracts on *Trogoderma granarium* in 1% concentration.

Table 3. Toxicity four plants extract and cypermethrin on larvae *Trogoderma granarium* after eight-day treatment.

Plant extracted	Concentration %				Mean extract plant
	0.5	1	2	4	
<i>Datura stramonium</i>	80.43	100	100	100	95.10
<i>Quercus infectoria</i>	30.76	43.86	66.31	76.54	54.36
<i>Solanum nigrum</i>	81.45	97.43	100	100	94.72
<i>Xanthium strumarium</i>	15.32	60.23	85.24	92.43	63.30
cypermethrin	100	100	100	100	100
Mean concentration	61.59	80.30	90.31	93.79	
L.S.D. of plant extract	1.2				
L.S.D. of concentration	15.08				
L.S.D. interaction between plant extract and concentration	23.11				

Toxicity on F1 progeny production

Pesticide and plant extracts applied to wheat grains using various concentrations were significant ($P < 0.01$) and the toxic influence against F1 progeny of *Trogoderma granarium* infesting wheat was shown in (Table 4). Adult emergence from the control was significantly higher than the rest of the other treatments at all concentrations irrespective of

plant extracts. However, the lower emergence was obtained from grains treated with *Datura stramonium*, *Solanum nigrum* and cypermethrin by using 4% concentration, after eight days of treatment was 8.34, 9.34 and 5.43 respectively. The data analysis showed higher significant differences in 4% concentration and gave 93.79% mortality.

Table 4. Effect plant extracts and cypermethrin on F1 progeny of *Trogoderma granarium* after eight day treatments.

Species Concentration	<i>Datura stramonium</i>	<i>Quercus infectoria</i>	<i>Solanum nigrum</i>	<i>Xanthium strumarium</i>	cypermethrin	Mean of concentration
zero	92.54	90.23	93.11	91.45	90.55	
0.5	34.75	28.60	33.23	25.21	21.89	28.73
1	17.36	25.90	19.50	24.45	20.65	21.57
2	11.56	20.56	16.87	20.45	15.29	16.94
4	8.34	12.42	9.43	14.67	5.43	11.05
Mean concentration	18.11	21.12	20.50	21.19	17.06	
L.S.D. (0.01) plant extract	0.82					
L.S.D. (0.01) concentration	18.22					

Repellents of cypermethrin and ethanol plant extracts against *Trogoderma granarium*

Pesticide and ethanol plant extracts repellent results have been tabulated in Table 5. It had been shown that the ethanol extract of *Datura stramonium* exhibited maximum percent repellents (91.87%) in 4% concentration, while the lowest

percent repellence (77.65%) was recorded in *Quercus infectoria*, and the cypermethrin gave 93.76% in the same concentration. Similar results were found by Mohiuddin et al. (1987) who observed that the repellents rate was 75% at the treatment of *Xanthium strumarium* against *Trogoderma castaneum*.

Table 5. Percentage repellents of cypermethrin and ethanol plant extracts against *Trogoderma granarium*.

Species Concentration	<i>Datura stramonium</i>	<i>Quercus infectoria</i>	<i>Solanum nigrum</i>	<i>Xanthium strumarium</i>	cypermethrin	control	Mean concentration
0.5	50.45	44.11	69.34	51.98	70.65	0	57.30
1	77.87	54.34	78.56	60.43	83.76	0	70.99
2	85.39	65.21	88.45	80.54	90.54	0	82.02
4	91.87	77.65	91.45	86.21	93.76	0	88.18
Mean concentration	76.39	60.32	81.95	69.79	84.67		
L.S.D. (0.01) plant extract	11.24						
L.S.D. (0.01) concentration	3.5						

Discussion

Ethanol plant extracts showed that the mortality rate increased with increasing concentration as well as the length of exposure times. The stored grains with high insect mortality could be attributed to the presence of toxic secondary metabolites. It has been reported that some secondary metabolites may act as insecticides or anti-feedants against *Trogoderma*

castaneum (Nawrot et al., 1989). *Datura stramonium* contains alkaloid compounds such as atropine, scopolamine in the leaves, stems and fruits in addition to essential oils, while *Solanum nigrum* contains solanine and solasodine (Chakravarty, 1976) which may be the direct reason of killing the insects. Furthermore, similar studies indicated that the effectiveness of insecticides

activity of *Acacia nilotica* extracts in controlling pests of *Trogoderma granarium*, *T. castaneum*, *Callosobruchus maculatus* and *Sitophilus zeamais* (Chairat et al., 2002). The present results showed that pesticide cypermethrin gave maximum mortality than plant extracts and the result supports the findings of Hasan et al. (2006) who reported that pesticide deltamethrin was more toxic to kill insects (25%) than the extract of the plant *Haloxylon recurvum* (17%) mortality. The activity of crude plant extracts on insects is composed of both toxic and anti-feedant effects (Koul, 1993; Akhtar and Isman, 2004). In some studies it has been found that the compound Azadirachtin, isolated from the neem tree (*Azadirachta indica*), is very important both as toxicant and anti-feedant and has been one of the most widely, tested and successfully implemented plant insecticides over the past two decades (Schmutterer, 1995). Moyin-Jesu (2010) report that the neem leaf extracts decrease the insect population, number of damaged leaves and number of holes per plant in maize plant.

Extract of *Datura stramonium* produced larvae mortality rates after one day compared with other plants extract *Quercus infectoria*, *Xanthium strumarium* and *Solanum nigrum*. Some of the larvae did not die after two days of exposure, but instead they died at the pupal or at the adult stage, this is due to the chronic effects of chemical compounds isolated from plant extract (Sukumar et al., 1991).

Conclusion

In general, it could be concluded that, plant extracts used in the present study act as larvicidal, adulticidal, and possess growth and emergence inhibiting, repellent activities against the *Trogoderma granarium*. Furthermore, this study suggested that ethanol extracts of plants belonging to families taxonomically unrelated possess toxic effects with significant insecticidal effect and could be a potential tool to protect stored grains against *T. granarium*, and this may contribute to a reduction in the application of synthetic insecticides, which in turn increases the opportunity for natural control of various medically important pests by plant pesticides. It is often active in specific target insects, less expensive, easily biodegradable to nontoxic products and potentially suitable for use in a *Trogoderma granarium* control program (Bream et al., 2010).

References

Abbott, C. I. 1925. A method of computing the effectiveness of an insecticide. J. Econ. Entomol. 13:65-67.

- Akhtar, Y. and M. B. Isman. 2004. Comparative growth inhibitory and antifeedant effects of plant extracts and pure allelochemicals on four phytophagous insect species. J. Appl. Entomol. 128:32-38.
- Akob, C. A. and F. K. Ewete. 2007. The efficacy of ashes of four locally used plant materials against *Sitophilus zeamais* (Coleoptera: Curculionidae) in Cameroon. Internat. J. Trop. Insect Sci. 40(1):21-26.
- Anonymous, L. 1980. Introduction to detia fumigation detia export GmH: 3 pp.
- Baser, K., C. B. Demircakmak, N. Ermin, F. Demirci and I. Boyday. 1998. The essential oil of *Bifora radians*. Bied. J. Essen. Oil. Res. 10:451-452.
- Bream, A. S., T. M. El-Sheikh, M. A. Fouda and M. I. Hassan. 2010. Larvicidal and repellent Activity of extracts derived from aquatic plant *Echinochloa stagninum* against *Culex pipiens*. Tunisian J. Plant Prot. 5:107-123.
- Chairat, C. T., J. Siripan, M. Suratwade and I. Somchai. 2002. Fumigation toxicity of *Eucalyptus* oil against three stored product beetle. Thai. J. Agric. Sci. 35(3):265-272.
- Chakravarty, H.L. 1976. Plant wealth of Iraq. Ministry of Agriculture and Agrarian reform, Iraq: 190-194 pp.
- Duke, S. O. 1990. Natural pesticides from plants. In: J. Janick and J. E. Simon (Eds.) pp.511-517. Advances in New Crops. Timber Press, Portland.
- Dwivedi, S. C. and B. Mathur. 1997. Screening of plant extracts as repellent against *Spodoptera litura* (Lepidoptera: Noctuidae). Indian Biol. 32(1):71-73.
- Dwivedi, S. C. and L. Bajaj. 2000. Repellent action of seven plant extracts against *T. granarium* (Coleoptera: Dermestidae). Uttar Pradesh J. Zool. 20(1):97-99.
- Dwivedi, S. C. and Y. Sharma. 2002. Investigation on repellent of Khapra beetle *Trogoderma granarium* (Coleoptera: Dermestidae) to five plant species. Indian Biol. 34(2):55-58.
- Dwivedi, S. C. and N. B. Shekhawat. 2004. Repellent effect of some indigenous plant extracts against *Trogoderma granarium* (Everts). Asian J. Exp. Sci. 18(1&2):47-51.
- Garcia, J. R. 1990. Bioassay of five botanical materials against the bean weevil *Callosobruchus*

- chinensis* L. on mung bean (*Vigna radiate* L.) Wilczek College, Laguna (Philippines): 67.
- Harris, D. L. 2009. Khapra Beetle, *Trogoderma granarium* Everts (Insecta: Coleoptera: Dermestidae). University of Florida, IFAS Extension (EENY -372 (IN667).
- Hasan, M., M. Sagheer, A. Ullah, W. Wakil and A. Javed. 2006. Response of *Trogoderma granarium* (Everts) to different doses of *Haloxylon recurvum* extract and deltamethrin. Pak. Entomol. 28(2): 25- 30.
- Hassan, A., W. Lwande, N. Olesitayo, L. Moreka, S. Nokoe and A. Chapya. 1990. Weevil repellent constituents of *Ocimum suave* leaves and *Eugenia caryophyllata* cloves used as the grain protectant in parts of Eastern Africa. Discovery Innovation 2:91–95.
- Irshad, M., A. Khan and U. K. Baloch. 1988. Losses in wheat in public sector storage in Rawalpindi region during 1984-1985. Pak. J. Agric. Res. 9(2):136-140.
- Isawunmi, M.A. 1984. The peppery fruits of Nigeria. Nigerian Field J. 49:37–44.
- Khalif, J. M. and I. M. Al-Farhani. 2008. Effect of some plants powder comparative in preference of Khapra Beetle *Trogoderma granarium* Everts (Coleoptera: Dermestidae). Basra J. Agric. Sci. 21(2):70- 93.
- Koul, O. 1993. Plant allelochemicals and insect control: an antifeedant approach. In: T. N. Ananthakrishnan and A. Raman (Eds.) pp.51-80. Chemical Ecology of Phytophagous Insects. Oxford and IBH, New Delhi.
- Lowe, S. M., S. Browne, S. Boudjelas and M. DePoorter. 2000. 100 of the World's Worst Invasive Alien Species: A selection from the Global Invasive Species Database. Invasive Species Specialist Group, World Conservation Union (IUCN). Available on=line at issg.org/booklet.
- McDonald, L. L., R. H. Guy and R. D. Speirs. 1970. Preliminary evaluation of new candidate materials as toxicants, repellents and attractants against stored product insects. Marketing research report number 882, Washington agriculture research service, United States department of Agriculture, Washington.
- Mohiuddin, S., R. A. Qureshi, M.A. Khan, M.K. Nassir and S.A. Qureshi. 1987. Laboratory investigations on the repellents of some plants oils to red flour beetle *Tribolium castenum*. *T. granarium* (Coleoptera: Dermestidae). Uttar Pradesh J. Zool. 20(1):97-99.
- Mordue, A. J. 2004. Present concepts of mode of action of azadirachtin from neem. In: O. Koul and S. Wahab (Eds.) pp.229-242. Neem: Today and in the New Millennium, Kluwar Academic Publishers, Dordresch, Boston, London.
- Moyin-Jesu, E. L. 2010. Comparative evaluation of modified neem leaf, wood ash and neem leaf extracts for seed treatment and pest control in maize (*Zea mays* L.). Emir. J. Food Agric. 22(1):37-45.
- Nadi, M. A. 2001. Toxicity of plant extracts to *T. granarium*. Pak. J. Biol. Sci. 4(12):1503-1505.
- Nawrot, J., J. Harmatha, I. Kosotova and I. Ognyanov. 1989. Antifeedant activity of rotenone and some derivatives towards selected insect storage pests. Biochem. System. Ecol. 7(1):55-57.
- Obeng-Ofori, D., C. H. Reichmuth, J. Bekele and A. Hassan. 1997. Biological activity of 1, 8-cineole, a major component of essential oil of *Ocimum kenyense* (Ayobangira) against stored product beetles. J. Appl. Ent. 121:237–243.
- Odeyemi, O. O., P. Masika and A. G. Aeolian. 2008. Insecticidal capensis against *Sitophilus zeamais* (Motschulsky) (Coleoptera: Curculionidae). Afri. Entomol. 16(2):220–225.
- Okonkwo, E. U. and W. I. Okoye. 1996. The efficacy of four seed powders and the essential oils as protectants of cowpea and maize grains against infestation by *Callosobruchus maculatus* (Fabricius) (Coleoptera: Bruchidae) and *Sitophilus zeamais* (Motsch) (Coleoptera: Curculionidae) in Nigeria. Internat. J. Pest Manage. 42(3):143–146.
- Owusu, E. O. 2001. Effect of some Ghanaian plant components on control of two stored- product insect pest of cereals. J. Stored Prod. Res. 37(1):85–91.
- Saxena, J. D. and S. R. Sinha. 1995. Evaluation of some insecticides against malathion resistant strains of red flour beetle, *Tribolium castaneum* (Herbs). Indian J. Entomol. 75(4):401- 405.
- Schmutterer, H. 1995. Properties and potential of natural pesticides from the neem tree, *Azadirachta indica*. Annu. Rev. Entomol. 35:271-297.

- Shantaram, H. 1958. The extension of larval duration in *Trogoderma granarium* Everts after DDT treatment. *Science* 23(5-6):145 -151.
- Sukumar K., M. J. Perich and L. R. Boobar. 1991. Botanical derivative in mosquito control. A review. *J. Am. Mosquito Control Associ.* 7:210-216.
- Sutherland, J., V. Baharally and D. Permaul. 2002. Use of the botanical insecticide, neem to control the small rice stinkbug *Oebalus poecilus* (Hemiptera: Pentatomidae) in Guyana. *Entomotropica* 17:97-101.
- Talukder, F. A. 2006. Plant products as potential stored –product insect management agents – A mini review. *Emir. J. Food Agric.* 18(1):17-32.
- Tsumura, Y. S., Y. Hasegawa, Y. Sekiguchi, Y. Nakamura and Y. Ito. 1994. Residues of post-harvest applied pesticides in buckwheat after storage and processing into noodle. *J. Food Hyg. Soci. Japan* 35(1):1-7.

PLANT SCIENCE

A study of medicinal plant species and their ethnomedicinal values in Caparo Barinas, Venezuela[◇]

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Abstract

The objective of this study is to create a database of medicinal plant species used in the treatment of the diseases that most commonly strikes the population in Canton-Limoncito, Andrés Eloy Blanco, south-western state of Barinas, in the western plains of Venezuela. Informal direct interviews were conducted randomly to 45 families, plant specimens in situ were collected, followed by subsequent identification, literature review, and digital camera photographic records. The best represented families are: Plantaginaceae, Labiatae, Asphodelaceae, Piperaceae, Gramineae, Chenopodiaceae, Fabaceae, Sterculiaceae, Costaceae, Euphorbiaceae, Malvaceae, Moraceae, Leguminosae, Adoxaceae, Annonaceae, Anacardiaceae, Solanaceae, Myrtaceae, Tiliaceae, Araceae. And the main therapeutic uses are: anti-inflammatory, antibiotic, antiseptic, analgesic, anticonvulsant, parasite, antirheumatic, influenza, astringent, diuretic, detoxifying, digestive, hepatic, laxative and sedative. The 95% of the people in the community use medicinal plants as primary treatment, the remaining 5% of the population are selecting conventional medicine from the nearest town. From this survey, it can be concluded that, majority of the population depends on medicinal plants from the environment to get better life conditions.

Key words: Ethnobotanical study, Caparo, Venezuela

Introduction

Caparo Experiment Station-ULA (EEC) (7000 Ha) is located in the town The Canton-Limoncito, Andrés Eloy Blanco Municipality, southwest of the State of Barinas, in the western plains of Venezuela. It belongs to The Caparo forest (170000 ha.) which is a unique relict of the original forest vegetation been preceded by the formation of the Colombo-Venezuelan trade winds (Hueck, 1978) (Figure 1). It was created by the University of Los Andes in 1968 and is being used in teaching, research and extension activities in co-operation with the Ministry of Environment. It consists of the forestry and agricultural activities that is associated with trees like cedar, mahogany and Linnet with papaya (Arends and Catalán, 1997). This program was established on the basis of a comprehensive attitude, so that all experience and research are

being covered by an eco-friendly approach which provides better results. The role of research would be to explore and establish ways to quantify forest policies and their implementation. Option was tested, documented and characterized in its results always refer to defined environments, the forest type. Caparo was all that could be supplied on tropical forest silvicultural treatment (Lawrence and Vicent, 2010).

However, several factors of political, economic and social aspects have influenced the dramatic decrease in tree diversity of the country, leading in turn a major loss of genetic diversity of tree flora and fauna in Venezuela (Guevara et al., 2010). Currently the number of families has increased to 45, establishing an active and organized community in the surroundings of the experimental station. These communities have empirical knowledge of native plants with therapeutic action for the most common disease conditions. This work aims to contribute to the knowledge and use of medicinal plants of the population of the Caparo Experimental Station, The Canton-Limoncito, Andrés Eloy Blanco, south-western state of Barinas, in the western plains of Venezuela. Through direct

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interviews, data were randomly collected about the main medicinal uses of plants in this area.

Materials and Methods

Open type surveys were conducted during a trip made to the Experimental Station of Caparo on November 2009. The observations were made in Painted Palma sector. People with greater knowledge of traditional medicine were identified by conducting short interviews. This study included all subjects who agreed to answer the survey. In all cases the people knew they were surveyed for this work. The information obtained was classified according to: main plant use, part of the plant used and methods of application to the diseases.

Results

Distribution of families in the Experimental Reserve of Caparo

The Caparo reserve has been divided in units Unit One (I) covers 56,358 Ha; Unit Two (II), 57,253 hectares; and Unit Three (III), 60,673 acres within the units, Unit One (I) is the experimental area in which the ULA-MARN Comodato (Universidad de los Andes, Ministry of Environment and Renewable Natural Resources) is located (pink area). Families are occupying 900 ha within the Experimental reserve. Their geographical distribution is: 32 families within the Reserve, 8 in Costa Caño sector and 5 on the road (Figure 1).



Figure 1. Distribution units of Caparo Experiment Station and location of the families.

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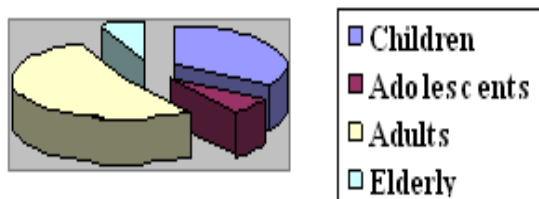


Figure 2. Distribution of individuals of the population according to age Caparo.

Test subjects and age distribution

A total of 45 families are occupying the Palma Pintada area whose age distribution is as follows: 52 children, of whom, 42 are from 0-15 years and 10 are adolescents, 83 adults and 10 elderly (Figure 2).

Medicinal plants used

Forty four medicinal plants used were found according to the survey in the Palma Pintada area of Caparo Experimental Station. Twenty eight of them were identified and shown in Table 1. The Anacardiaceae, Asteraceae, Leguminosae and Myrtaceae families are the most represented with the largest number of species.

Methods of administration of medicinal plants used in the community

The main application form is the cooking of the plant, followed by shredding and baths.

Cooking: It is made through the oral ingestion of plant infusions, either with or without the addition of other herbs.

Crushing: It is made through the direct application of previously crushed plant on the affected area.

Baths: It is made through the application to the skin of the leaf, bark or root infusion of the plant.

Table 1. Key plants and their ethnobotanical uses in Caparo Experiment Station.

Plant Common Name	Scientific name	Family	Main uses	How to use
Yanten	<i>Plantago major</i>	Plantaginaceae	Antibiotic, anti-inflammatory	Cooking
Yerbabuena	<i>Mentha piperita</i>	Labiatae	Influenza	Cooking
Media luna	<i>Piper peltatum</i>	Piperaceae	Anti-inflammatory	Bathing
Zábila	<i>Aloe vera</i>	Asphodelaceae	Any use	Crushed
Bejuco guaco	<i>Mikania guace</i>	Asteraceae	Rheumatism	Poultice
Cordoncillo	<i>Piper sp.</i>	Piperaceae	Fever	Cooking
Limonaria	<i>Cymbopogon citratus</i>	Gramineae	Influenza	Cooking
Paico	<i>Chenopodium ambrosioides</i>	Chenopodiaceae	Dewormer	Washed
Samán	<i>Samanea saman</i>	Fabaceae	Influenza	Cooking
Guásimo	<i>Guazuma ulmifolia</i>	Sterculiaceae	Dewormer, antiseptic	Cooking
Caña india	<i>Costus sp.</i>	Costaceae	Rheumatism	Cooking
Bejuco	<i>Kalanchoe gastonis-bonnieri</i>	Asteraceae/ Crassulaceae	Antibiotic	Cooking
Jabillo	<i>Hura crepitans</i>	Euphorbiaceae	Anti-inflammatory	Cooking and bathing
Escobilla blanca	<i>Scoparia dulcis</i>	Scrophulariaceae	Dewormer	Cooking
Higuerón	<i>Ficus sp.</i>	Moraceae	Dewormer	Cooking
Mucuteno	<i>Cassia spectabilis</i>	Leguminosae	Dewormer	Cooking
Sauco	<i>Sambucus</i>	Adoxaceae	Anti-inflammatory	Cooking
Guanábano	<i>Annona muricata</i>	Annonaceae	Antivenom	Cooking
Gateado	<i>Astronium graveolens</i>	Anacardiaceae	Antiepileptic	Cooking
Anís	<i>Pimpinella anisum</i>	Apiaceae	Antiseptic, antibiotic	Poultice
Yerba mora	<i>Solanum nigrum</i>	Solanaceae	Analgesic	Cooking
Mala madre	<i>Chlorophytum comosum</i>	Agavaceae	Healing	Cooking, Poultice
Guayabo	<i>Psidium guajava</i>	Myrtaceae	Abortive	Cooking y bathing
Eucalipto	<i>Eucalyptus</i>	Myrtaceae	Antiespasmotic	Crushed
Drago	<i>Pterocarpus sp.</i>	Leguminosae	Purifying	Cooking
Jobo	<i>Spondias mombin</i>	Anacardiaceae	Antiseptic	Poultice
Orumo	<i>Cecropia sp.</i>	Tiliaceae	Hernias	Cooking
Ocumo	<i>Xanthosoma sagittifolium</i>	Araceae	Diabetic	Cooking
				Cooking and bathing

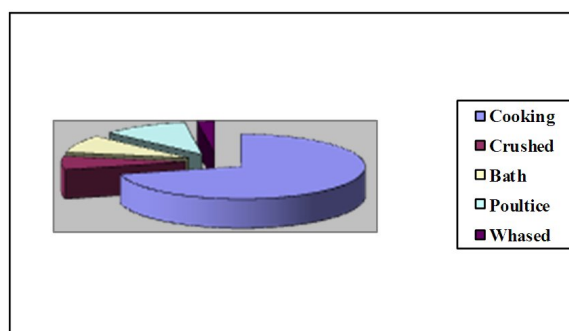


Figure 4. Management methods of medicinal plants used in the community in Caparo Experiment Station.

Discussion and Conclusions

Plants are an important source of medicines. Around half of the approved drugs produced are based on natural products (Harvey, 2008). For centuries, man has used plants as a source for drug development based on empirical knowledge, either in traditional preparations or drugs. It is currently estimated that 80% of the world's population uses traditional medicine to meet their primary healthcare needs (OMS/UICN/WWF, 1993). Since the sixteenth century until the late eighteenth century botanical and medical knowledge in Europe was well documented and disseminated. However, in some cases modern science has neglected this source of knowledge and ancient remedies, have not been yet evaluated systematic and pharmacologically. Adams et al. (2009) conducted a research on five of Europe's most important herbal gardens from XVI and XVII century, for terms related with rheumatic diseases, phytochemistry and possible anti-inflammatory activities of those species. It showed that from sixty-three plants, more than half proved to have antiphlogistic activity *in vitro* or *in vivo* (Adams et al., 2009).

Another research in Kenya, examined the farmers' ethnobotanical knowledge. This study suggests that community-based gene banks linked to formal government facilities, will allow to combine and intertwine indigenous knowledge and "modern" to a more realistic and sensitive understanding of natural environmental resources sustainable development (Lado, 2004).

In America, the results of surveys of the inhabitants of the province of Pinar del Rio, Cuba, allowed to establish the traditional use of 112 medicinal plants for different conditions. The results coincide with those obtained by other researchers in different areas of the country (Scull et al., 1998). A fieldwork also conducted in seven communities in the province of Camagüey, provides information on 111 species of plants belonging to 96 genera and 55 families where 173 indications were obtained for medicinal use, primarily for respiratory, gastrointestinal, hepatobiliary, and skin (Beyra et al., 2004). In Mexico, an evaluation of indigenous knowledge of plants from tropical rainforests has been performed based on scientific information and quantitative ethnobotanical research method. It recognizes 1,330 species from indigenous knowledge, from the primary and secondary forests (1052) and agricultural areas (maize fields) and indigenous gardens (278) yielding 3,173 "products" that

include medicines, food and others (Toledo et al., 1995).

For Venezuela, preliminary results of an ethnobotanical inventory of the Moron area, Carabobo State have been published. Of a total of 455 species collected, 46 were reported with different uses by the inhabitants, of which the vast majority are used in traditional medicine. It also includes a list of all species recorded so far in the study area (Díaz and Ortega, 2006). Likewise, Velazquez Bermudez documented the relative importance of species of medicinal use in Trujillo state. Thirty two species of medicinal plants, belonging to 25 botanical families were identified. Of these, 9 species are wild and 23 cultivated. The use, traditional methods of preparation and administration of plants in a rural community was evaluated by Bermúdez and Velázquez (2002).

Ethnobotanical studies are the basis for the development of programs to get the most knowledge about the use of traditional medicine, enriching the cultural heritage and the best use of medicinal plants. As a result of the survey applied, twenty eight medicinal plants, grouped into 24 families were found. Anacardiaceae (2%), Asteraceae (2%), Leguminosae (2%) and Myrtaceae (2%) families are the most represented with the largest number of species. It is important to note that due to the high biodiversity in the Caparo Forest Reserve, the number of families is diverse and found in greater proportion. According to the interviews, therapeutic properties were attributed to these species for 34 different conditions, where applications such as parasite (6%), analgesics (3%), antibiotics (3%), anti-inflammatory (3%) and the common cold (2%) are the most common ailments in the area. The main ways of applications are cooking (22%), baths (4%) and poultices (4%). In comparison with the previous ethnobotanical investigations, there are twenty eight matching applications and six species are presented which don't have any previous reported uses.

In relation to ethno pharmacological information, traditional uses were precisely those most cited for the most common health problems in the community. It took into account that most of the medicinal plants commonly used by the inhabitants of Palma Painted sector are mainly used as antibiotics, anti-inflammatory and antiseptic. The conditions treated with the medicinal plants recorded, reflects the importance of these plants in traditional

herbal medicine in the study site. This work will contribute primarily to safeguard the knowledge of the inhabitants of the area very rich in biodiversity of the flora and fauna of the region and could provide data for the study of botanical species that could contribute to the development of new drugs.

References

- Acero, A. 1980. Manejo de las Reservas Forestales Adscritas a la Zone 6 del MARNR. Publisher Ministerio del Ambiente y de los Recursos Renovables, Venezuela.
- Adams, M., C. Berset, M. Kessler and M. Hamburger. 2009. Medicinal herbs for the treatment of rheumatic disorders. A survey of European herbals from the 16th and 17th century. *J. Ethnopharmacol.* 121:343-359.
- Arends, E. and A. Catalán. 1977. Proyecto de investigación sobre el impacto ecológico. Reserva forestal de Caparo, unidad Y. Publisher Universidad de Los Andes, Venezuela.
- Bermúdez, A. and D. Velázquez. 2002. Etnobotánica médica de una comunidad campesina del estado Trujillo, Venezuela: un estudio preliminar usando técnicas cuantitativas. *Revista de la Facultad de Farmacia*, 44, s/p.
- Beyra, Á., M. León, E. Iglesias, D. Ferrándiz, R. Herrera, G. Volpato, D. Godínez, M. Guimaraes and R. Álvarez. 2004. Estudios etnobotánicos sobre plantas medicinales en la provincia de Camagüey (Cuba). *Anales del Jardín Botánico de Madrid*, 61, s/p.
- Casanova, R. V. 1989. La Universidad de los Andes ante el Problema de la Destrucción de las Reservas Forestales de Ticoporo y Caparo. Publisher Universidad de los Andes, Instituto Iberoamericano de Derecho Agrario y Reforma Agraria, Venezuela.
- Díaz, W. and F. Ortega. 2006. Inventario de recursos botánicos útiles y potenciales de la cuenca del río Morón, estado Carabobo, Venezuela. *ERNSTIA* 1:31-67.
- Guevara, J., O. Carrero, M. Llija, H. Reinthaler and L. Rodríguez. 2010. Importancia de la Estación Experimental Caparo ante la pérdida de biodiversidad en los llanos del Orinoco. Taller técnico "40 años de la Universidad de Los Andes en Caparo". Publisher Universidad de Los Andes, Venezuela.
- Harvey, A. 2008. Natural products in drug discovery. *Drug Discov. Today* 13:894-901.
- Lado, C. 2004. Sustainable environmental resource utilisation: a case study of farmers' ethnobotanical knowledge and rural change in Bungoma district, Kenya. *Appl. Geography* 24:281-302.
- Lawrence, Vicent. 2010. 40 años de Caparo. Taller técnico "40 años de la Universidad de Los Andes en Caparo". Publisher Universidad de Los Andes, Venezuela.
- OMS/UICN/WWF. 1993. Directrices sobre conservación de plantas medicinales. *Media Natura*, 55p.
- Scull, R., M. Miranda and R. Infante. 1998. Plantas medicinales de uso tradicional en Pinar del Río. Estudio etnobotánico. I. *Revista Cubana de Farmacia*, 32, s/p.
- Toledo, V., A. Batis, R. Becerra, E. Martínez and C. Ramos. 1995. La selva útil: etnobotánica cuantitativa de los grupos indígenas del trópico húmedo de México. *INTERCIENCIA* 4:177-187.

PLANT SCIENCE

Ethnobotanical knowledge of native plants in Santa Rita Estado Aragua, Venezuela

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Abstract

An ethnobotanical survey was conducted in a village, Santa Rita, parish capital of Francisco Linares Alcántara Municipality, Venezuela. There is an estimated population of 79,885 people by the year 2008 (INE). The main respondents of the survey were three diabetic patients and informants belong to the "Buena Vida's" club, where all members are diabetic. The validity and reliability of the data were given by the coherence between the narrations of the informants and the interpretation of the researchers. The diseases that have largely expressed by the informants were: levels of high blood sugar, nervousness, insomnia, flu, colic, parasites, burns and shingles. They revealed that they used the modern synthetic drugs prescribed by doctors along with plants, in the form of decoctions of leaves, stems, fruits, flowers and roots, others shaped like cayenne infusion to treat insomnia and nerves, external application as aloe to treat burns. The informants also commented on how they prepared the plants, amount used and the desired effects.

Key words: Medicinal plants, Diabetes, Ethnomedicine

Introduction

If health is defined as a social process, there arises the need to incorporate the popular knowledge of each region in healing process, represented by shamans, healers, piaches or midwives and to use homemade and/or natural medicines, as well as, to apply traditional healing practices to satisfy the daily illness of the communities (Martínez, 2006). This way traditional knowledge can become a powerful source of health promoting quality of life for overcoming disease.

Nowadays, when studying some people in context of their community and observing their ethnobotanical uses that present in the traditional healing process, there is a disposition to conserve and to practice knowledge which they have inherited

from their ancestors and antiquity and which are continued using at the moment. The present day knowledge are based on them, that even though human kind has managed to obtain great advances in the modern technology, there are still some diseases which do not respond to treatments. Therefore, there must be records for native healing practices and homemade treatments, that far from losing importance before the powerful pharmaceutical emporium, they are effective today, more than ever.

In traditional medicine, ethnobotanical practices are based on some mixtures, and are such, that it is not easy to differentiate what is original or native; it is not easy to know which of them are plants, animals, clothes and meals. There are a lot of documented evidences from Egyptian, Aramaic, Ottoman, Assyrians, Ethiopian, Mayan, the Aztecs, Incas, Latin American indigenous cultures, Tibetans etc., with a huge therapeutic potential to treat diseases suffered by them and that today the merits are granted only to science (Riveiro, 1977).

At the present moment, it is important to rescue the popular traditional knowledge of healing without contrasting medical technical-scientific advances and

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using it rationally, by means of its verified effectiveness. It is necessary to indicate that even though from the present state politics and the state reforms of health novel programs that stimulate the participation of the communities have been promoted, it has not been obtained the promotion of the traditional knowledge present in the communities and that is manifested through traditional “doctors” (healer, shamán, piache) and which are multiplied by means of the family heads. For that reason, today more than ever, it is important to rescue the popular beliefs of healing that exist in the community of the Santa Rita parish of the Aragua state as far as the use of the medicinal plants and to offer common therapeutic alternatives to treat pathologies. Because most of the diseases have poor answer to treatments with conventional medicines, as well as to use the therapeutic resources that are more accessible to the community and to take advantage of the traditional knowledge and medicinal properties that they confer to it. The importance of reconstructing the popular beliefs in health, resides in knowing from daily, the types of pathologies, ways and paths of how to cure them. In order to finish this analysis, it is of high priority to recognize forms, ways and pathways of treatment in the communities; to accept, and review.

With regard to Venezuela, WHO registered 583,000 people with diabetes for year 2000, considering for year 2030 around 1,606,000 diabetic patients. According to the epidemiologists numbers of Corposalud (Corporación de Salud del estado Aragua, 2005), in the Municipality of Francisco Linares Alcántara, the incidence of diabetes every year is 614.9 per each 100,000 inhabitants, being this pathology the most occurring disease in both municipal and state levels. The objective of the present investigation was to record the ethnobotanical values of native plants in Santa Rita Estado Aragua, Venezuela.

Materials and Methods

This investigation was conducted in the small town of Santa Rita, close to the Medical School, University of Carabobo. It is the capital parish of

the Francisco Linares Alcántara Municipality, Aragua State, Venezuela, which is the place of the informants who contributed their experiences to the survey.

The three informants were diabetic patients and belong to “Buena Vida’s club”, where all their members have the same pathology. This group was chosen because the number of morbidity and mortality that they showed in the different epidemiological archives, related to diabetes (Instituto Nacional de Estadística (INE) [citado 5 Mayo, 2009], Organización Mundial de la Salud (OMS) [citado 5 Mayo 2009], Corporación de Salud del estado Aragua, 2005), occupy the first positions. They frequently meet in an urban type II assistant center that belongs to the same Parish. They allowed us to get close to their social reality as for their popular beliefs and the different ways from healing that they use to cure their diseases.

It is conducted from the logic of the daily lived, informants, about their use of plants and treatments of their pathologies in the foundation of traditional knowledge. The registry of the survey was done by means of the focused interview gathering the narrations of life of the three informants related to the healing processes. It was all, considered from the epistemology of the complex thing and applying the method of the hermeneutics through the stories of life of the informants.

Informants were induced to narrate their experiences of life as for the processes of healing and the most common diseases suffered by them. The facts were developed fluidly, because all of them were interested in telling their experiences and the knowledge learned or transferred from their ancestors. The validity and trustworthiness were given by the coherence between the narrations of the informants and the interpretation of the investigators. Pathologies that the informants usually suffered were: high levels of sugar in blood, nervousness, insomnia, influenza, colic, parasites, and burns (Table 1).

Table 1. Diseases that suffer most the “Buena Vida’s” club informants.

Diseases	Traditional remedies
High level of blood sugar	Drugs (metformin, Glucophage, glyburide-diaformina)+ Plants (leaves, stems, flowers and roots)
nervousness/insomnia	Bitter melon, Cow’s hoof, <i>Eucalyptus</i> , bellyache bush, soursop.
Flu	Chinese hibiscus
Parasites	Lemongrass
Burns	Goosefoot, goat-weed
colics, upset stomach	Must not be used in Full Moon because the parasites get excited themselves
	<i>Aloe vera</i> , Onion slice
	Plants

Another aspect that was approached was related to the healing treatments. Obtained data revealed that the informants suffered a very high value of sugar in blood, used to take medicines prescribed by physicians to them accompanied with some plants, in the form of boiled extracts of leaves, stems, fruits, flowers and roots; others in infusion as in the case of the chinese hibiscus (*Hibiscus rosasinensis* L.), to treat insomnia and nervousness; other plants are externally used like crystals of *Aloe vera* to treat burns. Regarding the form of preparation, amount, and produced effects, it was presented as follows:

Treatment for blood sugar (diabetes)

Cook 2 to 3 handfuls of tender leaves of bitter melon (*Momordica charantia*), Cow's hoof (*Bauhinia purpurea*) or Eucalyptus (*Eucalyptus globulus*), separately. In the case of the bitter melon, fruits and flowers can also be cooked. It is recommended to take one cup three times a day, the informants referred that they felt better after treatment and posterior examinations values of sugar in the blood have lowered. Onion is also good to treat this disease; therefore it can be eaten in all its forms.

Treatment for nervousness and insomnia

They use chinese hibiscus (*Hibiscus rosasinensis* L.) infusion. One or two flowers of chinese hibiscus were selected. One cup of boiling water is added, kept for 5 minutes and drink. They assume a tranquilizer effect for this drink.

Treatment for flu

Cook 2 or 3 chopped leaves of lemongrass in boiling water for 5 to 10 minutes, sieved to separate the debris because they can produce injuries at the throat level. Drink 1 cup twice a day until the influenza is cured.

Treatment for parasites

The informants used the goosefoot (*Chenopodium ambrosioides* L.) plant, of which 2 to 3 shoot cooked with a cup of water, it does not have to be liquefied nor to use too many leaves, caution must be taken because when used very concentrated can produce death in the children. Take one cup daily to the illness during 3 days, stop and repeat a week later for three days. Only if the child shows persistence of parasites, it is possible to repeat within a month.

Treatment for Burns

It is recommended to remove 2 to 3 crystals from the *Aloe vera* leave and place it directly on the burned skin.

Treatment for colic or upset stomach in children

Collect 2 to 3 shoots of orange and boil in a cup of water and it is administrated to the child in small quantities. At the same time, heat is transmitted to the child at the navel level by hand imposition.

Treatment for shingles (Herpes zoster)

The treatment that is used is with blackshade (*Solanum nigrum*) dressings.

Shared Knowledge

When the natural medicine is visualized at the community level, it is expected that the settlers know other ways of treatments of some pathologies that do not respond to conventional treatments and those plants that can cultivate themselves in the yards of their houses making them more accessible. The knowledge transmitted by the tradition in oral form and the medicine practiced throughout many years plays an important role in Ethnomedicine. There is a shared vision by the family, where each one gives solution to own health problems by using house or the "garden" pharmacy which never stops serving (Correa, 1995).

When plants are used to treat a certain disease, a set of dimensions are handled where the economic, cultural, daily, the traditional thing, the inherited knowledge, the social and familiar consequences of the perceived facts are combined. The ritual practices that accompany the process by healing are assumed accepting that the important thing is the interpretation between the allopathic medicine (scientific knowledge) and the ethno-plants (popular traditional knowledge) because it allows the use of both in complementary form. The treatments are conditional to the influence of the tradition and the transmitted knowledge of generation to generation (Portillo, 1993).

The therapeutic treatment is clarified by their social-cultural frame, techniques, and procedures that compose their preventive and curing practices. From this one optics, the healing processes are effective when accompanied with different traditional plants from the Venezuelan population in these events of healing (Portillo, 1993). Therefore it is necessary that the professionals in the health sector must be aware about the knowledge on different therapeutic alternatives to which the communities resort to cure their diseases. Each human group has a perception of the plants that is in agreement with other aspects that confirm its culture such as language, history and traditions, as well as therapeutic properties attributed to them by the original inhabitants of that community. And when obtaining an approach between the popular

knowledge and the scientific knowledge, there must exist a complementarity and not a clash among them, since we have grown in a culture with reducing and linear thought, whereas at the present time, there is a global world where the problems are more linked one another every day (Morin, 2008).

Conclusions

Ethnobotanical and ethno-pharmacological research is very crucial in the development of drugs from natural sources. An ethnobotanical survey was carried out in the village, Santa Rita, parish capital of Francisco Linares Alcántara Municipality, Venezuela. The information obtained on identification, preparation, clinical use, gathering, and preservation of medicinal plants dramatically facilitates the search for new drugs, and the time needed for drug development programs. Since many plant species are indicated as potential resource for treating various diseases such as diabetes, nervousness, insomnia, flu, colic, parasites, burns, and shingles, this should encourage further research in these fields. Such fieldwork surveys document the traditional medical use of plants before the dyeing out of such informative resources. The preservation of the know-how and plant species is a fundamental step toward developing efficient remedies for various diseases.

References

- Briceño A. Dioses, Raíces y Sortilegios. 1993. Villa de Cura (Ven): Editorial Miranda.
- Corporación de Salud del estado Aragua. 2005. Estadísticas de Epidemiología estado Aragua.
- Correa, R. 1995. Farmacopea del Parque Henry Pittier [Tesis Maestría]. Maestría Cultura Popular. Universidad de Carabobo sede Aragua.
- Instituto Nacional de Estadística (INE) [citado 5 Mayo 2009]. Disponible en: URL: <http://www.ine.gov.ve>.
- Martínez, N., E. Cayama, N. León and L. Pérez. 2008. Phytotherapy and adverse reactions. In: Toxines et fonctions cholinergiques neuronales et non neuronales. 16th Rencontres en Toxinologie E-Book: p.111-118. Disponible-en: <http://sfet.asso.fr/images/stories/SFET/pdf/EBook-RT16-2008-signets.pdf>
- Martínez, N. 2008. Dialogando con el Jardín. Comunidad y Salud 1(6):37-47. Disponible :<http://servicio.cid.uc.edu.ve/fcs/index1.htm>.
- Martínez, N. 2006. Elementos culturales en relación con la Utilización de las plantas Medicinales en Venezuela desde una Teoría de acción Humana con un enfoque Socioeducativo. [Trabajo de Ascenso. Honor al mérito y mención Publicación]: Universidad de Carabobo sede Aragua.
- Martínez, N. 2003. Las plantas medicinales no son tan inofensivas como parecen. En: Latin American Society for Nutrition. Venezuelan Chapter. p.17-29. www.slam.org.ve/publicaciones/completas/plantas_medicinales_1.asp
- Morin, E. Romper con el desarrollo. Traducción de Rigoberto Lanz. [Publicado 17 Mar 2008]. Disponible en: URL: <http://debatesso.breelsocialismo.blogspot.com/2008/03/romper-con-el-desarrollo.html>
- Municipio Francisco Linares Alcántara. Disponible en: URL: http://es.wikipedia.org/wiki/Municipio_Francisco_Linares_Alc%C3%A1ntara
- Organización Mundial de la Salud (OMS) [citado 5 Mayo 2009]. Disponible en: URL: <http://www.who.int/diabetes/facts/en/index.html>.
- Portillo, G. 1993. Preventive Medicine in Medical Schools. Report of Colorado Springs Conference. J. Med. Education 28(10):3.
- Riveiro, D. 1977. Historia de las Civilizaciones Antiguas de América. Madrid (Es): Editorial Itsmo 36.
- Tratamientos naturales para los males del cuerpo humano. Herpes zoster o culebrilla. Disponible en: URL: <http://www.chispaisas.info/alternativa35.htm>

PLANT SCIENCE

Chemical profile analysis of three different species of *Plectranthus*[◇]

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Abstract

The genus *Plectranthus* comprises about 300 species distributed over tropical Africa, America, Asia and Australia. In this work we have studied the most common *Plectranthus* species used as tea drinks known in the Brazil as “Boldo”, where it is very popular for gastric spasms and liver disorders. Since the same trivial name is used for different *Plectranthus* species, ethnobotanical information becomes very difficult and confusing. The essential oils from *Plectranthus barbatus*, *P. grandis*, *P. ornatus* were obtained by HS-SPME. Their analyses were performed by GC-MS.

Key Words: Essential oil, *Plectranthus barbatus*, *Plectranthus grandis*, *Plectranthus ornatus*, SPME

Introduction

The genus *Plectranthus* (Family: Lamiaceae) comprises about 300 species distributed over Tropical Africa, America, Asia and Australia. Many lamiaceae species have been investigated for their high content of essential oils, which are widely used in pharmaceutical preparations, perfumery and cosmetics (Ascensão; 1999). The *Plectranthus* species are of ornamental, economic and medicinal interest and they show a rich diversity of ethnobotanical uses. Several species are used all around the world as vermicides, antiseptic and purgative, or for the treatment of ear infection, toothache and stomach ache, as well as a remedy for vomiting and nausea, and against a vast array of other diseases (Cousins, 1994). The genus is plagued with numerous nomenclatural disharmonies that make it difficult to collect accurate data about its uses. Since different trivial names are used for the same *Plectranthus* species, ethnobotanical information becomes very difficult

and confusing. The fact that most taxonomic revisions of *Plectranthus* have tended to be regional rather than global, this could have contributed to the confusion that has led the use of multiple taxonomic names to describe the same species (Lukhoba, 2006). *P. barbatus*, previously known as *Coleus barbatus* (Andr.) Benth, is a native species from India and Tropical Africa, but well adapted in the Brazilian Northeast and Southeast regions, from which several medicinal uses have been reported, such as: for gastrointestinal disorders, skin affections, pain, respiratory and genito-urinary diseases, gastric spasms and liver disorders (Camara, 2003.). In Kenya and the Democratic Republic of Congo, *P. barbatus* is used in the treatment of wounds and ringworms (Chifundera, 2001; Githinji and Kokwaro, 1993;). In Brazil, *P. grandis* and *P. barbatus* are usually used in folk medicine to treat many purposes, but the most popular use being for gastric lesions. The increased use of these phytomedicines and the frequent mix-up of similar Lamiaceae species have brought strong health problem to the population (Soares, 2006). Many of the phytochemical studies on *Plectranthus* species have focused on the volatile fractions. Plants of this genus contains a wide range of mono- and sesquiterpenoids, including humulene and β -caryophyllene, which are known for their

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antimicrobial properties (Ascensão, 1998), borneol, camphene, β -phellandrene, α -thujene δ -selinene, α -ionone and humulene normally found in *P. barbatus* (Mathela, 1986; Maia, 1988; Camara, 2003). Studies about the chemical composition of *Plectranthus ornatus* Codd. (syn. *Coleus comosus* Hochst.), reported the isolation of diterpenoids which, according to an ethnopharmacological survey, are often used for stomach, liver diseases and also for the analgesic, anti-inflammatory, antipyretic and diuretic activities (Corrêa, 1998; Oliveira, 2005). In this work, Solid Phase Microextraction was used to characterize the volatile composition of the three most common traditional used species of *Plectranthus* in Brazil. SPME has been successfully used for the chemical characterization of aromas and fragrances and it is now favored over other procedures due to its simplicity, speed, low cost and solventless operation (Araújo, 2007; Dias-Maroto, 2002; Sartoratto, 2003). This technique has been proposed also for analysis of aroma related to biogenic organic compounds released *in situ* from live parts of plants (Loi, 2008).

Materials and Methods

Plant Material

The aerial parts of 100g of each *Plectranthus* species were collected on the campus of the Rio de Janeiro Federal University in Rio de Janeiro, RJ, in April 2008.

SPME

Fresh and dried leaves of the three studied *Plectranthus* species (300mg) were separately reduced to small pieces and placed in the 4ml vials

and extracted by HS-SPME using a carboxen-divinylbenzene 75 μ m - fiber (CAR-DVB) at 80°C, for a sample/headspace equilibration time of 15 min. The extracted materials were subsequently desorbed and analyzed in the GC-MS apparatus.

GC-MS Analysis

The quantitative and qualitative analyses were carried out on a CG-MS QP5000 Shimadzu with a ZB-5MS fused silica capillary column (30m x 0,25mm, 0,25 μ m film thickness). The operating temperatures were: injector 260°C; detector 290°C and column oven 60°C up to 290°C (3°C/min). Chromatographic grade helium (1.0mL/min) was used as carrier gas for GC-MS. Identification of the detected analytes was accomplished by matching their mass spectra with the available databases NIST 2006 and confirmed by comparing their retention indices with literature data (Adams, 2004).

Results and Discussion

GC/MS analyses and comparison of the MS and RI values for the separated compounds with those record in the literature permitted, allowed the identification of monoterpenes (acyclic and monocyclic) and sesquiterpenes (acyclic and monocyclic) in the volatile fractions. In total, 17 different compounds were identified in the volatile fractions from *P. barbatus*, 17 from *P. grandis* and 25 from *P. ornatus*. In total, 26 different compounds were identified (Tables 1 and 2), accounting for 77.41 – 93.40% of the chemical composition of the corresponding oils.

Table 1. SPME chemical composition of the volatile fraction from fresh aerial parts of the three *Plectranthus* species^a.

Compounds	RI	<i>P. barbatus</i> %	<i>P. grandis</i> %	<i>P. ornatus</i> %	Identification
(<i>E</i>) 3-hexen-1-ol	856	-	1.06	0.41	RI, GCMS
(<i>Z</i>) 3-hexen-1-ol	858	7.75	6.67	0.94	RI, GCMS
α -thujene	929	-	-	4.40	RI, GCMS
α -pinene	933	1.29	1.69	2.39	RI, GCMS
β -pinene	977	-	-	1.13	RI, GCMS
1-octen-3-ol	984	2.47	2.68	11.85	RI, GCMS
3- methyl-2-heptanone	988	1.02	1.10	0.59	RI, GCMS
β - myrcene	990	3.24	4.58	0.64	RI, GCMS
3-octanol	1000	-	-	4.31	RI, GCMS
(<i>Z</i>) acetato de 3-hexen-1-ol	1006	4.47	9.38	0.88	RI, GCMS
α -cimene	1029	-	-	0.46	RI, GCMS
limonene	1032	-	-	0.56	RI, GCMS
(<i>Z</i>) β -ocimene	1040	1.27	1.41	0.49	RI, GCMS
(<i>E</i>) β -ocimene	1051	-	-	0.89	RI, GCMS
α -cubenene	1342	0.82	1.02	3.60	RI, GCMS
β -bourbonene	1381	-	-	8.07	RI, GCMS

α -caryophyllene	1417	-	1.39	1.61	RI, GCMS
β -farnesene	1458	24.86	16.22	29.83	RI, GCMS
α -copaene	1371	12.68	12.38	3.60	RI, GCMS
γ -muurolene	1474	-	-	0.60	RI, GCMS
D-germacrene	1481	21.40	25.48	6.40	RI, GCMS
α -zingiberene	1493	4.44	4.67	0.45	RI, GCMS
δ -cadinene	1516	1.93	2.39	2.01	RI, GCMS
β -sesquifelantrene	1522	1.18	1.28	-	RI, GCMS
caryophyllene oxide	1581	0.83	-	3.99	RI, GCMS
Total identified peaks	88.83		93.40	90.10	

RI: Experimental Retention Indices

aData are the means of three experiments performed in triplicate. Standard deviations were below 10%.

Table 2. SPME chemical composition of the volatile fraction from dried aerial parts of the three *Plectranthus* species^a.

Compounds	RI	<i>P. barbatus</i> %	<i>P. grandis</i> %	<i>P. ornatus</i> %	Identification ^c
α -thujene	929	-	-	17.60	RI, GCMS
α -pinene	933	10.92	8.56	10.83	RI, GCMS
sabinene	975	-	-	3.44	RI, GCMS
β -pinene	977	-	-	3.67	RI, GCMS
β -myrcene	990	4.33	3.73	1.67	RI, GCMS
α -cimene	1029	-	0.94	5.71	RI, GCMS
limonene	1032	-	-	1.07	RI, GCMS
(<i>Z</i>) β -ocimene	1040	1.30	0.94	-	RI, GCMS
(<i>E</i>) β -ocimene	1051	-	-	1.22	RI, GCMS
α -cubenene	1342	0.82	1.47	2.00	RI, GCMS
β -bourbonene	1381	0.89	-	7.98	RI, GCMS
α -caryophyllene	1417	2.05	0.52	1.07	RI, GCMS
β -farnesene	1458	6.90	4.31	18.50	RI, GCMS
α -copaene	1371	17.12	17.85	3.40	RI, GCMS
D-germacrene	1481	1.99	3.75	4.43	RI, GCMS
α -zingiberene	1493	1.17	1.25	-	RI, GCMS
δ -cadinene	1516	1.93	-	-	RI, GCMS
β -sesquifelantrene	1522	1.18	-	-	RI, GCMS
caryophyllene oxide	1581	36.41	35.03	10.73	RI, GCMS
Total identified peaks	87.01		77.41	93.32	

RI: Experimental Retention Indices.

aData are the means of three experiments performed in triplicate. Standard deviations were below 10%.

The chromatographic profile of *P. barbatus* and *P. grandis* showed great similarity in qualitative and quantitative composition of the fresh parts analyzed in both mono and sesquiterpenes. The monoterpenes found in these species were the same, in fresh and dry leaf stages, α -pinene, β -myrcene and (*Z*) β -ocimene being in superior quantity in the dry stage. This can be explained due to the protection of the volatile fractions offered by specific structures, remaining in the leaves for a longer time, while the low molecular alcohols evaporate, thus increasing the percentage of the monoterpenes in the composition. The fresh leaves of *P. ornatus* showed the richest volatile profile of them all. The volatile fractions of this species are composed by a greater number of monoterpenes and sesquiterpenes, the α -thujene (4.40 - 17.60%) being the most remarkable

monoterpene in the fresh and dried stages, while the farnesene (4.31 - 29.83%), copaene (3.40 - 17.85%) and germacrene D (1.99 - 25.98%) are the major sesquiterpenes found in the leaves in both fresh and dried stages. The *P. ornatus* yielded the richest terpene profile of them with 25 and 15 compounds in the fresh and dried leaf volatile fractions, respectively. The compound distribution in the analyzed aerial fresh parts of the three species has shown that majority of the volatile fraction is composed by the sesquiterpenes compounds, as it represents close to 50 % of the composition in the three species, Table 3. *P. barbatus* and *P. grandis* have a similar percentage distribution between monoterpenes and sesquiterpenes. This similarity in the chemical profile is remarkable between these species: both have α -copaene and D-germacrene in dried stage

and α -copaene, D-germacrene and farnesene in fresh stage as main constituents. However, all three species have roughly 70% of their fresh volatile from fractions leaves composed by terpenes.

Although the pattern of volatiles fractions between *P. barbatus* and *P. grandis* is showed as similar, the presence of one oxygenated sesquiterpene in *P. barbatus* approach this species to *P. ornatus*.

Table 3. Compound distribution in the analyzed fresh aerial parts from *Plectranthus* species.

Species	Monoterpene (%)		Sesquiterpene (%)		Others ^a (%)	N ^c
	Non-oxy	Oxy	Non-oxy	Oxy		
<i>Plectranthus barbatus</i>	20.00	-	46.67	6.67	26.67	15
<i>Plectranthus grandis</i>	18.75	-	50.00	-	31.25	16
<i>Plectranthus ornatus</i>	33.33	-	37.50	4.17	25.00	24

^a Other compounds as n-alkanes and alcohols; ^c Number of identified compounds.

Table 4. Compound distribution in the analyzed dried aerial parts *Plectranthus* species.

Species	Monoterpene (%)		Sesquiterpene (%)		Others ^a (%)	N ^c
	Non-oxy	Oxy	Non-oxy	Oxy		
<i>Plectranthus barbatus</i>	23.07	-	69.23	7.69	-	13
<i>Plectranthus grandis</i>	36.36	-	54.55	9.09	-	11
<i>Plectranthus ornatus</i>	53.33	-	40.00	6.67	-	15

^a Other compounds as n-alkanes and alcohols; ^c Number of identified compounds.

Table 4 shows the compound percentage distribution in the analyzed aerial dried parts. The percentage of sesquiterpenes in all the species was high, representing more the 40% in number of constituents in the mixture for all the samples. However, just the unique oxygenated compound, the caryophyllene oxide was found as major component for *P. barbatus* and *P. grandis* in concentration over 35% in both cases. We can see a distinct distribution among the three plants, the comparison between *P. barbatus* and *P. grandis* is closer qualitatively than to *P. ornatus*. Nevertheless, the presence of caryophyllene oxide shows a qualitative approach between the species, the similarity among *P. barbatus* and *P. grandis* remain even in this case. The presence of α -copaene is found for high level in the three species, while the most important sesquiterpene in *P. ornatus* is β -caryophyllene, representing almost 19% of the composition. The monoterpene α -pinene is the main monoterpene common to volatile fraction from dried aerial parts of all studied species. Among the three compared species we showed that they don't have the same volatile composition. *P. barbatus* has its secondary metabolites closer to *P. grandis* than to *P. ornatus*, mainly the sesquiterpenes α -copaene and D-germacrene in the fresh stage. In the dried leaves the volatile composition has a significant change, being α -copaene the most important metabolite in *P. grandis*. The monoterpene α -pinene was found as a major monoterpene in the volatile composition

of the dried parts in all studied species. It was found that *P. barbatus* chromatographic profile is more similar to *P. grandis* than to *P. ornatus*. However, the analysis of the EO's of dried material of the three species display a great increasing of caryophyllene oxide in comparison to fresh materials. Over 35.0% of this compound was found in *P. barbatus* and in *P. grandis*, being about 10.0% in *P. ornatus*. The presence of this sesquiterpene in fresh material of all species did not achieve 2.0 % of the composition. Some studies have report that β -caryophyllene started to oxidize immediately when air exposed and after 5 weeks almost 50% of the original compound was consumed. Caryophyllene oxide was shown to be an allergen of moderate strength and β -caryophyllene air exposed for 10 weeks showed a weak sensitizing capacity in the local lymph node assay (Sköld, 2006). Despite of the caryophyllene oxide was found to be the major oxidation products in dried parts of these plants, the chemical markers of these species are not the same. Thus, is important to evaluate the risks of these terpenes to people that use these plants in traditional medicine. This work enhances the importance of acknowledgment about taxonomy instead of trivial popular names for the species and call attention for the material stage used in folk preparations in order to avoid loss of the active compounds or chemical degradation processes and risk to the health.

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References

- Adams, R. P. 2004. Identification of Essential Oil Components by Gas Chromatography/Mass Spectroscopy. Allured, Carol Stream, IL, USA.
- Araújo, H. C., M. E. G. Lacerda, D. Lopes, H. R. Bizzo and M. A. C. Kaplan. 2007. Studies on the aroma of maté (*Ilex paraguariensis* St. Hil.) using headspace solid-phase microextraction. *Phytochem. Anal.* 18:469-474.
- Ascensão, L., A. C. Figueiredo, J. G. Barroso, L. G. Pedro, J. Schripsema, S. G. Deans and J. J. C. Scheffer. 1998. *Plectranthus madagascariensis*: morphology of the glandular trichomes, essential oil composition and its biological activity. *Int. J. Plant Sci.* 159:31-38.
- Ascensão, L., L. M. Mota and L. Castro. 1999. Glandular trichomes on the leaves and flowers of *Plectranthus ornatus*: Morphology, distribution and histochemistry. *Ann. Bot.* 84:437-447.
- Camara, C. C., N. R. Nascimento, C. L. Macedo-Filho, F. B. Almeida and M. C. Fonteles. 2003. Antispasmodic effect of the essential oil of *Plectranthus barbatus* and some major constituents on the guinea-pig ileum. *Planta Med.* 69:1080-1085.
- Lukhoba, C. W., A. Simmonds and J. Paton. 2006. *Plectranthus*: A review of ethnobotanical uses. *J. Ethnopharmacol.* 103:1-24.
- Chifundera, K. 2001. Contribution to the inventory of medicinal plants from the Bushi area, South Kivu Province, Democratic Republic of Congo. *Fitoterapia* 72:351-368.
- Codd, L. E. 1985. Lamiaceae. In: Leistner OA, ed. *Flora of soltem Africa*. Pretoria: Botanical Research Institute, Department of Agriculture and Water Supply 28:137-172.
- Corrêa, A. D., R. S. Batista and L. E. M. Quintas. 1998. Plantas Medicinais; Vozes: Rio de Janeiro 72-73.
- Dias-Maroto M. C., M. S. Coelho and M. D. Cabezudo. 2002. Headspace solid-phase microextraction analysis of volatile components of spices. *Chromatograph.* 55:723-728.
- Githinji, C. W. and J. O. Kokwaro. 1993. Ethnomedicinal study of major species in the family Labiatae from Kenya. *J. Ethnopharmacol.* 39:197-203.
- Loi, R. X., M. C. Solar and J. D. Weidenhamer. 2008. Solid-phase microextraction method for in vivo measurement of allelochemical uptake. *J. Chem. Ecol.* 34(1):70-75.
- Maia, J. G. S., L. S. Ramos, A. I. R. Luz, M. L. Da Silva and M. G. B. Zoghbi. 1988. Uncommon Brazilian essential oils of the Labiatae and Compositae. *Dev. Food Sci.* 18:177-188.
- Mathela, D. K., H. B. Kharkwal and C. S. Mathela. 1986. Terpenoids of roots of *Coleus forskohlii*. *Fitoterapia* 57:299-301.
- Oliveira, P. M., A. A. Ferreira, D. Silveira, S. B. Alves and D. S. Raslan. 2005. Diterpenoids from the Aerial Parts of *Plectranthus ornatus*. *J. Nat. Prod.* 68:588-591.
- Sartoratto, A. and F. Augusto. 2003. Application of headspace solid phase microextraction and gas chromatography to the screening of volatile compounds from some Brazilian aromatic plants. *Chromatograph.* 57:351-356.
- Sköld, M., A. T. Karlberg, M. Matura and A. Börje. 2006. The fragrance chemical – caryophyllene air oxidation and skin sensitization. *Food Chem. Toxicol.* 44:538-545.
- Soares, H. C. P., D. Felix, M. A. Kaplan, P. M. Margis and R. Margis. 2006. Authentication of Medicinal Plant Botanical identity by Amplified Fragmented Length Polymorphism Dominant DNA Marker:inferences from *Plectranthus* genus. *Planta Med.* 72:929-931.

PLANT SCIENCE

Cytotoxic activity in *Tagetes lucida* Cav. [◇]

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Abstract

Tagetes lucida (common name Santamaria) is a native herb widely used in the area with an important economic potential. It has several uses, such as, food, antiseptic, to control diseases infectious and spirituals. The objective of this work was to determine the agent of the cytotoxic activity from *T. lucida*. 17 straw extracts from different organs of *T. lucida* were obtained by cold maceration (methanol:chloroform), successive maceration (hexane, dichloromethane and methanol), infusion and extraction Soxhlet (ethyl acetate). The mostly of the plant extract were citotoxic to the crustacean *A. salina*. Hexanic extracts were notable for their cytotoxic effect. They were purified the 7-methoxycoumarin and 6,7-dimethoxycoumarin from leaf hexanic extract and both compounds caused citotoxicity in *A. salina*, with LC50 values of 28 and 238 µg/ml, respectively. We find that the cytotoxic effect observed in *T. lucida* is due to the coumarins.

Key words: Citotoxicity, coumarins, *T. lucida*

Introduction

It is common that any societies of any latitude have a rich tradition in medicinal plant utilization among its varied folk healing practices. A total of 3,000 species have been compiled in a database of medicinal plants employed by several Mexican ethnic groups (Lozoya and Martinez, 1994). Incredibly, of this plant group only approximately 1% of them have been studied in depth regarding their potential medicinal properties (Argueta, 1994). Into this medicinal plant core *Tagetes* spp, have been described as microbiocide and used for the treatment of gastrointestinal disorders.

Of the *Tagetes* genus there are several reports on its biocide activity. Some of them are related with their bactericide activity that including enterobactericide activity (Caceres et al., 1990, 1991). In the *Tagetes* genus, it has been research for new insecticides with specific characteristics, such as the terpenes specially limonene, caryophyllene and ocimene, which are directly responsible for this

larvicide activity against *Aedes aegypti*. All of them are not harm for the environment and they have a similar or a better effect than the conventional insecticides (Rai and Acharya, 1999; Hitmi et al., 2000; Dharmagadda et al., 2004; Caballero Gallardo et al., 2011). Also, it was reported that peperitone and piperitenone obtained from *T. patula* had antifungal activity against *Botrytis cinerea* and *Penicillium digitatum*. However, in this antifungal effect it was reported a synergy of these and others components of the essential oil (Mares et al., 2004). Likewise, in the effect antifungal observed in the extracts from *T. filifolia* were involved the piperitone and piperitenone with other antifungal metabolites detected in this plant such as terpinolene, dihydrotagetone, cis-tagetone, limonene and allo-ocimene (Kishore et al., 1993; Cespedes et al., 2006).

T. lucida (synonymous: *T. florida* Sweet, *T. schiedeana* Less.) (Santamaria, one of Mexican common names) is a native herb widely used in the area and has an important economic potential. It has several traditional uses, such as, food, antiseptic, to control diseases infectious, emotional and spirituals. *T. lucida* is one of the plants most used by the Latin-American population for the treatment of gastrointestinal disorders (Giron et al., 1991; Damian Badillo et al., 2008a). Sometimes, *T. lucida* is not possible differentiate it of *T. filifolia* or *T. micrantha* or even of *Artemisia drucunculus*, therefore is necessary to confirm the specie on

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which is working (Linares and Bye, 1987). *T. lucida* is an aromatic herb distributed naturally from Mexico to Honduras, at altitude between the 1000 and 2000 m. It has sessile, glabrous, oblong-lanceolate and opposite leaves (5-10 cm long) with yellow terminal flowers. It is known that *T. lucida* is an important source of antioxidant and antifungal compounds (Aquino et al., 2002; Damian Badillo et al., 2008b). In *T. lucida* thirty compounds were identified, the methyl chavicol (95-97%) was the major constituent and, from flower oil, two bithienyls were detected as minor constituents (Ciccio, 2005; Marote et al., 2010). Recently, it was reported that stems and leaves extracts ethanolic and aqueous from *T. lucida* were toxic to T47D and HeLa cell lines, also the leaves and root hexanic extracts were toxic to crustacean *A. salina* (Vega-Avila et al., 2009; Mejia Barajas et al., 2011). These authors only reported the cytotoxic activity of the plant extracts and not identified the biologically compound actives. In a continuation of our phytochemical work and of search of biological activities in *T. lucida*, the aim of this work was to determine the causal agent of its cytotoxic activity observed *in vitro*.

Materials and Methods

Plant extracts obtaining. Plants were collected at the 3.5 km of Tiripetio-Acuiztio road in Michoacan, Mexico at October of 2008 and 2010. A voucher specimen was deposited at the Herbarium of the Institute of Ecologia, A.C., Centro Regional del Bajio, Patzcuaro, Michoacan, Mexico. The plant organs (leaves, flowers, stems and root) were dried and each one was successively macerated for 3 days with solvents of ascendant polarity at temperature room, started with hexane, then ethyl acetate and finally with methanol. Also, it was done a cold maceration for 3 days of roots and flowers with mixture of methanol:chloroform (2:1 v:v). Soxhlet extracts were obtaining from leaves and stems with ethyl acetate at 79°C for 2 h. All these extracts were filtered and concentrated with evaporator rotary. Aqueous extracts were obtaining by infusion of each plant part and then they were concentrated by lyophilization.

Extraction, isolation and identification of pure compounds

The compounds isolation from *T. lucida* was done by chromatography column technic. 0.1 g leaves hexanic extract was chromatographed in a glass column (2 cm diameter x 8 cm large) was packed with 8 g of silica gel (70X230 Merk) as stationary phase and as mobile phase was a mixture of hexane and ethyl acetate in ascendant polarity.

Two chromatographic running were done; they were collected fractions of 10 mL each; the first were 134 and the second 87 fractions. Those fractions with some residue were analyzed by ¹HMRN. Compounds 1 and 2 were isolated from leaf hexanic extracts.

¹H and ¹³C magnetic nuclear resonance spectra were obtained in a Varian Mercury Plus 400 spectrometer. The ¹H and ¹³C NMR spectra of pure compounds were obtained at 400 MHz. Deuterated chloroform (CDCl₃) and tetramethylsilane (TMS) were the solvents of the plant metabolites and reference, respectively.

Compound 1. (7-methoxycoumarin). Yellow crystals, (hexane-ethylacetate); mp 110-112°C, (7% yield); ¹H-NMR (400MHz,CDCl₃), δ ppm: 7.63 (d, *J* = 9.4 Hz, 1H, H-4), 7.36 (d, *J* = 8.5 Hz, 1H, H-5), 6.81 (dd, *J* = 2.4, 8.5 Hz, 1H, H-6), 6.80 (d, *J* = 2.5 Hz, 1H, H-8), 6.23 (d, *J* = 9.4 Hz, 1H, H-3), 3.85 (s, 3H, OMe). ¹³C-NMR (400MHz CDCl₃), δ ppm: 162.23 (C-2), 162.05 (C-8a), 160.64 (C-7), 155.32 (C-4a), 142.84 (C-4), 128.16 (C-5), 112.51 (C-3), 112.03 (C-6), 100.24 (C-8), 55.20 (OMe).

Compound 2. (6,7-dimethoxycoumarin). Pale yellow needles (hexane-ethylacetate); mp 140-142°C, (2.5% yield); ¹H-NMR (400MHz,CDCl₃), δ ppm: 7.63 (d, *J* = 10.0 Hz, 1H, H-4), 6.86 (s, 1H, H-5), 6.84 (s, 1H, H-8), 6.30 (d, *J* = 10.0, 1H, H-3), 3.95 and 3.90 (s, 3H, OMe). ¹³C-NMR (400MHz CDCl₃), δ ppm: 161.35 (C-2), 152.80 (C-7), 149.96 (C-6), 146.29 (C-8a), 143.31 (C-4), 113.02 (C-4a), 111.38 (C-3), 107.94 (C-5), 99.95 (C-8), 56.31 (OMe), 56.31 (OMe). NMR data in agreement with those reported (Huang et al., 2006).

Citotoxicity assay

Eggs of *A. salina* [Salt Creek CoTM] and synthetic sea water Red Sea SaltTM [Red Sea Fish Pharm] were obtained in the local market. It was performed in accordance with the Meyer method (1982). Phototrophic nauplii (instar I and II) were collected from sea water with a light source (1000-4000 lux) at 25 °C for 24 h. 10 – 15 nauplii (100 µL) were placed in wells of an ELISA plate and 3 µL of the extracts dissolved in dimethyl sulfoxide (DMSO) were added (1, 10, 100 and 1000 µg). Berberine sulfate was used as a positive control and DMSO as negative control. The nauplii were incubated at room temperature for 24 h and the dead and live nauplii were counted. 100 µL of methanol were added to annihilate the surviving nauplii. Survival percentage was determined by the ratio of live nauplii between total nauplii (Geran et al., 1972).

Statistical analysis

In order to determinate the cytotoxic activity of the extracts plant and pure compounds, at least ten nauplii were tested. Concentration-response data were analyzed with the PROBIT program and a factorial analysis with $P < 0.01$ was used for determinate the statistical significance, and they are showed as mean $\pm$ standard error.

Results

It was obtained 17 extracts from *T. lucida* using several extraction methods. It was observed that the flower, leaf and root hexanic extracts, leaf and root dichloromethanic extracts and root metanolic extract, all of them had a lethal effect on the survival of *A. salina*, (Table 1). Ethyl acetate and aqueous extracts were innocuous for the crustacean. However, to determining the LC_{50} values on the crustacean, it was highlighted the hexanic extracts, see Table 2.

Table 1. *T. lucida* extracts tested against the survival of the crustacean *A. salina*.

Conc. $\mu\text{g}/\text{mL}$	Survival (%)															
	¹ Hexanic				¹ Dichloromethanic				¹ Methanolic				² Methanol/ Chloroform		³ Ethyl acetate	
	F	L	R	S	F	L	R	S	F	L	R	S	F	R	L	S
1000	3	0	0	0	0	0	0	0	44	92	0	0	3	0	0	3
100	3	10	0	89	30	5	0	92	100	95	52	19	38	73	57	97
10	63	59	76	100	82	80	97	100	100	100	85	70	100	100	93	100
1	93	88	100	100	92	100	100	98	100	100	92	90	100	100	92	100
0	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

R = roots, S = stems, L = leaves, F = flowers. 1 = Maceration with successive extraction, 2 = Cold maceration, 3 = Soxhlet extraction

Table 2. Median lethal concentration for *A. salina* of the *T. lucida* extracts.

Plant organ	LC_{50} ($\mu\text{g}/\text{mL}$)				
	¹ Hexane	¹ Dichloromethane	¹ Methanol	² Methanol/Chloroform	³ Ethyl acetate
Flowers	26	88	877	88	>1000
Leaves	16	52	>1000	>1000	223
Roots	40	64	96	386	>1000
Stems	509	672	46	>1000	590

1,2,3 = same annotation that in Table 1

By its lethality the leaves hexanic extract were selected for the purification of the major compounds with cytotoxic activity. They were isolated two major compounds; one was obtained of the chromatographic fraction 45 to 48 with polarity 80:20 and another was obtained of chromatographic fraction 19 to 22 with polarity 20:30, both with regard to the mixture of solvents hexane:ethyl acetate. They were identified by ^1H and ^{13}C NMR spectra as 7-methoxycoumarin and 6,7-dimethoxycoumarin (Figure 1).

It was observed that the coumarins had a lethal effect dependent of the concentration on the crustacean *A. salina* (Figure 2). 7-methoxycoumarin presented a LC_{50} value of 28 $\mu\text{g}/\text{mL}$ on *A. salina*, while the 6,7-dimethoxycoumarin had a LC_{50} value of 238 $\mu\text{g}/\text{mL}$.

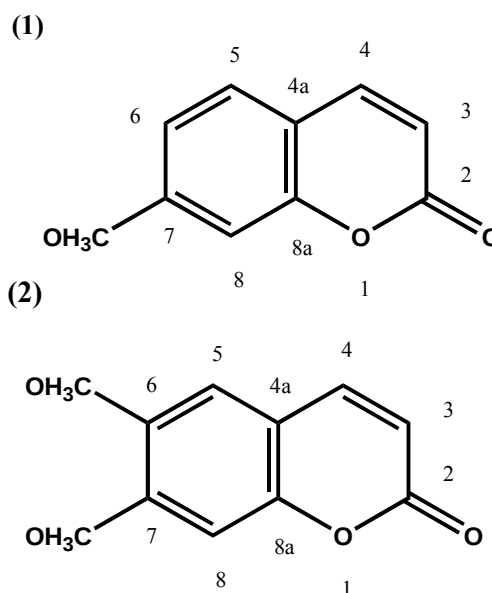


Figure 1. Coumarins from *T. lucida*. Major compounds isolated from *T. lucida* were 7-methoxycoumarin (1) and 6,7-dimethoxycoumarin (2).

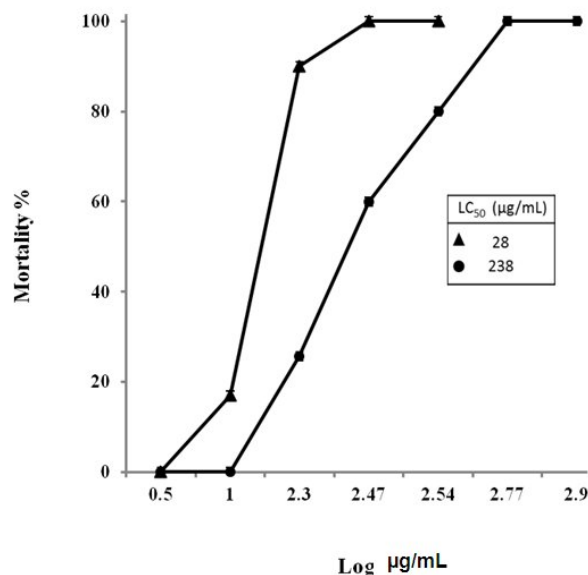


Figure 2. Response of *A. salina* to the coumarins purified from *T. lucida*. Concentration-response curves of 7-methoxy coumarin (▲) and 6,7-dimethoxy coumarin (●). Experiments were done for triplicate with 10 nauplii per replicate. Data are expressed as means of the percent of mortality $\pm$ s.e; $P < 0.01$ versus control.

Discussion

Forever, the plants are renewable resources and had been a valuable natural resource to human being, both for its feed, to cure its ills and several human uses. A lot native plants are used for medicinal properties, however, it is unknown their bioactive components. This fact is an opportunity area to search bioactive plant metabolites in the medicinal plants of ethnical pharmacopeias, specify bioactive metabolites with cytotoxic, antitumoral and anticancer activities.

T. lucida is one of the 28 species of *Tagetes* that habit in Mexico (Turner, 1996). In this country, it is used as medicinal, insecticide and ornamental plant. The economic importance of this plant is given for its potential as spice, nutraceutical and flowering herb. Today, this plant is used as a spice in the Southern states of USA, Costa Rica and Southern states of Mexico. It is cultivated commercially in Costa Rica as a spice herb and sold in the supermarket as a substitute of tarragon (*A. dracuncululus*). In Guatemala, the extracts of this plant are sold as infusion, tincture and elixir. These products are used for stomach pains, gastritis, menstrual pains, to treat infections and dysmenorrhea (Caceres, 1996). This species has shown an important chemical variability in according to geographic and ecologic distribution. This fact is an advantage chemical and agronomic

to select varieties plant and it is possible found novel secondary metabolites. Major secondary metabolites obtained of *T. lucida* collected from different sites belong to chemical families, such as; flavonoids, terpenes, phenolic compounds and coumarins (Abdala, 1999; Aquino et al., 2002; Ciccio, 2005; Cespedes et al., 2006).

Initially, it was observed that the plant extract obtaining by maceration successive process allowed detecting the cytotoxic activity form *T. lucida*. In this work we screened in the core plant extracts from *T. lucida* taken in count the Meyer criterion, which considered those plant extracts biologically actives that have an effect on the survival of *A. salina* at 1000 $\mu\text{g/mL}$ or less (Meyer et al., 1982). With this criterion all plant extracts had a cytotoxic effect but the exception was the leaves methanolic extract. However, all of them the most lethal were the flower, leaves and root hexanic extracts, leaves and root dichloromethanic extracts and finally the stem methanolic extract. A criterion second of scrutiny was taken in count. This is accorded for the United States National Cancer Institute plant screening program, it advice that a plant extract is generally considered to have a cytotoxic effect at IC_{50} value, following incubation between 48 to 72 h, of 20 $\mu\text{g/mL}$ or less (Geran et al., 1972). *T. lucida* leaf hexanic extract was selected to be fractioned for to search its compounds with cytotoxic activity. They were isolated from it, two coumarins; 7-methoxycoumarin and 6,7-dimethoxycoumarin. Coumarin such as 7-hydroxy coumarin and 7-methoxycoumarin had been reported in *T. lucida* and others *Tagetes* spp. The coumarins have different pharmacologic effects. It has been reported properties like antiinflammatory, antioxydants, antiallergics, hepatoprotectors, antivirals, cytotoxic, antitumoral, anticancer and sensibilizers to ultraviolet light (Mohler et al., 1992; Thornes et al., 1994; Maucher and Von, 1994; Kofinas et al., 1998; Kasahara et al., 2002; Kostova, 2005; Uchiyama et al., 2002).

Pure 7-methoxycoumarin and 6,7-dimethoxycoumarin from *T. lucida* had a cytotoxic effect that depended of the concentration. The cytotoxic effect was different between the pure coumarins, because that 28 $\mu\text{g/mL}$ 7-methoxycoumarin cause the mortality of the 50% of the nauplii, while the 238 $\mu\text{g/mL}$ 6,7-dimethoxycoumarin cause the same effect. This result is agreed with the report of Watanabe et al. (2005). They mentioned that the oxygen-containing functional group at the 7-position of the coumarin core was advantageous for the cytotoxic activity.

The US NCI plant screening program has established that, a pure compound is generally considered as candidate molecule to continue investigating it as potential anticancer agent if it has a cytotoxic effect with a LC_{50} value, following between 48 to 72 h of 4 $\mu\text{g/mL}$ or less (DTP NCI/NIH). In accord to this criterion broadly accepted and based on our results, it is conclude that the 6,7-dimethoxycoumarin and 7-methoxycoumarin from *T. lucida* had of lower to moderate cytotoxic effect on *A. salina*, respectively.

However, the coumarins from *T. lucida* are susceptible of chemical modification for increase their cytotoxic activity on *A. salina* and tumor human cell lines, but innocuous to normal human cell lines. Currently, we are working on this aspect of the semi synthesis chemical of coumarins from *T. lucida* with these biological activity characteristics.

Nutritional aspect of *T. lucida* is a pharmacologically and nutriaceutiacally important if it is used as spice; because this plant could contributes to maintenance of health human being. For all this, *T. lucida* is proposal as an alternative plant culture new with advantages with regard to other regional and conventional crops. This work contributes to basic information for to promote it as crop and its use as herbal remedy, nutraceutical and food reinforcement in accord to official standard.

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References

Abdala, L. R. 1999. Flavonoids of the aerial parts from *Tagetes lucida* (Asteraceae). Biochem. Syst. Ecol. 27:753-754.

Aquino, R., A. Caceres, S. Morelli and L. Rastrelli. 2002. An extract of *Tagetes lucida* and its phenolic constituents as antioxidants. J. Nat. Prod. 65:1773-1776.

Argueta, A. 1994. Two decades of Mexican ethnobotany and research on plant derived drugs. Atlas de las plantas de la medicina tradicional mexicana. 3:172.

Caballero Gallardo, K., J. Olivero Verbel and E. E. Stashenko. 2011. Repellent activity of essential oils and some of their individual

constituents against *Tribolium castaneum* Herbst. J. Agric. Food Chem. 59:1690-1696.

Caceres, A., O. Cano, B. Samayoa and L. Aguilar. 1990. Plants used in Guatemala for the treatment of gastrointestinal disorders. 1. Screening of 84 plants against enterobacteria. J. Ethnopharmacol. 30:55-73.

Caceres, A., A. V. Alvares, E. Ovando and B. E. Samayoa. 1991. Plants used in Guatemala for the treatment of respiratory diseases. 1. Screening of 68 plants against gram-positive bacteria. J. Ethnopharmacol. 31:93-208.

Caceres, A. 1996. Plantas de Uso Medicinal en Guatemala. Editorial Universitaria, Guatemala. 305-307.

Cespedes, C. L., J. G. Avila, A. Martinez, B. Serrato, J. C. Calderon-Mugica and R. Salgado-Garciglia. 2006. Antifungal and antibacterial activities of Mexican tarragon (*Tagetes lucida*). J. Agric. Food Chem. 54:3521-3527.

Ciccio, J. F. 2005. A source of almost pure methyl chavicol: volatile oil from the aerial parts of *Tagetes lucida* (Asteraceae) cultivated in Costa Rica. Rev. Biol. Trop. 52:853-857.

Damian-Badillo, L. M., R. Salgado-Garciglia, R. E. Martinez-Muñoz and M. M. Martinez-Pacheco 2008a. Antifungal properties of some Mexican medicinal plants. The Op. Nat. Prod. J. 1:27-33.

Damian-Badillo, L. M., R. M. Espinosa-Madrigal, R. E. Martinez-Muñoz, O. A. Ron-Echeverria, R. Salgado-Garciglia, A. Flores-Garcia, D. Raya-Gonzalez and M. M. Martinez-Pacheco. 2008b. The Mexican medical plants with antifungal properties are an economic and health opportunity area. Pharmacologyonline 3:61-77.

Dharmagadda, V. S., S. N. Naik, P. K. Mittal and P. Vasudevan. 2004. Larvicidal activity of *Tagetes patula* essential oil against three mosquito species. Biores. Technol. 96:1235-1240.

Geran, R. I., H. M. Greenberg, M. McDonald and B. J. Abbot. 1972. Protocols for screening chemical agents and natural products against animal tumors and other biological systems. Cancer Chemoth. Rep. 33-117.

Giron, L. M., V. Freire, A. Alonzo and A. Caceres. 1991. Etnobotanical survey of the medicinal

- flora used by the Caribs of Guatemala. J. Ethnopharmacol. 34:173-187.
- Huang, C. G., C. H. Ma, W. Ke, Z. L. Sun, J. Y. Peng, Z. X. Li, X. Zhou, G. R. Fan. 2006. Large-scale isolation and purification of scoparone from herba artemisiae scopariae by high-speed counter-current chromatography. Chromatograph. 64:83-87.
- Hitmi, A., A. Coudret and C. Barthomeuf. 2000. The production of pyrethrins by plant cell and tissue cultures of *Chrysanthemum cinerariaefolium* and *Tagetes* species. Crit. Rev. Biochem. Mol. Biol. 35:317-337.
- Kasahara, Y., K. Yasukawa, S. Kitanata, M. Taufiq-Khan and F. Evans. 2002. Effect of methanol extract from flower petals of *Tagetes patula* L. on acute and chronic inflammation model. Phytother. Res. 16:217-222.
- Kishore, N., A. K. Mishra and J. P. Chansouria. 1993. Fungitoxicity of essential oils against dermatophytes. Mycoses 36:211-215.
- Kofinas, C., I. Chinou, A. Loukis, C. Harvala, C. Roussakis, M. Maillard and K. Hostettmann. 1998. Cytotoxic coumarins from the aerial parts of *Tordylium apulum* and their effects on a non-small-cell bronchial carcinoma line. Planta Med. 64:174-176.
- Kostova, I. 2005. Synthetic and natural coumarins as cytotoxic agents. Anti-Cancer Agents 5:29-46.
- Linares, E. and R. A. Bye. 1987. A study of four medicinal plant complexes of Mexico and adjacent United States. J. Ethnopharmacol. 19:153-183.
- Lozoya, X. 1994. Two decades of Mexican ethnobotany and research on plant derived drugs. Ethnobotany and the search for new drugs. Ciba Foundation Symposium. 185.
- Mares, D., B. Tosi, F. Poli, E. Andreotii and C. Romagnoli. 2004. Antifungal activity of *Tagetes patula* extract on some phytopathogenic fungi: ultrastructural evidence on *Phytium ultimum*. Microbiol. Res. 159:295-304.
- Martinez, M. 1993. Las Plantas Medicinales de Mexico. Botas Eds, Mexico. 6:656-662.
- Maucher, A. and E. Von Angerer, 1994. Antitumour activity of coumarin and 7-hydroxycoumarin against 7,12-dimethylbenz[a]anthracene-induced rat mammary carcinomas. J. Cancer Res. Clin. Oncol. 120:502-504.
- Marote, I., M. Marotti, R. Piccaglia, A. Nastri, S. Grandi and G. Dinelli. 2010. Thiophene occurrence in different *Tagetes* species: agricultural biomasses as sources of biocidal substances. J. Sci. Food Agric. 90:1210-1217.
- Meyer, B. N., N. R. Ferrigni, J. E. Putnam, L. B. Jacobsen, D. E. Nichols and J. L. McLaughlin. 1982. Brine shrimp: a convenient general bioassay for active plants constituents. Planta Med. 45:31-34.
- Mejia-Barajas, J. A., J. M. Cajero, R. E. del Rio and M. M. Martinez-Pacheco. 2011. Escrutinio de la actividad toxica de *Tagetes lucida* en *Artemia salina*. Rev. Latinoamer. Quim. 38:95.
- Mohler, J. L., L. G. Gomella, E. D. Crawford, L. M. Glode, C. D. Zippe, W. R. Fair and M. E. Marshall. 1992. Phase II evaluation of coumarin (1,2-benzopyrone) in metastasis prostatic carcinoma. Prostate 20:123-31.
- Rai, M. K. and D. Acharya. 1999. Screening of some Asteraceous plants for antimycotic activity. Compositae Newslet. 34:37-43
- Thornes, R. D., L. Daly, G. Lynch, B. Breslin, H. Browne, H. Y. Browne, T. Corrigan, P. Daly, G. Edwards and E. Gaffney. 1994. Treatment with coumarin to prevent or delay recurrence of malignant melanoma. J. Cancer Res. Clin. Oncol. 120:32-34.
- Turner, B. 1996. The Comps of Mexico: A systematic account of the family Asteraceae. Phytol. Memoirs 6:1-93.
- Vega-Avila, E., A. Espejo-Serna, F. Alarcon-Aguilar and R. Velasco-Lezama. 2009. Cytotoxic activity of four Mexican medicinal plants. Proc. West. Pharmacol. 52:78-82.
- Uchiyama, T., S. Hara, M. Makino and Y. Fujimoto. 2002. Seco-adianane-type triterpenoids from *Dorstenia brasiliensis* (Moraceae). Phytochem. 60:761-764.
- Watanabe, J., H. Shinmoto and T. Tsushida, 2005. Coumarin and flavone derivates from estragon and thyme as inhibitors of chemical mediator release from RBL-2H3 cells. Biosci. Biotechnol. Biochem. 69:1-6.

PLANT SCIENCE

Public health and heavy metals in urban and periurban horticulture

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Abstract

The health effects of heavy metals can be complex and severe. Exposure to heavy metals has been linked with developmental retardation, various cancers, kidney damage, and even death in some instances of exposure to very high concentrations. Soils in urban and suburban areas are transformed by human activities, they are characterized by a strong spatial heterogeneity resulting from the various inputs of exogenous materials and the mixing of original soil material, and they often hold pollutants that may be a threat to human health. The objective of this work is to present a study of heavy metals occurrence in 33 urban and periurban soils dedicated to horticulture in Buenos Aires (Argentina). Total heavy metal content in soils (cadmium, chromium, copper, nickel, lead and zinc) was evaluated using Sequential Plasma Emission Spectrometer (ICP-ES). Data were analyzed using multivariate analysis showing a primary separation in two groups: eight sites with high contents of one or more of the analyzed heavy metals and the second group with lower levels of heavy metals. Maximum values of Cd, Pb, Zn and Cu in horticultural soils resulted problematic with reference to public health.

Key words: Heavy metals, Public health, Urban horticulture

Introduction

Heavy metals are substances that can contaminate our environment and damage our health. The addition of heavy metals to the cultivated soil during the 20th century caused an increase of cadmium content of 30%, and for lead of 15% in soil (Logardt, 2007).

The health effects of heavy metals can be complex and severe. Exposure to heavy metals has been linked with developmental retardation, various cancers, kidney damage, and even death in some instances of exposure to very high concentrations. Exposure to high levels of mercury and lead has also been associated with the development of autoimmunity, in which the immune system starts to attack its own cells, mistaking them for foreign invaders. Autoimmunity can lead to the development of diseases of the joints and kidneys, such as rheumatoid arthritis, or diseases of the circulatory or central nervous systems (WRI, 1999).

Heavy metals pose a major concern in public health from acute and chronic toxicity and the wide

variety of sources of exposure. The metals with environmental exposure, primarily through food, are lead (Pb), chromium (Cr), cadmium (Cd), and mercury (Hg). Cr and Cd are considered carcinogenic for World Health Organization, while Pb and Hg are especially concerned by their neurotoxicity (Zubero Oleagoitia et al., 2008).

Current guidelines for toxicity are based on earlier attempts to predict the soil concentrations of heavy metals where a measurable effect can influence human health. Trace elements in soils may or may not enter the root tissues and then move to the xylem. Some are often transported to the leaves (e.g. Zn, Cd, Co, B, Mo) whilst others (e.g. Cr, Pb, Hg and Cu) typically have limited mobility to aerial parts of the plant. It is particularly difficult to identify critical contamination limits for concentrations of heavy metals in soils, due to physico-chemical and biological variables which control bioavailability and potential toxicity (Dickinson et al., 2000).

Soils in urban and suburban areas are transformed by human activities; they are characterized by a strong spatial heterogeneity resulting from the various inputs of exogenous materials and the mixing of original soil material. The evolution of urban soils is controlled by the same factors as natural soils, but the human factor imposes extremely rapid transformation cycles in comparison with those dominant under natural

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conditions. They often hold pollutants that may be a threat to human health (Morel et al., 2005).

The rate of absorption of heavy metals by vegetables seems to be linked with their levels in the soil. Lead is taken up by the plant roots and is then transported to the leaves. Lead from traffic fumes in the air settles on the leaves. It can be washed away by watering the leaves, especially when the leaf surface is waxy (cruciferous plants, Alliums). Cadmium can be taken up by plants through roots and leaves. For these two very poisonous heavy metals with no positive biological functions, their presence in plants is controlled by respecting the soil standards (Tixier and de Bon, 2006).

The objective of this work is to present a study of heavy metals values in 33 urban and periurban soils dedicated to horticulture in Buenos Aires, Argentina.

Materials and methods

Soil Samples

Three soil samples were taken per location, in an acre homogeneous area, geo-referenced with GPS. Each sample was composed of three sub-samples that were taken at a depth of 0-20 cm. The soil samples were extracted with a stainless steel borehole, were air dried for 24 to 48 hours, passed by the mortar and sieved by 2 mm. Sampling situations are presented in Figure 1.

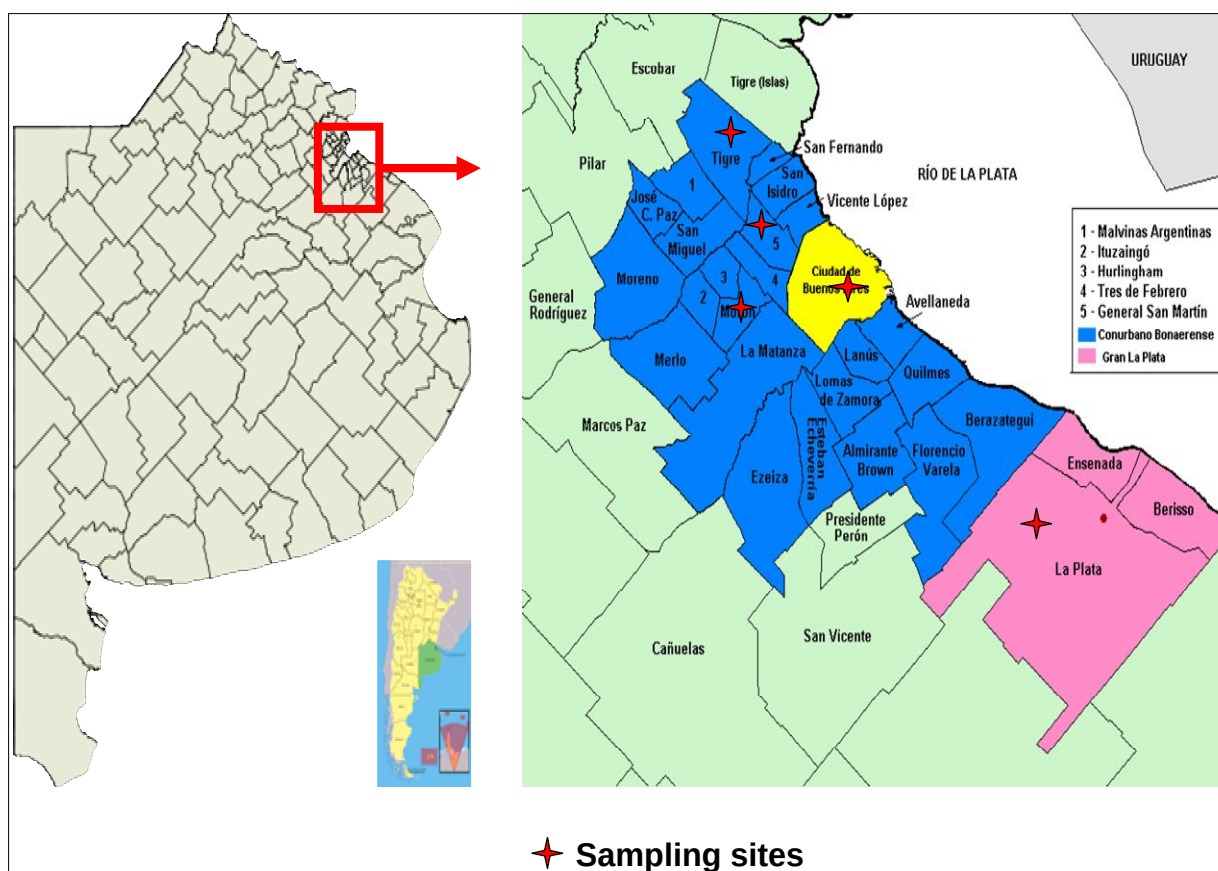


Figure 1. Argentine and Buenos Aires province, periurban samples of La Plata (# 1-22), periurban samples of Buenos Aires (# 23-29) and urban samples of Buenos Aires (# 30-33).

Chemicals

A dried and ground sample (0.5 gram) and 10 ml of aqua regia acid solution (3:1 HCl 37%:HNO₃ 70%) were added into a 50 ml teflon digestion vessel. The mixture was heated at 120-130 °C until nearly dryness and then was treated with 7 ml of 20% HCl (37%) and re-warmed at 80° C for 20

min. After cooling, the solution was hand mixing and filtered through Whatman 40 filter paper into a 10 ml volumetric flask, rinsed and made up to volume with deionised water (McGrath et al. 1994).

Apparatus

This solution was analysed by ICP-ES Baird 2070 to evaluate heavy metal content in soils (cadmium, chromium, copper, nickel, lead and zinc). The calibration solution was prepared by diluting Certipur® ICP multi-element standard solution IV, and in order to monitor the effectiveness of method, reference material NIST 2704 with a recovery of 88 to 91%, was used.

Statistical

Data were processed using the Infostat statistics program (2007). The data were firstly analysed using multivariate analysis (cluster and principal component analysis). The cluster analysis grouped the treatments according to the values of a set of measured soil variables. This analysis is often used as exploratory data method in order to obtain more knowledge about the structure of the observations under study. This technique allows grouping the study units with the maxima similarity under some criterion. To have a better understanding of the cluster separation, a principal component analysis provide a graph where both,

treatments and soil variables, can be displayed to study the correlation among the soil variables, and the association between treatments and soil measurements. Then, to compare the soil heavy metals content, the data were firstly checked for normality and then subjected to analysis of variance (ANOVA). The separation of treatments means was carried out by the Tukey test ($P < 0.05$).

Results and discussion

Heavy metals data were analysed using multivariate analysis with cluster analysis, which allows grouping objects described by a set of values of several variables. The grouping of objects is often used as exploratory data method in order to obtain more knowledge about the structure of the observations in the study. This technique brings together units of study with maximal similarity.

Figure 2 shows a primary separation in two groups. The first one includes all the horticulture sites with high contents of one or more of the analysed heavy metals. The second group considers the situation with lower levels of heavy metals.

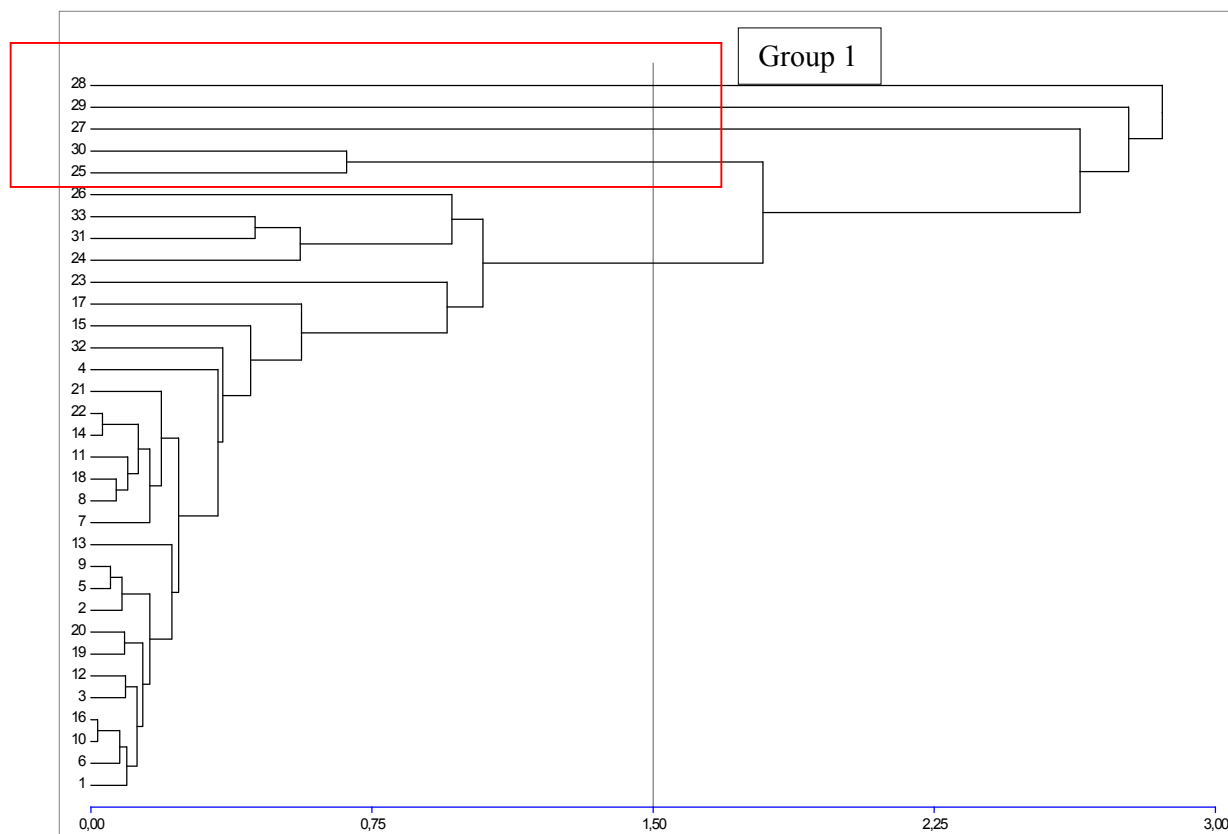


Figure 2. Cluster analysis of the heavy metals for the horticulture sites.

In Figure 3, as a result of the principal component analysis, it can be noticed that one of the studied situations (29) presents the highest level of Cu. The horticultural site number 27 presented the highest value for Pb, while the situation 28 had the maximum value for Cr. Both situations 25 and

30, presented the highest levels for Zn and Ni. The Cd values were maximum in four of the studied situations: 24, 25, 30, and 31, with higher values than 1.90 mg kg^{-1} . All samples with elevated levels of the mentioned heavy metals were from periurban and urban sites of Buenos Aires.

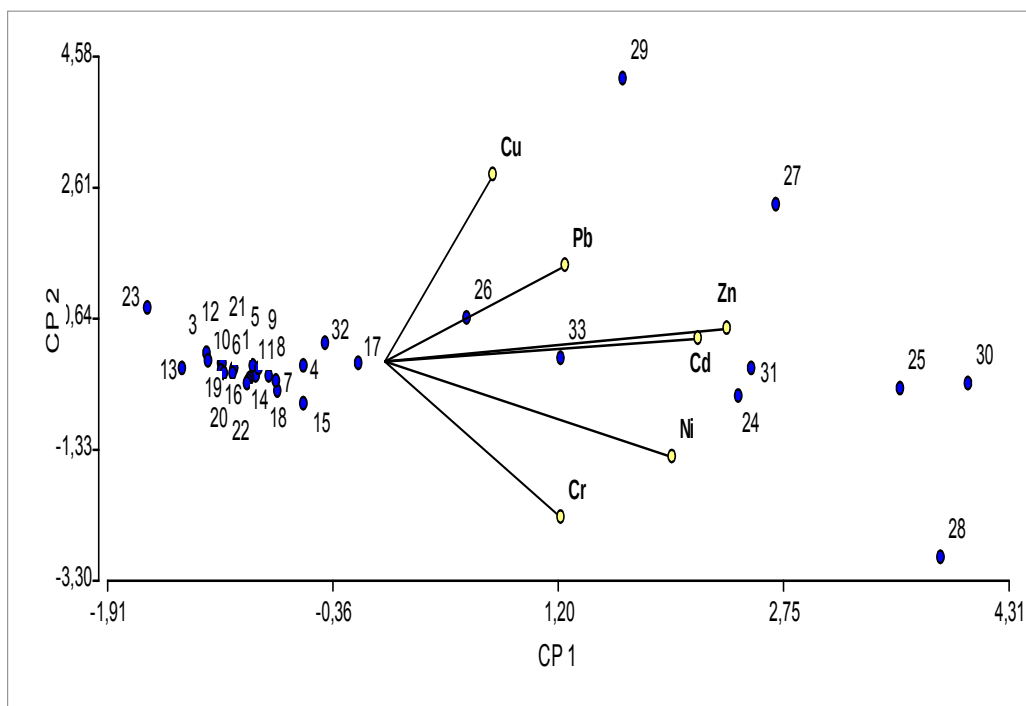


Figure 2. Bi-plot from the principal component analysis considering the heavy metals analyzed for all the horticulture sites.

Toxic metals, including "heavy metals," are individual metals and metal compounds that negatively affect people's health. Cadmium is an extremely toxic metal commonly found in industrial workplaces, particularly where any ore is being processed or smelted. Cadmium hazards may be present in a number of seemingly unrelated operations and materials, as phosphate fertilizers. Due to its low permissible exposure limit, overexposures may occur even in situations where trace quantities of cadmium are found (OSHA, 2009).

Soil samples presented low values of Cd, sometimes non detectable, for that reason are included in Group 2, but as it presents a low permissible exposure level, overexposures may occur with trace quantities. Maximum values, in a range of $1.90\text{--}2.16 \text{ mg kg}^{-1}$, are lower than the guideline soil value of 3 mg kg^{-1} presented by the Hazardous Waste Argentine law (Ley 24051, 1992). These cadmium levels, although lower than those supported by law, could indicate anthropogenic

contributions according to the limit of 0.8 mg kg^{-1} proposed by Vegter (1995), so these samples should be considered in terms of abnormal concentration and subsequent risk. Cadmium concentrations in soils not contaminated by anthropogenic sources range from 0.06 to 1.1 mg/kg, with a minimum of 0.01 mg/kg and a maximum of 2.7 mg/kg as established by Alloway and Steinnes (1999).

According to Zubero Oleagoitia et al. (2008), higher levels of Cd and Pb were found in resident population of urban areas indicating that vegetable consumption of local products and soil contamination could be associated with this increase in heavy metals.

Lead adversely affects numerous body systems and causes forms of health impairment and disease that arise after periods of exposure as short as days (acute exposure) or as long as several years (chronic exposure). Long term (chronic) overexposure to lead may result in severe damage to the blood-forming, nervous, urinary, and

reproductive systems. In general populations, lead may be present in hazardous concentrations in food, water, and air (OSHA, 2009).

Studies have shown that lead does not readily accumulate in the fruiting parts of vegetable and fruit crops (e.g., corn, beans, squash, tomatoes, strawberries, and apples). Higher concentrations are more likely to be found in leafy vegetables (e.g., lettuce) and on the surface of root crops (e.g., carrots). At high concentrations, lead is a potentially toxic element to humans and most other forms of life. For this reason, there is a need to be concerned about elevated lead levels in the

environment, particularly in metropolitan areas. The Minnesota State Legislature has established a soil standard of 100 mg kg^{-1} , currently lower than the levels used by the United States Environmental Protection Agency and most other States (Rosen, 2010).

Figure 4 presents lead content of the studied soils. It was found a maximum value of Pb of 676 mg kg^{-1} , high for quality soil guide of Argentina, and considered an excessive level as Angelone and Bini (1992). They are also worrying, as stated above, four soil samples in the range of $100\text{-}137 \text{ mg kg}^{-1}$ Pb.

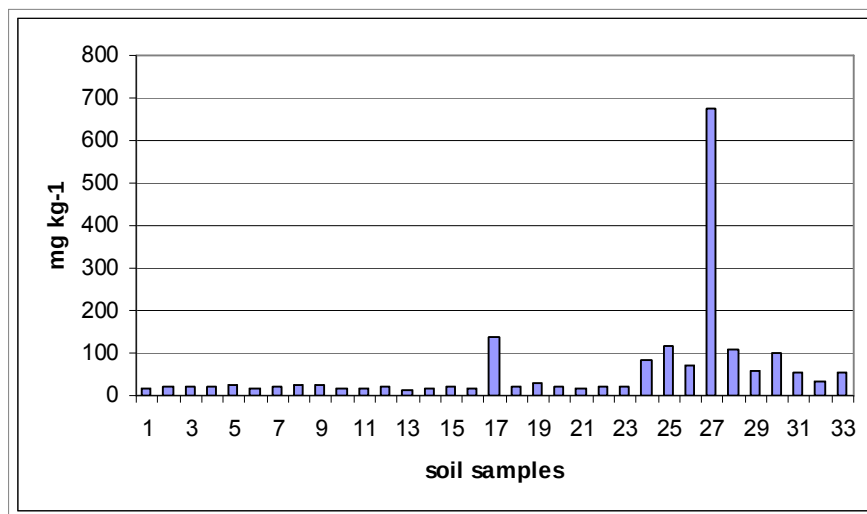


Figure 4. Lead content in horticultural soils.

With regard to heavy metal chromium, trivalent Cr has a low order of toxicity, but hexavalent form is extremely toxic. There is no evidence, however, that Cr normally present in food is a danger to health (Reilly, 2002). The maximum content of Cr found in horticultural soils: 116 mg kg^{-1} , presented a non-alarming value, is acceptable according to Argentine law, and is within the normal range considered by Alloway (1995).

Copper is an essential micronutrient involved in a variety of biological processes indispensable to sustain life. At the same time, it can be toxic when present in excess, the most noticeable chronic effect being liver damage (de Romaña et al., 2011). Copper is readily absorbed from the stomach and small intestine. Although copper homeostasis plays an important role in the prevention of copper toxicity, exposure to excessive levels of copper can result in a number of adverse health effects including liver and kidney damage, anemia,

immunotoxicity. One of the most commonly reported adverse health effect of copper is gastrointestinal distress (ATSDR, 2008). Maximum value of Cu found in horticultural soil was elevated (688 mg kg^{-1}), indicates contamination for Argentine law, and along with another value of 103 mg kg^{-1} , are considered as excessive levels for Angelone and Bini (1992).

Nickel normally occurs at very low levels in the environment, so very sensitive methods are needed to detect nickel in most environmental samples. A lot of nickel released into the environment ends up in soil or sediment where it strongly attaches to particles containing iron or manganese. Under acidic conditions, nickel is more mobile in soil and might seep into groundwater. Food contains nickel and is the major source of nickel exposure for the general population, but the most serious harmful health effects from exposure to nickel, as reduced lung function and cancer, have occurred in people who have breathed dust

containing certain nickel compounds while working in nickel refineries or nickel processing plants (ATSDR, 2005 a). In this study, maximum value of Ni: 17 mg kg⁻¹ resulted very low for argentine law and in a normal range for Alloway (1995).

Zinc enters the air, water, and soil as a result of both natural processes and human activities. Food is a source of human exposition to zinc compounds. If large doses of zinc are taken by mouth even for a short time, stomach cramps, nausea, and vomiting may occur. Ingesting high levels of zinc for several months may cause anemia, damage the pancreas, and decrease levels of high-density lipoprotein (HDL) cholesterol (ATSDR, 2005b). Maximum value of 220 mg kg⁻¹ found in horticultural soils, is considered low for argentine regulations, but near to excessive as Angelone and Bini (1992).

As a basis to identify and assess soil contamination processes at regional level Mic  et al (2010) proposed baseline values for heavy metals in Alicante (Spain), a representative agricultural area of the European Mediterranean region. Taking into consideration the heavy metals considered in this work, the baseline values were : 0.7 mg kg⁻¹ for Cd , 36 mg kg⁻¹ for Cr, 28 mg kg⁻¹ for Cu, 31 mg kg⁻¹ for Ni, 28 mg kg⁻¹ for Pb, and 83 mg kg⁻¹ for Zn. These low baseline values for agricultural soils remark the importance to perform this work for different soils in urban and periurban environments.

De Zeeuw and Lock (2000) suggested a number of prevention and control measures that can be applied in urban horticultural systems to help produce safe and healthy products: test soils and irrigation water for heavy metals, establish minimum distances between field and main roads, increase pH to immobilise heavy metals, wash contaminated crops to reduce metal content.

Urban horticulture has positive social impacts for the inclusion of different urban sub-communities into a social organisation. Horticulture is practised for home-consumption but very often also for the market as high-value cash crops. In developing countries, the consumption of vegetables is generally lower than the FAO recommendation of 205 g/day/capita (Tixier and de Bon, 2006).

As vegetable consumption should be increased and urban and periurban soils are relevant for horticulture production, an interdisciplinary approach must be focused in order to avoid contamination of natural resources soil and water

and protect public health limiting the exposition to metals through related food chain.

Conclusions

According to Argentine regulations, maximum values of Pb and Cu in soils are considered excessive. Both corresponded to soil samples in periurban sites of Buenos Aires that have received industrial waste disposal, and are not suitable for orchard production.

There are also other samples referring to Cd, Zn and Pb in urban and periurban sites of Buenos Aires, which could result problematic considering more exigent international guides, regarding to human exposure through food consumption in contaminated soils and potential environmental problems related to migration to groundwater.

In periurban soils of La Plata, total heavy metal content in soil resulted at a low level, the lowest ones corresponding to organic farms.

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References

- Alloway, B. J. and E. Steinnes. 1999. Anthropogenic additions of cadmium to soils. In: M. J. Singh and B. R McLaughlin (Eds.), pp.97-123. Cadmium in soils and plants. Kluwer Academic Publishers, Dordrecht, The Netherlands.
- Alloway, B. J. 1995. Heavy Metals in Soils. p. 368. 2nd Ed. Blackie, London.
- Angelone, M. and C. Bini. 1992. Trace element concentrations in soils and plants in Western Europe. In: D. C. Adriano (Ed.) pp.19-60. Biogeochemistry of Trace Metals, Lewis Publishers, Boca Raton.
- ATSDR. 2005a. Toxicological profile for nickel. Pp.397 pdf [http://tobacco-information.bhp.doh.gov.tw/toxicfolder/ http://www.atsdr.cdc.gov/es/phs/es_phs13.html](http://tobacco-information.bhp.doh.gov.tw/toxicfolder/http://www.atsdr.cdc.gov/es/phs/es_phs13.html)
- ATSDR. 2005b. Zn. pp.7 <http://www.atsdr.cdc.gov/toxprofiles/tp60-c1-b.pdf>
- ATSDR. 2007. Agency for Toxic Substances and Disease Registry. Plomo. http://www.atsdr.cdc.gov/es/phs/es_phs13.html
- ATSDR. 2008. Agency for Toxic Substances and Disease Registry. Toxicological profile for

- Cu. <http://www.atsdr.cdc.gov/toxprofiles/tp132-c2.pdf>
- De Romaña, D. L., M. Olivares, R. Uauy and M. Araya. 2011. Risks and benefits of copper in light of new insights of copper homeostasis. *J. Trace Elem. Med. Biol.* 25:3-13.
- De Zeeuw, H. and K. Lock. 2000. Urban and periurban agriculture, health and environment. Discussion paper for FAO-ETC/RUAF electronic conference "Urban and Periurban Agriculture on the Policy Agenda", 21 August–30 September 2000. 10p.
- Dickinson, N. M., J. M. Mackay, A. Goodman and P. Putwain. 2000. Planting trees on contaminated soils: issues and guidelines. *Land Contam. Reclam.* 8:87-101.
- InfoStat. 2007. InfoStat, versión 1.1. Manual del Usuario. Grupo InfoStat, FCA, Universidad Nacional de Córdoba. Primera Edición, Editorial Brujas Argentina.
- Ley 24051. 1992. Residuos peligrosos. Decreto 831/93. Reglamentación de la Ley N° 24.051. Bs. As. 23/4/93. <http://www.infoleg.gov.ar/>
- Logardt, S. 2007. Heavy metals in organic vegetable production connected to KRAV in the south of Skåne. Dept. of Landscape management, Design and Construction, SLU. Bachelor Project in the Danish-Swedish Hort. Program Vol. 2007: 5.
- McGrath, S. P., A. C. Chang, A. L. Page and E. Witter. 1994. Land application of sewage sludges: scientific perspectives of heavy metal loadings limits in Europe and the United States. *Environ.* 2:108-118.
- Micó, C., M. Peris, L. Recatalá and J. Sánchez. 2010. Baseline values for heavy metals in agricultural soils in a European Mediterranean region. *Sci. Total Env.* 409:9-18.
- Morel, J. L., C. Schwartz, L. Florentin and C. de Kimpe. 2005. Urban soils. In: Elsevier Ltd. Daniel Hillel (Ed.) Vol 4:202-208. *Encyclopedia of Soils in the Environment*.
- OSHA. Occupational Safety and Health Administration. 2009. <http://www.osha.gov/SLTC/metalsheavy/index.html>
- Page, L. 1982. Methods of soil analysis. Chemical and microbiological properties. American Society of Agronomy, Soil Science Society of America, Madison, Wisconsin. pp.1159.
- Reilly, C. 2002. Metal contamination in food. 3rd Ed. Blackwell Science UK. pp.266.
- Rosen C. J. 2010. Lead in the home garden and urban soil environment. Univ. Minnesota extension, <http://www.extension.umn.edu/>
- Tixier, P. and H. de Bon. 2006. Urban Horticulture. In: René van Veenhuizen (Ed.) pp.313-346. *Cities farming for the future. Urban agriculture for Green and Productive Cities. IIRR/RUAF/IDRC 2006*.
- Vegter, J. J. 1995. Soil protection in the Netherlands. In: W. Salomons (Ed.). Pp. 79-99. *Heavy metals. Problems and solutions*. Springer –Verlag.
- WRI. World Resources Institute. 1999. <http://www.wri.org/publication/content/8375>.
- Zubero Oleagoitia, M. B., J. J. Aurrekoetxea Agirre, J. M. Ibarluzea Maurologoitia, M. J. Arenaza Amezaga, M. Basterretxea Irurzun, C. Rodríguez Andrés and J. R. Sáenz Domínguez. 2008. Metales pesados (Pb, Cd, Cr y Hg) en población general adulta próxima a una planta de tratamiento de residuos urbanos de Bizkaia. *Rev. Esp. Salud Publica.* 82:481-492.

ANIMAL SCIENCE

Some productive traits of crossbred dairy cows in the farm of University of Khartoum, Sudan

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Abstract

This study was carried out on some productive traits of two different crossbred dairy cows having 62.5% and 50% foreign blood at University of Khartoum farm, Sudan. The effect of lactation length on total milk yield was found to be highly significant ($P < 0.01$). Effect of foreign blood percentage on total milk yield was found to be significant ($P < 0.05$). Age at first calving had non-significant effect on total milk yield. The regression analysis of lactation length showed that there is non-significant effect of age at first calving and foreign blood percentage on lactation length. Crossbred dairy cows which have 62.5% Friesian blood performed better than 50% Friesian blood and secured advanced age at first calving.

Key words: Age at first calving, Cattle, Lactation length, Milk yield

Introduction

The demand for liquid milk and its products increased sharply nowadays with the increase in population worldwide. There are many constraints facing dairying in Sudan, which can be classified as genetic and environmental attributes and a wide array of shortcomings including endemic diseases, poor nutrition and competent veterinary services (Elemam, 2002).

The selective breeding and crossbreeding are the main tools to enhance the milk production potentiality of tropical indigenous cattle. Marked improvement in production in dairy cattle has been reported, through crossbreeding (Ali et al., 1988).

In developing countries, the performance of high-yielding breeds imported from countries with highly advanced production systems is often negatively affected due to genotype-environment interactions (Smith, 1988; Bondoc et al., 1989; Rege, 1991; Ojango et al., 2005).

It was obvious that the crossbred cattle can perform satisfactorily under local Sudanese environment, and their yields were higher than that

of pure local breeds.

This study was undertaken in a herd of Friesian / local crossbred reared for milk production under Sudan condition; in particular at the University of Khartoum farm, with objective to evaluate the productive performance of this herd in term of milk yield, lactation length, age at first calving and foreign blood percentage in the herd.

Materials and Methods

Animals and farms

Khartoum University farm is located in Khartoum North, Shambat. This farm was established in 1940 by purchasing of local breeds and adopted in 1972 a breeding program with the introduction of Holstein-Friesian. It maintains the level of foreign blood ranging from 25-62.5%. The cows are kept in partial shaded constructed from iron bars, the floor is of concrete.

Feeding regime

Cows were fed on forage (home-raised *Sorghum bicolor*), Abu 70 (*Medicago sativa*), Berseem and concentrates. They were allowed to graze two hours daily from 7-9 am and the major green fodder is given in the yard. The concentrate is provided during milking and it is composed of cotton seed cake, wheat bran, molasses and salt.

Data collection

40 cows at different physiological status for three lactation season (1999-2002) were randomly selected and their records were obtained.

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Statistical analysis

The data was statistically analyzed by (SAS 1994).

Results and Discussion

Milk yield

The average milk yield for the two experimental groups (62.5% and 50% Friesian blood cows) was shown in Table 1. These values obtained were lower than those obtained by Ibrahim (1983) who reported 2598 kg as an average milk yield for crossbred having 62.5% Friesian inheritance and 2347 kg as an average milk yield for crossbred having 50% Friesian inheritance. Also these values were lower than those obtained by Ali et al. (1988), Rao and Nagarcenkar (1978) and Verma et al. (1973) for crossbred having 62.5% and 50% Friesian inheritance in different countries. They found that for 62.5% and 50% Friesian inheritance were (2867 and 2135 kg), (2668 and 2870 kg) and (2498 ± 137 and 2037 ± 309 kg) as an average milk yield respectively. However the result for 50% Friesian inheritance was higher than the value reported by Ample and Jain (1967), who reported 1872 ± 405 kg as an average milk yield. The results for 62.5% Friesian blood were higher than those reported by Madalena et al. (1990), who found 1401 ± 205 kg as an average milk yield. However the results were lower than those obtained by Fawi (1994). The variations between the different results may be attributed to the management system adopted or to the environmental conditions prevailing during the different studies.

Lactation length

The average lactation length for the two experimental groups (62.5% and 50% Friesian blood cows) was shown in table. 1. The data revealed that the lactation length was longest in the 50% Friesian blood, which recorded 316 ± 28 days. While the 62.5% Friesian blood secured 313 ± 18 days. These results were higher than those reported by Fadel-Elmoula (1989), Madalena (1990) and Rana (1991), who stated values of (298 days for 50% and 301 days for 62.5% Friesian blood), (294 ± 12 for 50% Friesian blood) and (305 ± 18 days for 50% Friesian blood and 191 days for 62.5% Friesian blood) respectively. The values in this

study may be attributed to lack of proper management, miss adoption of certain programme in drying of lactating cows, lower fertility level of the herd, absence of proper heat detection and miss timed insemination.

Table (3) showed the regression analysis of the lactation length, which indicated that neither the level of foreign blood percentage nor age at first calving affected the lactation length ($P < 0.05$). Linear regression analysis of total milk yield showed a highly significant effect of lactation length on total milk yield figure (1) and this in agree with the data reported by Ample and Jain (1967), but quadratic regression analysis of total milk yield showed non-significant effect of lactation length on total milk yield Table 2.

Age at first calving

The average age at first calving for the two experimental groups (62.5% and 50% Friesian blood cows) was shown in table (1). The data indicated that the 62.5% Friesian blood secured advanced age at first calving than the 50% Friesian blood. The results reported here were significantly greater than the findings of Verma et al. (1973) for 50% and 62.5% Friesian blood, who found that 1149 ± 67.4 days and 1117 ± 34 days respectively. Also the results differed from the findings of Danassoury and Bayoumi (1962), who reported 42.9 ± 8.9 months as an average age at first calving. The results reported in the present study were also lower than those obtained by Asker et al. (1965), Ali et al. (1988) and Ibrahim (1990), who found 42, 42.2 and 34.2 months respectively. The reported results pertinent to age at first calving in this study were in line with the findings of Arora and Sharma (1981) and Elhabeeb (1991). In the present study, age at first calving had no effect on the total milk yield Figure 2, which agrees with Fangaly (1980). The effect of age at first calving on lactation length was found to be statistically non-significant, which comply with results reported by Mahmoud (1989). The variation in age at first calving might be attributed to the genetic an environmental difference encountered in the different study.

Foreign blood percentage had a significant effect on the total milk yield ($P < 0.05$) and had no effect on lactation length.

Table 1. Mean and standard error of milk yield (kg), lactation length (days) and age at first calving (days) for percentage of two pedigrees.

Friesian blood	Milk yield	Lactation length	Age at 1 st calving
62.5	$2231^a \pm 124$	$313^a \pm 18$	$1606^a \pm 79.7$
50	$1847^b \pm 175$	$316^b \pm 28$	$1412^b \pm 80.5$

a-b means with different superscripts in the same column were significantly different ($P < 0.05$).

Table 2. Regression analysis of total milk yield.

Source	Degree of freedom	Sum square	Mean square	F. ratio
Regression	4	56283479		
Foreign blood	1	4704135	4704135	3.46*
Age at 1 st calving (L)	1	3699669	3699669	2.72
Lactation length (L)	1	41865195	41865195	30.8**
Lactation length (Q)	1	2490711	2490711	1.83
Error	36	48906890	1358525	
Total	40	105190369		

** : Highly significance differences ($p < 0.01$).

* : Significant difference ($p < 0.05$).

L: Linear, Q: Quadratic

Table 3. Regression analysis of lactation length

Source	Degree of freedom	Sum square	Mean square	F. ratio
Regression	4	25199		
Foreign blood	1	61.6	61.6	0.01
Age at 1 st calving (L)	3	24758	8253	0.92
Error	36	324314	9009	
Total	40	349512		

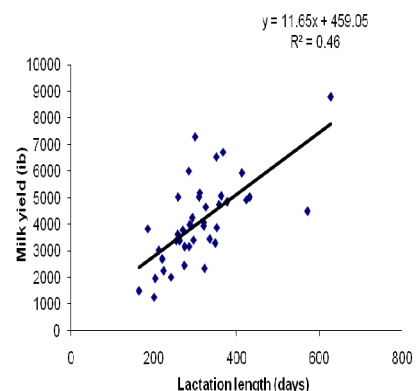


Figure 1. Effect of lactation length on milk yield.

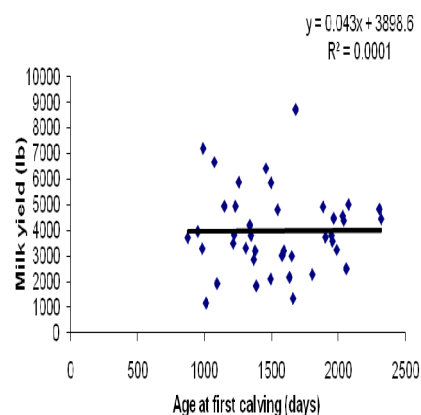


Figure 2. Effect of age at first calving on milk yield.

Conclusion

From the results obtained in this study it could be concluded that the crossbred dairy cows which have 62.5% Friesian blood performed better than 50% Friesian blood and secured advanced age at first calving.

References

- Ali, T. E., F. A. Ahamed and Y. A. Ombabi. 1988. Productivity of crossbred zebu cattle with different levels of Friesian blood. Sudan J. Anim. Prod. 1(2):69-79.
- Ample, V. N. and J. P. Jain. 1967. Comparative performance of different grades of crossbred cows on military farms in India. J. Dairy Sci. 50(10): 1695-1702.

- Arora, D. N. and J. S. Sharma. 1981. Factors affecting some of the economic traits in Holstein-Friesian cattle. Indian Vet. J. 60(10):820-823.
- Asker, A., A. K. H Juma and S. A. Kassir. 1965. Dairy characters of Friesian, Ayrshire, native and crossbred cattle in Iraq. J. Agric. Sci. University of Ainshams (10):17-26.
- Bondoc, O. C., C. Smith and J. P. Gibson. 1989. A review of breeding strategies for genetic improvement of dairy cattle in developing countries. Anim. Breed. Abstract 57:819-829.
- Danssoury, M. S. and M. S. Bayoumi. 1962. Age at first calving and its effect on first lactation period, first dry period, first lactation interval

- and first milk yield. *Indian J. Dairy Sci.* 15:131-138.
- Elemam, M. B. 2002. Study of some productive traits of crossbred dairy cows in University of Khartoum farm, Sudan. M. Sc. Thesis. University of Khartoum, Sudan.
- Elhabeeb, A. E. 1991. Variation in reproductive milk production traits in Kenana and Butana dairy cattle in Sudan. Ph.D. Thesis. University of Khartoum, Sudan.
- Fadel-Elmoula, A. A. 1989. Factors affecting reproductive and productive performance of crossbred dairy cattle in Sudan. M. Sc. Thesis. University of Khartoum, Sudan.
- Fangaly, O. A. 1980. Reproduction and milk yield of Kenana and Butana cattle herds in the Sudan. M. Sc. Thesis. University of Khartoum, Sudan.
- Fawi, N. M. 1994. Study of some aspects of the performance of Friesian crossbred dairy cattle under University of Khartoum Farm Conditions. M.Sc. Thesis, University of Khartoum, Sudan.
- Ibrahim, M. T. 1990. Study of milk composition and some production traits of Friesian and Sudanese grade dairy cattle. M. Sc. Thesis. University of Khartoum, Sudan.
- Madalena, F. A., A. M. Lemos, R. L. Tedoro and R. T. Barposa. 1990. Dairy production and reproduction in Holstein-Friesian and Guzera crosses. *J. Dairy Sci.* 73:1872-186.
- Mahmoud, T. I. 1989. Study of milk composition and some production traits of Friesian and Sudanese grade dairy cattle. M. Sc. Thesis. Institute Of Animal Production University of Khartoum, Sudan.
- Ojango, J. M. K., V. Ducrocq and G. E. Pollott. 2005. Survival analysis of factors affecting culling early in the productive life of Holstein-Friesian cattle in Kenya. *Livestock Prod. Sci.* 92:317-322.
- Rana, P. S. 1991. A note on the performance of crossbred cows at military dairy farms. *Anim. Breed. Abstract.* 60(12):929.
- Rao, G. and R. Nagarcenkar. 1978. First lactation performance of crossbred cows and purebred exotic cattle in lando-Gangetic plains. *Indian J. Dairy Sci.* 32(4):365-370.
- Rege, J. E. O. 1991. Genetic analysis of reproductive and productive performance of Friesian cattle in Kenya. 1. Genetic and phenotypic parameters. *J. Anim. Breed. Gen.* 108:412-423.
- SAS. 1994. Statistical Analytical Systems, Users guide (Version 6), SAS Institute Inc., Cary, North Carolina, USA.
- Smith, C. 1988. Genetic improvement of livestock using nucleus breeding units. *World Anim. Rev.* 65:2-10.
- Verma, S. K., R. N. Singh, H. S. Singh and J. P. Verma. 1973. A study on the performance of the crossbred grades of Friesian and Sahiwal. *Ind. Vet. J.* 50:251-256.

ANIMAL SCIENCE

Toxic effects of *Abrus precatorius* L. seeds on laboratory rats[◇]

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Abstract

In traditional medicine people uses the seeds of *Abrus precatorius* L. plant (commonly known as peony) for multiple purpose. The objective of this investigation was to study the toxic effects of the seeds from the *A. precatorius* in experimental rats. An experimental study of diagnostic type with analytical, explanatory, and descriptive phases was made. The study consisted of two phases: Phase 1: diagnostic phase where a survey was conducted with 30 people without sex distinction. Phase 2: experimental phase where 15 rats of the Sprague-Dawley race, from both sexes and 300g of weight, were used, distributed in 2 groups. Group 1 (control): it consisted of 3 rats to which distilled water was administered; Group 2 (experimental): consists 12 rats to which the watery extract of the peony seeds was administered. The 83% of the 30 surveyed people (Phase 1), knows the peony seed; 40% uses it; and 67% ignores that the seed is toxic. The pathological and anatomic findings revealed that the extract of peony seeds had a poisonous effect on the endothelial wall in the rats of Group 2. These findings could be correlated with the observed clinical manifestations.

Key words: Traditional medicine, *Abrus precatorius*, Toxoalbumin

Introduction

From time immemorial, the knowledge about plants and their medicinal values were existing, but only a little bit is known on their active principles, attributing them with nonexistent properties. All these knowledge have been transmitted from generations to generations assuming them as if they were certain, not knowing the ways to prepare them, toxic properties neither how to dose them to obtain a therapeutic effect. In extreme cases, they produce adverse reactions that in some cases, can cause very serious sequels and even, ultimately, death.

At the moment, there have been implemented health policies that have stimulated the use of medicinal plants as a therapeutic alternative. According to the World Health Organization (WHO), 80% of the people from developing countries trust on traditional medicine for primary medical and health care (García et al., 2001).

Venezuela has a very rich medicinal flora and is widely used by populations that, in some occasions, do not know the properties of the plants (Perrinet al., 1977). Latin America has many communities that depend on herbal medicine and traditional healers for the care of their health (Siles et al., 2004).

Peony (*Abrus precatorius* L.) is a perennial climbing vine from the mountains of India that easily reaches 5 meters in length. It has ligneous stems in the base and herbaceous in its top. Its leaves are complex, displaying an even number of opposite leaflets. Its flowers are papilionaceous and are grouped in a cluster, with purple pink or reddish corollas, and also white in rare occasions. Fruits are flattened seedcases, which contain three to seven hard seeds, scarlet-red color and a peculiar, dark spot at one end (Sisa, 2009).

The seeds of the peony have been recognised for their toxicity since times immemorial. And so many poisonous plant materials including their seeds have been extensively used in folk medicine against a variety of illnesses and also for criminal purposes (Olsen, 2004; Martínez, 2003, 2006, 2008).

The presence of amino acids in the seeds of peony has been described and they could be responsible for their toxic and hem-agglutinating properties (Jurado, 1989; Ganem et al., 2000). Abrin, a highly toxic product from *A. precatorius* is

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shown to exhibit antitumor activity on Yoshida sarcoma (solid and ascites forms) in rats and a fibrosarcoma in mice (Subba Reddy, 1969). Moreover isoflavanquinones named abruquinones have been isolated from the roots of *A. precatorius* and exhibited with remarkable inhibitory effects on the platelet aggregation (Kuo et al., 1995).

It has been demonstrated that phytotoxins produced by the seeds of *A. precatorius* have high toxic activity and it was verified that the abrin can cause gastrointestinal problems, circulatory collapse, comma, and death, depending on the amount of completely chewed and ingested seeds (Villaseñor, 2004).

For the previous given reasons, the objective of this work was to determine the knowledge that people has about the seed of *A. precatorius* and to evaluate the poisonous effects of this seed in experimental rats.

Materials and Methods

Diagnosis phase

The investigation was made in two phases, a diagnosis phase that allowed obtaining the data of the traditional knowledge that people has about this plant toxicity, which are the basis this study. The second phase was a pure experimental part being the independent variable the administration of the peony seed to the experimental rats, and the dependent variables where the anatomic pathological findings that observed in the rats, after the administration of the referred seed.

In first stage, the sample was thirty (30) people without age or sex distinction, who were close to perfumery and plant sales located in Maracay, Municipality of Girardot, Aragua State, between May and July 2009, to determine their knowledge about the use of the seeds of *A. precatorius* to whom a survey was applied to know what information level they had on the use of the seeds of this plant.

Pharmacological experiments

In the second phase, 15 rats of the Sprague-Dowley stock were used for the experiments. They had a weight of 300g and 3 months old, and obtained from the Instituto Venezolano de Investigaciones Científicas (IVIC) and lodged in the animal station of the Institute of Biomedical Investigations of the University of Carabobo (BIOMED-UC). They were distributed as follows: a control group of three (3) rats signed 1 to 3 respectively, which were sacrificed for pathologic anatomy control and later comparison with the experimental group, and a second group of 12 rats, signed from 1 to 12, that constituted the

experimental group. The watery extract of peony was administered through a nasogastric cannula, and subsequent to their death or sacrifice, the necropsy was performed and their organs properly preserved. The experiments are conducted in our lab under permitted ethical regulations and rules.

Rats from the control group and those that did not die during the experiment, were sacrificed by the method of cervical dislocation. Necropsy was performed to each animal and the dissected organs were placed in 10% formaldehyde water solution for preservation. Later, each piece was placed in a properly prepared slide for microscopic pathological anatomy evaluation. This phase was conducted in the Department of Pathological Anatomy, Faculty of Veterinary Sciences, of the Universidad Central de Venezuela at Aragua State.

Biological Material

The authentication of the plant was made in the herbarium "Victor M. Badillo" in the Institute of Agricultural Botany, Faculty of Agronomy, Universidad Central de Venezuela at Aragua State. 12 seeds from *A. precatorius* were taken and their weights were individually registered, verifying that the average weight of the seeds was 0.1g. The seeds were crushed to dust in a mortar. 0.1g of dust was weighed for each rat, equivalent to a seed. A watery extract was prepared by adding 7 mL of distilled water to each dust aliquot.

Results

The surveyed people, in the diagnosis phase, were asked if they knew the seed of peony (Figure 1).

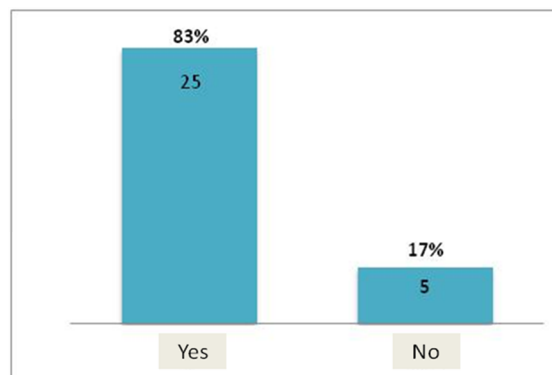


Figure 1. The survey results for - Do you know peony seed?

It can be observed that twenty five (25) people referred to know the seed of peony, which is 83% of the surveyed people.

The second question asked, if they ever heard about the use of seeds of peony. Twenty-six (26) people declared to know what the seed was used for. This represents 87% of the total surveyed

people, and four (4) people answered to ignore the use of those seeds, which represents 13% of the surveyed people.

The third question asked was, if they had ever used the seed, to which thirteen (13) people (43%) said they had used it, and 17 people (56%) referred they had not used it.

The fourth question enquired about the uses that surveyed people gave to the seed of peony (Figure 2).

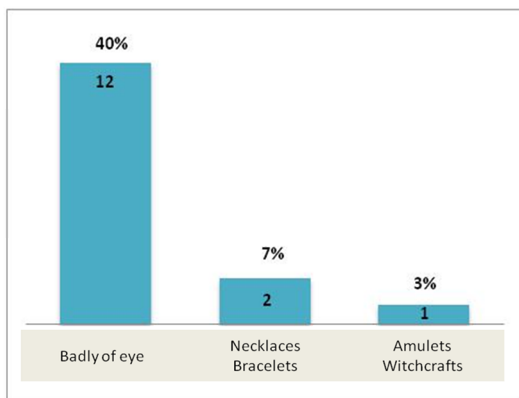


Figure 2. The survey results for – What are the uses of seed of peony?

Here it is observed that twelve (12) people use the seed against religious beliefs which represents 40% from the total, two (2) people (7%) use them as necklaces or bracelets, and finally one (1) person (3%) declared to use it as an amulet.

Question 5 enquired if the surveyed people knew the toxicity of these seeds (Figure 3).

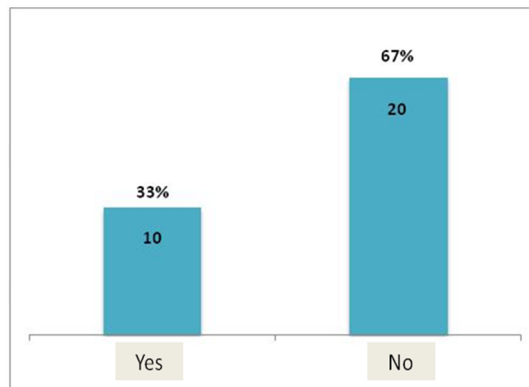


Figure 3. The survey results for – Have you ever heard that the seed of peony is toxic?

It was discovered that 20 people (67%) do not know the poisonous effects of peony seeds, whereas 10 people did know it, which represents 33%.

Results from Phase 2

Rats from the control group turned out to be in good conditions, with an approximate weight of 350g and without suggestive findings of being carrying some type of pathology. The results of the microscopic examinations are shown in Figure 4-8.

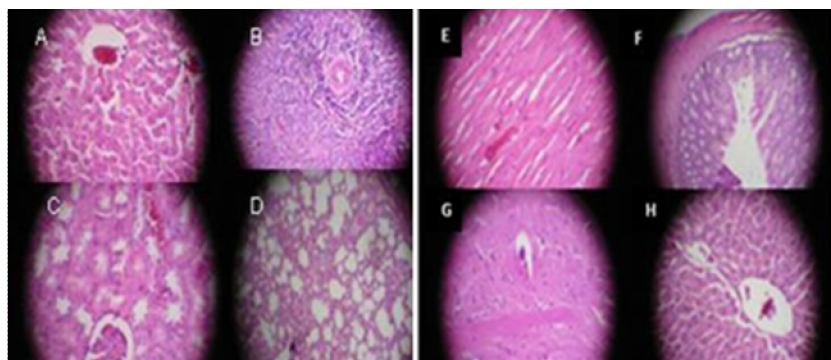


Figure 4. Histological sections in control rat group.

In the histological sections of the rat N°2 (control group) it was observed: A. Liver: fatty intra cytoplasmic degeneration of the hepatocyte. B. Spleen: presents no alterations. C. Kidney: increased Bowman space. D. Lungs: mixed focal polymorphoneutrophilic peribronchitis. E. Heart: showed no injuries. F. Small intestine: showed no

alterations. G. Stomach: presented no alterations. H. Brain: presented no alterations.

In the experimental group the most severe injuries were observed in rats 2, 3, 11 and 12.

Rat N°2 of the experimental group died on the second day of the experiment. The necropsy was performed, in which it was microscopically observed

distension of thin loops and pulmonary hemorrhagic dots in multiple areas. The microscopic examination is described in Figure 5.

Like rat N°2 rat from the experimental group, rat N°3 rat died after 24 hours to have initiated the experiment. Microscopically it was observed

distension of intestinal loops with microgranulations, congested lungs with hemorrhage in multiple areas and hepato splenomegaly. The microscopic findings can be observed in Figure 6.

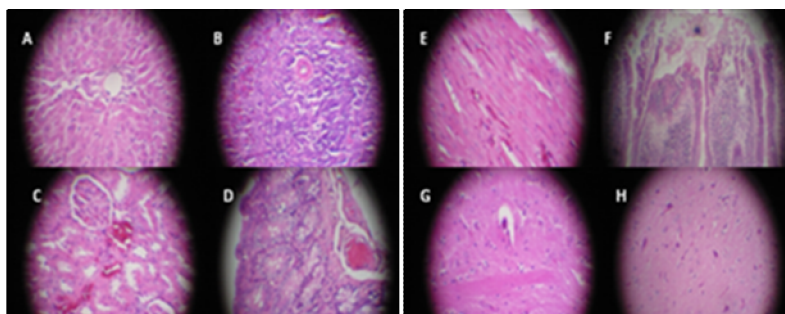


Figure 5. Histological sections from rat N°2 in the experimental group.

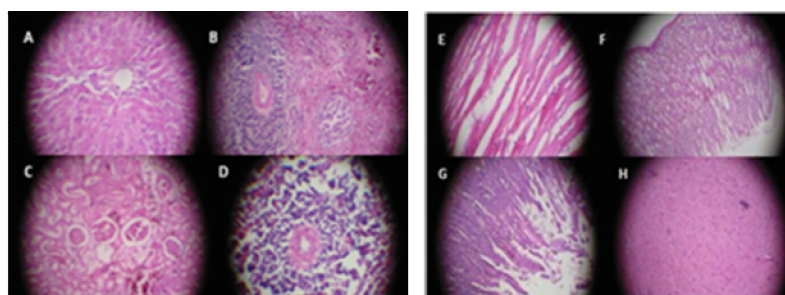


Figure 6. Histological sections from rat N°3 in the experimental group.

Histological sections of the rat N°3, from the experimental group, were observed: A. Liver: Noticeable congestion. Periacinar Necrosis. Slight focal hemorrhage. B. Spleen: severe lymphatic depletion, hemorrhage, and hemosiderosis. C. Kidney: It did not showed hemorrhage. Presence of hydropic degeneration. D. Lungs: Congestive, Diffuse edema. E. Heart: slight interstitial focal hemorrhage and myofibrillar edema. F. Small intestine: severe duodenal hemorrhagic enteritis. G. Large intestine: necrotic and hemorrhagic enteritis. H. Brain: severe brain edema.

Rat N° 11 started to have severe respiratory difficulties, intercostal retractions, and loud expiratory grunt from the experimental group, after 72 hours under experimental conditions. There was evident the presence of an episode of moderated epistaxis. Soon after, the rat was sacrificed by cervical dislocation with an immediate brilliant strong bleeding through the nostrils. Necropsy showed severe pulmonary hemorrhage, liver with a

accented lobular pattern and a severe hepatomegaly, friable kidney, spleniccontraction, presence of gaseous colitis because the rat was hiporhemic, and brain con hemorrhagic aspect (Figure 7).

Observations of the histological sections from rat N°11 in the experimental group were: A. Liver: Severe fat degeneration and slight focal hemorrhage. B. Spleen: amyloidosis, thrombosis of the splenic artery, focal slight lymphoid depletion. C. Kidney: glomerular synechiae, thrombosis, vascular rexis. D. Lungs: thrombosis and slight focal edema. E. Heart: intermyofibrillar edema. F. Submucous congestion, slight focal hemorrhagic enteritis. G. Large intestine: it did not show apparent injuries. H. Brain: severe edema. Espongiosis.

Rat N° 12 remained active till the fifth day reason why it was sacrificed for posterior necropsy. Microscopic findings were: delimited and slight pulmonary hemorrhage, hepatosplenomegaly.

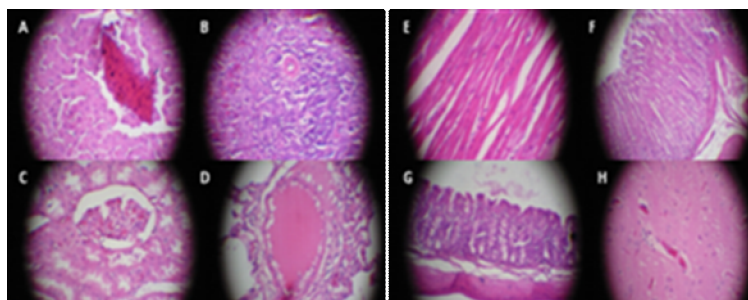


Figure 7. Histological sections from rat N°11 in the experimental group.

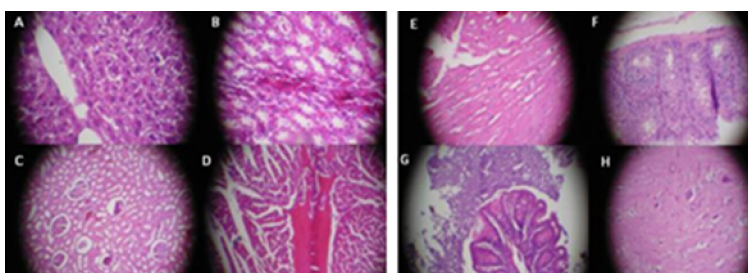


Figure 8. Histological sections from rat N°12 in the experimental group.

The histological sections from rat N°12 in the experimental group, presented: A. Liver: severe focal fat degeneration. Zonal severe hepatic necrosis. B. Spleen: amyloidosis, thrombosis of splenic arteriole. Necrosis. C. Kidney: severe interstitial hemorrhage, hydropic degeneration. D. Lungs: thrombosis, congestion and pulmonary hemorrhage. E. Heart: severe thrombosis. F. Slight focal hemorrhagic enteritis. G. Large intestine: It did not have apparent injuries. H. Brain: Slight neuroaxonal edema.

Discussion

The seed of *Abrus precatorius* L. is referred as protective amulet in the children in traditional knowledge. Having this in mind, a diagnostic phase was evaluated, which revealed that 40% of the surveyed population have used the seed as an amulet while 67% expressed not to know the toxicity of the seed and related lethality over ingestion of the peony seed. The obtained results in the second phase, demonstrated that the 12 rats that constituted the experimental group, presented poisoning which was manifested in the clinical studies and was corroborated by the posterior anatomic pathological analyses.

These analyses allowed observation of the of vascular hemodynamic changes present, clinically characterized by epistaxis, microscopically and histologically by edema, congestion and pulmonary hemorrhage, sub-epicardial hemorrhage, brain and splenic hemorrhage, brain edema and hemorrhagic

gastroenteritis, with variable degrees of intensity. Vascular changes were observed by rhexis.

The rest of the rats from the experimental group showed similar injuries but less intense. The anatomic pathological study finally revealed that the macroscopic and histological findings show a poisonous effect of the studied substance on the endothelial wall of the rats in the experimental group.

Conclusion

The ethno botanical use of different parts of *Abrus precatorius* is common in Venezuela for the treatment of various bacteria-related diseases. The results substantiate that the ingestion of the watery extract of *A. precatorius* seeds had poisonous effects in experimental animals which were feed with it during the study. This demonstrates that the active principles present in the seed of *A. precatorius* are highly toxic and how the use of this seed is common in Venezuela, because it is a part of the cultural heritage. This implies that this cultural practice is putting in risk health and wellness of the infantile population. For this reason, it becomes mandatory to form the health personnel so that they could contribute to the diffusion of these poisonous effects at community levels, and ultimately to diminish the risk of poisonings by the ingestion of this seed.

Reference

- Ganem, F., J. Lagomasino, Y. Álvarez. 2000. Obtención de la abrina y abrina aglutinina. *Universo Diagnostico* 1(1):17-23.

- García, M., D. Sáenz, L. Rojas, Z. Tinoco and J. Bonilla. 2001. Exploración del uso de plantas medicinales en zona urbana de Costa Rica. [Online and cited July 30th, 2010]. Available from: URL: <http://www.binasss.sa.cr/revistas/farmacos/v15n2/art7.htm#8>.
- Jurado, R. 1989. Toxicología veterinaria. 2nd ed. Barcelona: Salvat Editores, S.A.
- Kuo, S. C., S. C. Chen, L. H. Chen, S. J. B. Wu, J. P. Wang and C. M. Teng. 1995. Potent Antiplatelet, Anti-Inflammatory and Antiallergic Isoflavanquinones from the Roots of *Abrus precatorius*. *Planta Med.* 61(4):307-312.
- Martínez, N. 2003. Las plantas no son tan inofensivas como parecen Latin American Society for Nutrition Venezuelan Chapter. [Online, consulted September 18th, 2009] Available from: URL: www.slam.org.ve/publicaciones/completas/plantas_medicinales_1.asp.
- Martínez, N. 2006. Elementos culturales en relación con la utilización de las plantas medicinales en Venezuela desde una teoría de acción humana con un enfoque socioeducativo. Ph.D. Thesis. Maracay: Universidad de Carabobo Sede Aragua.
- Martínez, N. 2008. Dialogando con el jardín. *Comunidad y Salud* 1(6):37-47.
- Olsnes, S. 2004. The history of ricin, abrin and related toxins. *Toxicon*, 44(4):361-370.
- Perrin, M., S. Tillett, J. Steyermark and N. Ferrigni. 1977. Estudio preliminar de la farmacopea goajira. *Rev. Fund. José María Vargas* 1(4):21-25.
- Reddy, V. V. and M. Sirsi. 1969. Effect of *Abrus precatorius* L. on experimental tumors. *Cancer Res.* 29:1447-1451.
- Siles, J., C. Campbell, A. Martin, L. Aguilar, S. Lara, M. Vargas. 2004. Plantas medicinales. [Online, consulted July 3rd 2009] Available from: URL: <http://www.generoyambiente.org/arcangel2/documentos/155.pdf>.
- Sisa, J. 2009. *Abrus precatorius*. [Online, consulted February 25th, 2010]. Available from: URL: http://www.ecoaldea.com/plmd/regaliz_america.no.htm
- Villaseñor, J. and F. Espinosa-Garcia. 2004. The alien flowering plants of Mexico. *Divers. Distrib.* 113-123.

ANIMAL SCIENCE

A study on persistent organochlorine pesticide residues in fish tissues and water from an agricultural fish pond

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Abstract

Organochlorine pesticides (OCPs), (α - BHC, β - BHC, γ - BHC, Heptachlor, Aldrin, Chlordane, p, p' - DDE, o, p' - DDD, p, p' - DDD, Dieldrin, Endrin, Endosulfan and p, p' - DDT) were determined in water and fish samples from the fish pond of Oke-Osun Farm Settlement, Osogbo, Nigeria that receive runoffs from agricultural lands. The aim of the study was to investigate the pollution status of the pond water and fish samples. Qualitative and quantitative analysis of the OCPs after clean-up on silica gel adsorbent were carried out using Gas Chromatography equipped with Electron Capture Detector (GC-ECD). The results showed higher levels of OCPs in the pond water during the rainy season than during the dry season. Higher levels of OCPs were found in the fish samples in the dry season than during the rainy season. Significant positive correlations at $p \leq 0.05$ level were observed for β - BHC and aldrin, chlordane, p, p' - DDE, dieldrin and endrin; γ - BHC and heptachlor, p, p' - DDE, dieldrin and endrin; heptachlor and p, p' - DDD, dieldrin and endrin; heptachlor and p, p' - DDD, dieldrin and endrin; p, p' - DDE and dieldrin and endrin; and dieldrin and endrin. The mean seasonal levels of OCPs in the fish were higher, in most cases, than the recommended WHO Maximum Residue Levels (MRLs) in food items and should give cause for concern. Due to possible toxicity and bioaccumulation tendency of the OCPs by the predators higher in the food chain, the levels of OCPs detected in the fish samples could be a source of future health problems. The OCPs detected are consistent with the agricultural activities of the study area due to pesticide usage.

Key words: Farm settlement, Fish tissues, Organochlorine pesticides, Pond, Water

Introduction

Organochlorine pesticides (OCPs) are synthetic, non-polar, toxic and environmentally persistent dichlorodiphenylethanes, cycodienes or chlorinated benzenes that are used for pests control. Synthetic pesticides were introduced into the Nigerian markets in the 1950s (Adeyeye and Osibanjo, 1990) and farmers have been using them for control of weeds; weevils of cotton, beans and cereals; borers of plant stalks and yams; and disease vectors like tsetse flies and mosquitoes. The persistent nature of the OCPs is advantageous for the control of pests such as termites around buildings. Pesticides applications as enumerated above have brought about increased output of

agricultural products and better life quality generally. The use of pesticides and copper fungicides for the treatment of diseases by farmers over the years would no doubt have led to the accumulation of their residues in soils, water and fish of the agricultural areas, as have been reported elsewhere in literature (Merry et al., 1983; Osibanjo and Bamgbose, 1990; Akinnifesi et al., 2006).

However, the challenges of environmental degradation with its associated health problems provoked by the ubiquitous OCPs are daunting owing to their persistent nature and high lipid solubility. In turn, these factors have led to the problems of bioconcentration and biomagnification of the OCPs in animal tissues that live within the environment where the OCPs are found. For example, in the aquatic environment, OCPs are not readily metabolized and excreted by fish hence, they "biomagnify" up the food chain.

When pesticides reach water bodies, they are rapidly absorbed and accumulated by the bottom sediment, plankton, algae, aquatic invertebrates, aquatic vegetation and fish. Greater concentrations

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of organochlorine residues were reported in fish from higher trophic levels even when movement of the residues through a food chain was impossible (Hannon et al., 1970; Amakwe, 1984; Hamelink et al., 1986). Different species of fish vary greatly both in their susceptibility to organochlorine insecticides and in their ability to store residues in their tissues, but it is certain that very large numbers of fish have been killed by these chemicals (Johnson et al., 1973).

Ecological effects of OCPs include interference with reproductive success of organisms high on the food chain, especially fish eating birds such as osprey, pelicans, falcons and eagles. OCPs especially DDT have estrogenic and enzyme inducing properties. The adverse effects of DDT demonstrated in experimental animals include infertility (Jonsson et al., 1975), a decrease in the number of implanted ova (Lundberg, 1974), intrauterine growth retardation (Fabro et al., 1984), cancer (Cabral et al., 1982; Muino et al., 1995), neurologic developmental disorders (Eriksson et al., 1990; Muino et al., 1995) and mortality (Clement and Okey, 1974). Ortho and para isomers of DDT compete with estradiol for binding to estrogen receptors in uterine cytosol thus causing changes in steroid metabolism and alter the ability of birds to mobilize calcium to produce strong egg shells (Close, 1996; Allen and Otis, 1998; Cohn, 1999; Creekmore et al., 1999; O'Shea et al., 2001). This has led to the prescription of tolerances such as maximum residue level (MRL) and no observable adverse effect level (NOAEL) for various pesticides in food and water, especially by the Codex Alimentarius Commission (CODEX, 2004).

Due to their adverse health effects, the use of some of the OCPs such as DDT have been placed under legal restrictions in places like the USA as far back as 1972, but they still continue to enjoy increasing patronage in places like Nigeria and are sold to farmers under different trade names. The high efficacy and lower cost of OCPs compared with alternative pesticides are some of the reasons for their increasing patronage in Nigeria (Adeyemi et al., 2008). Whereas in the developed countries, strategic surveillance programme is put in place to evaluate the OCPs levels in the environment (Luke et al., 1988; MAFF, 1989; Kaphalia et al., 1990), there is a dearth of baseline data on the levels of OCPs particularly in the Nigerian agricultural environments located in remote or not-easily-accessible areas. Past studies were concentrated on city centres and places of easy access in which case there have been reported pollution stresses in such environmental matrices as soil, water, sediments,

air and biological materials (Suzuki et al., 1977; Skaare et al., 1990). Osibanjo and Bamgbose (1990) reported the contamination by chlorinated hydrocarbons of Nigerian marine fish and shellfish, based on the analyses of 94 samples of 25 marine fish species during the period 1983 to 1985 and 14 samples of 7 shellfish species in 1987. Ajayi and Osibanjo (1981) noted that rivers and streams in Nigeria within the catchment of major cities and agricultural areas have become grossly contaminated with pesticide residues. Pesticides are among the priority pollutants that has been monitored in a wide variety of matrices such as honey (Muino et al., 1995), waters (Ruiz-Gil et al., 2008; López-Blanco et al., 2006), soils (Arias-Estévez et al., 2008), stream sediments (Gonzalez-Lopez et al., 2005), crops especially leafy vegetables (Rosa et al. 2008) and potato crops (Lopez-Perez et al., 2006) and their levels may represent a serious hazard to human health. In one study on management of pesticides in the United Arab Emirates, Kaakeh et al. (2004), reported that the misuse, overuse and unnecessary use of insecticides have been the most important factor in the growth of interest in pest management and that the application of insecticides represents environmental contamination that constitutes adverse effects on non-target organisms. The greatest potential of unintended adverse effects of pesticides is through the contamination of hydraulic system; hence water is one of the primary media through which pesticides are transported from the application area to other locations and pesticide contamination of ground water is a global issue (Kaakeh et al., 2004; López-Blanco et al., 2005). Of all the various kinds of pesticides used by the farmers in the study area, Gammalin 20 (Lindane) is the most popular (used for protecting kola nut from pest, fishing and also used for formulating local insecticides and rodenticides). Others are DDT, DD-force, Weed-Off, Termicot, Atrazin, Glyphosphate, Metaclors-plus, Alaclhor 2-4 Amcine and aldrin.

The present study focused on the Oke-Osun Farm Settlement, Osogbo, Nigeria, one of the farm settlements established in 1960 where intensive agriculture is practiced till date. In this paper, we report the investigations carried out on the pollution status of the pond water and fish samples from the farm settlement with respect to levels of α - BHC, β - BHC, γ - BHC, Heptachlor, Aldrin, Chlordane, p, p' - DDE, o, p' - DDD, p, p' - DDD, Dieldrin, Endrin, Endosulfan and p, p' - DDT. This study was motivated by the fact that the farm settlement has not, since its establishment, been subjected to a

study of this nature and there is evidence that this group of chemicals is still used in agriculture clandestinely under unknown trade names in developing countries such as Nigeria (Sosan et al., 2008; Awofolu and Fatoki, 2003). The dependence of people of the study area and its environs on fish for their protein needs is steadily increasing because fish is relatively cheap and the resulting adverse ecological effects these compounds may have on the aquatic biota and the possible bioaccumulation in food chains, make the monitoring program important.

Materials and methods

Description of the sampling location

Oke-Osun farm settlement is located about 3.5 km South of Osun Groove at the outskirt of Osogbo metropolis between the longitudes 007.231 and 007.236°E and latitudes 04.080 and 04.150°N (Figure 1). The location is a rural setting stretching over 220 ha. A large percentage of the total farm land is used mainly for the cultivation of food crops

like maize, vegetables and cassava. Crops harvested from this settlement enjoy wide patronage by consumer intermediaries from Osogbo and its environs. The farmers here actively and regularly apply agrochemicals for the control of pests and weeds for the purpose of improving crop yields as deemed necessary without a follow-up assessment of how these agrochemicals (fertilizer, herbicides and germicides) affect the environment of the settlement.

Reagents

The reagents used in this study were of spectra purity. They include analytical grade dichloromethane (DCM) – Rochelle Chemicals, SA; HPLC grade n – hexane – Ultrafine Limited, Marlborough House, London; HNO₃ (AR) – Riedel-deHaën, Germany; Silica gel 60 PF₂₅₄ – MERCK, Germany; anhydrous sodium sulphate - Rochelle Chemicals, SA; and nitrogen (99.999% purity) - Rochelle Chemicals, SA.

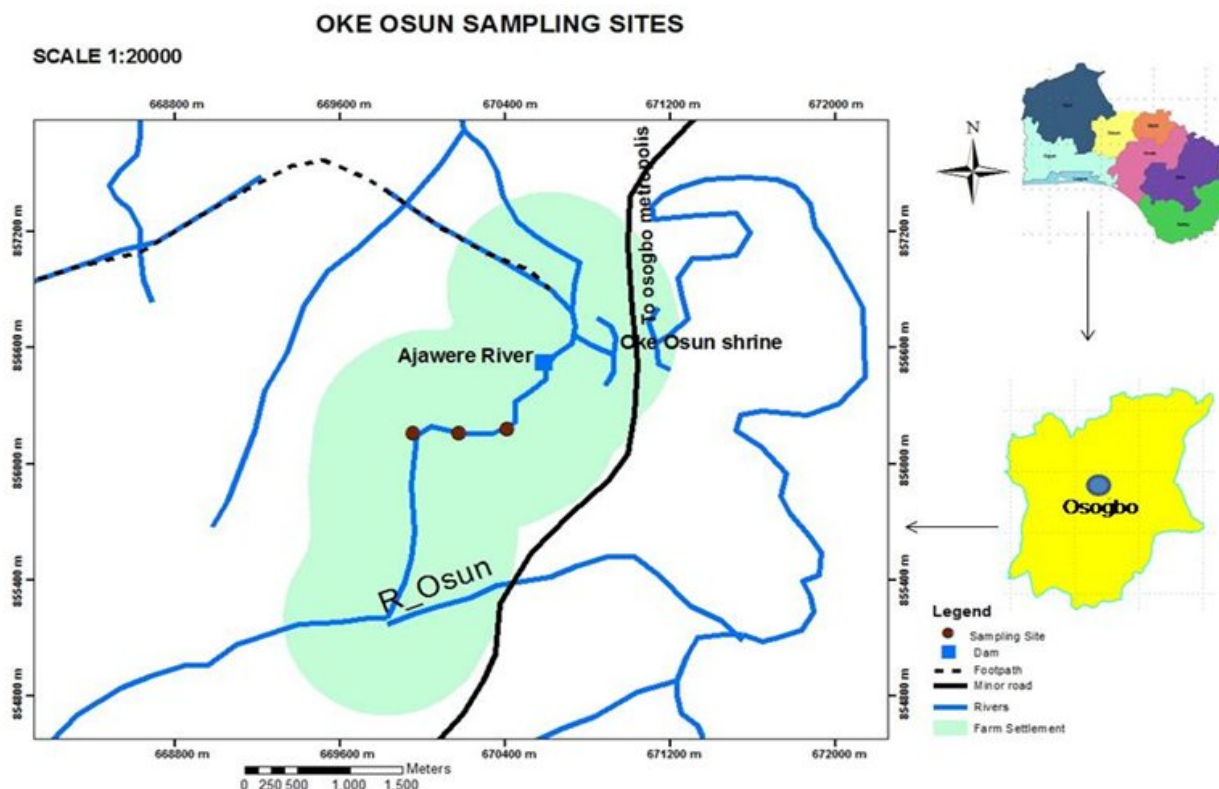


Figure 1. Map showing the sampling sites on the fish pond along Ajawere River.

Sample collection and pretreatment

For each month, water samples from three spots at 20 m apart within the fish pond were collected in pre-treated 2.5 L Winchester glass

bottles. Water from the spot of collection was used to rinse the bottles three times before sampling was done. Alteration of the organics due to microbial activities was prevented by acidification of the

samples to pH 2 by adding about 5 mL concentrated HNO_3 immediately after collection. The samples were stored in a refrigerator at 4°C prior to analysis (Fatoki and Vernon, 1990; Ogunfowokan et al., 2006). Macrophagous tilapia fish samples (*Tilapia zillii*) for each month were caught from the pond using drag net method with the help of trained personnel from the Department of Fisheries, Osun State Ministry of Agriculture and Natural Resources, Osogbo, Nigeria. The fish samples were wrapped in aluminum foils, kept in an all-glass container and stored in a deep freezer prior to analysis. *T. zillii* is a top to middle feeder fish species (Ize-Iyamu et al., 2007).

Extracting OCPs from water and fish samples

Liquid-liquid extraction method was used to extract OCPs from the 2 L water samples earlier collected. About 500 mL of water sample was put into a 1 L separatory funnel at a time. This was extracted with 10 mL (3 portions) of DCM by vigorous shaking for 30 minutes for each of the triplicate extraction. The extracts were combined, dried with anhydrous sodium sulphate and concentrated to about 2 mL under a stream of nitrogen of 99.999% percentage purity. The reduced extract was preserved for chromatographic clean-up prior to GC-ECD analysis.

The frozen composite whole-body tissue samples were homogenized using an agate mortar and pestle (Leiker et al., 1996; Fatoki and Awofolu, 2003). Approximately 10 g of the properly chopped sample was further mixed with 5 g of anhydrous sodium sulphate. This was soxhlet extracted for 5 hours using DCM/n-hexane mixture. The extract was dried over anhydrous sodium sulphate and concentrated to about 2 mL as described earlier in readiness for the clean-up procedure.

Clean-up procedure

A column of about 15 cm (length) $\times$ 1 cm (internal diameter) was packed with about 5 g activated silica gel prepared in a slurry form in n-hexane. About 0.5 cm^3 of anhydrous sodium sulphate was placed at the top of the column to absorb any water in the sample or the solvent. The column was pre-eluted with 15 mL of n-hexane without the exposure of the sodium sulphate layer to air. The reduced extract was placed in the column and allowed to sink below the sodium sulphate layer. Elution was done with 2×10 mL portions of the extracting solvent (DCM). The eluate was collected, dried with anhydrous sodium sulphate and then evaporated to dryness under a stream of analytical grade nitrogen (99.999%).

Gas chromatographic (GC) analysis

The dried eluate above was reconstituted with 1 mL spectra grade n-hexane and 0.5 mL of 20 ppm hexachlorobenzene was added as an internal standard. A PERKIN ELMER GC Autosystem (XL) available at the Department of Chemistry, University of Botswana, Garborone, Botswana, was used to profile the organochlorine pesticides in the sample using an electron capture detector (ECD). The GC was run under the following conditions: injector temperature: 250°C ; detector temperature: 300°C (held for 5 minutes); capillary column: Zebron ZB-1701: 30 m $\times$ 0.25 mm i.d. $\times$ 0.25 μm f.t.; oven temperature programme: 280°C starting from 50°C for 1 minute and continued at $20^\circ\text{C}/\text{minute}$ to 150°C and at $5^\circ\text{C}/\text{minute}$ to 280°C held for 4 minutes; injected sample volume: $1\mu\text{L}$; mode of injection and flow rate: splitless mode at the flow rate $19.6\text{ mL}/\text{minute}$; carrier gas: N_2 at $30\text{ mL}/\text{min}$.

Recovery experiment

Since no certified fish and water pesticide reference materials were available to us during the course of this study, recovery analysis was performed in order to ascertain the precision and efficiency of the analytical procedures using standard addition method. 1000 mL aliquot of ultra-pure water, obtained by reverse osmosis using Milli-Q Gradient A10 Water Deionizer, was measured into a 2000 mL beaker and acidified with concentrated HNO_3 to pH 2. This was spiked with 10 mL of 1000 ppm standard mixture consisting of thirteen different organochlorine pesticides (OCPs) purchased. The mixture was thoroughly shaken together to ensure maximum homogenization. This was extracted and cleaned up following the procedures outlined earlier. In addition, 10 mL of the 1000 ppm mixture of the OCPs, in spectra grade n-hexane, was put into a clean oven dried sample bottle. This was dried at ambient temperature by purging with high purity nitrogen gas and redissolved in 1.0 mL n-hexane. $1.0\mu\text{L}$ of the mixture was injected into the GC column for GC-ECD analysis. The recoveries of OCPs were determined by comparing the peak areas of the OCPs after spiking with those obtained from the evaporated standard residues and the percentage recovery (%R) was evaluated (Fatoki and Awofolu, 2003).

Response factor (RF) and limits of detection determinations

The response factor of the standard OCPs was determined by analyzing $1.0\mu\text{L}$ of 1000 ppm stock solution of the standard mixture containing the

internal standard (I.S.) on the GC-ECD. The response factors (RF) were obtained from the relationship:

$$RF = \frac{\text{Peak area of OCPs}}{\text{Peak area of I.S.}}$$

(Fatoki and Vernon, 1990; Ogunfowokan et al., 2003 and 2006).

The limits of detection (LOD) were evaluated by the determination of concentrations that give signals equal to the blank signal plus three standard deviations of the blank using the equation $y_C = y_B + 3s_B$; where y_C = analyte signal equivalent to detection limit; y_B = blank signal; and s_B = standard deviation of the blank. From the value of y_C , the analyte concentration corresponding to the detection limit was evaluated for GC-ECD determination of OCPs.

Statistical analysis

Both descriptive and inferential statistical analyses were used to interpret experimental data in this study. Data were analysed using SPSS for Windows, version 16. The matrix correlation coefficients of OCPs in pond water and fish from Oke-Osun Farm settlement was carried out to show

if significant correlations at $P < 0.05$ level occurred between individual OCPs in the matrices. Cluster analysis was also used to classify a group of the OCPs into similar classes that are associated.

Results and discussion

The results of recovery experiment are presented in Table 1 as mean percentage recoveries. Based on the percentage recovery values that ranged from 82.11 to 99.27 % in water, the analytical procedures outlined for OCPs assessment in this study are adjudged reliable, reproducible and efficient. The range of response factors of 0.657 to 1.892 showed the separation efficiency of the GC-ECD equipment used for the identification and quantification of OCPs. The LOD values for the OCPs ranged from 0.056 to 2.107 µg/L. The percentage relative standard deviation (%RSD) values for the recoveries of OCPs in water ranged from 2.57 to 6.99 %. These values showed that precision was better than 10 % RSD. The gas chromatogram of a mixture of the 13 OCPs plus the internal standard is shown in Figure 2. The 13 pesticides were well resolved and eluted within 25 minutes.

Table 1. Response factors, relative retention times, limits of detection and mean % recovery of OCPs in water from the fish pond of Oke-Osun farm settlement, Osogbo, Nigeria.

OCP	Response Factor	Retention Time (min)	% Recovery [#] in Water	Calculated limits of detection (µg/L)
HCB	-	8.58±0.25	92.31±5.81	0.673
α-BHC	1.772±0.011	9.93±0.21	85.16±5.04	1.087
γ-BHC	1.161±0.022	11.39±0.33	99.27±3.66	0.097
Heptachlor	1.286±0.101	12.26±0.42	92.03±6.42	1.090
Aldrin	1.344±0.021	13.28±0.34	86.72±5.14	2.107
β-BHC	0.672±0.005	14.01±0.44	89.62±4.84	0.100
Chlordane	0.603±0.006	17.29±0.26	91.21±3.76	0.483
p, p'- DDE	0.890±0.010	17.58±0.13	93.44±3.26	0.183
Dieldrin	0.934±0.040	18.21±0.29	83.98±3.18	0.895
o, p' - DDD	1.276±0.059	18.72±0.41	96.67±5.40	1.110
Endrin	1.076±0.021	19.00±0.52	84.59±2.57	0.307
p, p'- DDD	1.892±0.276	20.43±0.36	94.81±4.92	2.003
β-Endosulfan	0.657±0.055	20.72±0.29	82.11±4.77	0.067
p, p'- DDT	0.942±0.019	25.44±0.38	93.18±6.99	0.056

[#]Values are reported as mean of triplicate analysis ± % RSD.

Table 2. (a-d). Rainy Season Levels of OCPs ($\mu\text{g/L}$) in Pond Water from Oke-Osun Farm Settlement, Osogbo, Nigeria.

(a) May 2004

	α - BHC	β - BHC	γ - BHC	Heptachlor	Aldrin	Chlordane	p, p' - DDE	o, p' - DDD	p, p' - DDD	Dieldrin	Endrin	Endosulfan	p, p' - DDT	Total burden per sampling site
Pond water 1	4.88	8.46	9.73	10.96	8.40	6.60	8.18	8.27	3.44	5.30	5.94	7.67	7.03	82.18
	± 1.02	± 1.18	± 1.93	± 0.29	± 2.05	± 1.25	± 1.87	± 0.72	± 1.04	± 1.53	± 1.79	± 2.04	± 3.46	± 20.17
Pond water 2	1.84	1.85	3.21	1.96	1.75	1.29	4.13	5.65	4.59	2.14	3.60	1.22	8.02	41.25
	± 0.21	± 0.12	± 0.15	± 0.11	± 0.17	± 0.11	± 0.01	± 0.13	± 0.11	± 0.10	± 0.15	± 0.10	± 0.17	± 1.64
Pond water 3	3.88	ND	ND	ND	ND	ND	6.74	6.72	ND	6.39	5.56	10.32	7.06	46.67
	± 1.04						± 1.43	± 0.17		± 0.71	± 0.34	± 0.38	± 0.37	± 4.44
Mean	3.53	3.44	4.31	4.31	3.38	2.63	6.35	6.88	2.68	4.61	5.03	6.40	7.37	57.24
$\pm$ s.d.	± 1.55	± 4.45	± 4.96	± 5.85	± 4.43	± 3.50	± 2.05	± 1.32	± 2.39	± 2.21	± 1.26	± 4.68	± 0.56	± 22.25
Range	1.84-4.88	ND-8.46	ND-9.73	ND-10.96	ND-8.40	ND-6.60	4.13-8.18	5.65-8.27	ND-4.59	2.14-6.39	3.60-5.94	1.22-10.32	7.03-8.02	41.25-82.18

Value = mean of triplicate analysis $\pm$ s.d.; ND = Not detected

(b) June 2004

	α - BHC	β - BHC	γ - BHC	Heptachlor	Aldrin	Chlordane	p, p' - DDE	o, p' - DDD	p, p' - DDD	Dieldrin	Endrin	Endosulfan	p, p' - DDT	Total burden per sampling site
Pond water 1	16.69	18.40	24.86	54.29	64.15	4.17	90.14	100.88	41.07	44.47	38.79	63.28	78.60	629.79
	± 0.18	± 0.12	± 0.94	± 1.86	± 1.42	± 0.13	± 2.33	± 0.97	± 1.21	± 2.93	± 1.62	± 1.10	± 1.53	± 16.34
Pond water 2	15.73	44.69	42.85	24.67	25.11	9.32	56.63	61.99	24.41	11.35	34.88	58.97	59.29	469.89
	± 0.27	± 0.62	± 0.70	± 0.45	± 0.46	± 0.53	± 1.05	± 0.93	± 0.28	± 0.14	± 0.92	± 1.51	± 0.81	± 8.67
Pond water 3	27.76	ND	31.54	23.40	58.54	6.79	108.60	52.63	54.44	56.05	46.61	83.18	62.08	611.62
	± 0.49		± 1.14	± 0.24	± 1.82	± 0.37	± 2.14	± 0.17	± 1.96	± 1.16	± 3.22	± 2.10	± 1.29	± 16.10
Mean	20.06	21.03	33.08	34.12	49.27	6.76	85.12	71.83	39.97	37.29	40.09	68.48	66.66	579.28
$\pm$ s.d.	± 6.69	± 22.46	± 9.09	± 17.48	± 21.11	± 2.58	± 26.35	± 25.59	± 15.05	± 23.20	± 5.97	± 12.91	± 10.44	± 87.54
Range	15.73-27.76	ND-44.69	24.86-42.85	23.40-54.29	25.11-64.15	4.17-9.32	56.63-108.60	52.63-100.88	24.41-54.44	11.35-56.05	34.88-46.61	58.97-83.18	59.29-78.60	469.89-629.79

Value = mean of triplicate analysis $\pm$ s.d.

(c) July 2004

	α - BHC	β - BHC	γ - BHC	Heptachlor	Aldrin	Chlordane	p, p' - DDE	o, p' - DDD	p, p' - DDD	Dieldrin	Endrin	Endosulfan	p, p' - DDT	Total burden per sampling site
Pond water 1	29.97 ± 0.55	ND	70.44 ± 2.91	35.76 ± 1.08	ND	31.76 ± 2.10	36.22 ± 0.93	44.45 ± 1.93	24.37 ± 0.44	45.64 ± 1.14	39.90 ± 0.60	51.76 ± 1.06	55.41 ± 1.12	465.68 ±13.86
Pond water 2	30.12 ± 0.46	86.78 ± 0.51	34.69 ± 0.70	63.29 ± 1.88	78.82 ± 1.33	39.72 ± 0.22	63.67 ± 1.32	110.91 ± 1.23	34.34 ± 0.12	99.57 ± 0.39	49.12 ± 0.87	90.19 ± 2.34	69.56 ± 1.09	850.98 ±12.46
Pond water 3	6.48 ± 0.13	3.31 ± 0.09	9.90 ± 0.24	2.04 ± 0.15	8.42 ± 0.91	4.64 ± 0.51	8.44 ± 0.31	3.05 ± 0.06	11.61 ± 0.11	18.05 ± 0.16	13.56 ± 0.10	18.45 ± 0.15	12.35 ± 0.14	120.30 ±3.06
Mean	22.19	30.03	38.34	33.70	29.08	25.34	36.11	52.80	23.44	54.42	34.19	53.47	45.77	478.99
± s.d.	±13.61	±49.17	±30.43	±30.68	±43.28	±18.38	±27.62	±54.41	±11.39	±41.46	±18.45	±35.90	±29.80	±365.52
Range	6.48- 30.12	ND- 86.78	9.90- 70.44	2.04- 63.29	ND- 78.82	4.64- 39.72	8.44- 63.67	3.05- 110.91	11.61- 34.34	18.05- 99.57	13.56- 49.12	18.45- 90.19	12.35- 69.56	120.30- 850.98

Value = mean of triplicate analysis ± s.d.

(d) August 2004

	α - BHC	β - BHC	γ - BHC	Heptachlor	Aldrin	Chlordane	p, p' - DDE	o, p' - DDD	p, p' - DDD	Dieldrin	Endrin	Endosulfan	p, p' - DDT	Total burden per sampling site
Pond water 1	16.19 ± 0.14	5.23 ± 0.12	26.54 ± 1.23	14.17 ± 0.62	25.17 ± 0.15	25.35 ± 0.25	21.78 ± 1.26	19.37 ± 1.01	16.16 ± 0.24	12.48 ± 1.10	13.21 ± 0.48	13.71 ± 1.20	15.51 ± 0.20	224.87 ±8.00
Pond water 2	6.65 ± 0.35	11.86 ± 0.58	23.84 ± 0.19	15.26 ± 0.31	10.30 ± 0.24	11.93 ± 0.68	17.76 ± 1.38	37.39 ± 0.11	17.87 ± 0.13	5.74 ± 0.20	8.75 ± 0.41	1.35 ± 0.07	25.90 ± 0.86	194.30 ±5.51
Pond water 3	7.57 ± 0.42	ND	22.58 ± 1.50	10.77 ± 0.81	14.54 ± 0.13	7.59 ± 0.26	13.39 ± 1.20	23.94 ± 0.22	19.19 ± 0.57	22.44 ± 0.19	14.67 ± 0.25	8.37 ± 0.15	34.24 ± 0.18	199.29 ±5.88
Mean	10.14	5.60	24.32	13.40	16.67	14.96	17.64	26.90	17.74	13.55	12.21	7.81	25.22	206.15
±s.d.	±5.26	±5.79	±2.02	±2.34	±7.66	±9.26	±4.20	±9.37	±1.52	±8.40	±3.08	±6.20	±9.38	±16.40
Range	6.65- 16.19	ND- 11.56	22.58- 26.54	10.77- 15.26	10.30- 25.17	7.59- 25.35	13.39- 21.78	19.37- 37.39	16.16- 19.19	5.74- 22.44	8.75- 14.67	1.35- 13.71	15.51- 34.24	194.30- 224.87
Rainy Season mean ± s.d.	13.98 ±10.46	15.02 ±25.95	25.02 ±19.28	21.38 ±20.41	24.60 ±27.33	12.42 ±12.75	36.31 ±35.48	39.60 ±36.61	20.96 ±16.14	27.47 ±29.05	22.88 ±17.44	34.04 ±33.13	36.26 ±25.64	330.42 ±240.88
Seasonal range	1.84- 30.12	ND- 86.78	ND- 70.44	ND- 63.29	ND- 78.82	ND- 39.72	4.13 108.60	3.05- 110.91	ND- 54.44	2.14- 99.57	3.60- 49.12	1.22- 90.19	7.37- 66.66	57.24- 579.28

Table 3 (a-d). Dry Season Levels of OCPs ($\mu\text{g/L}$) in Pond Water from Oke-Osun Farm Settlement, Osogbo, Nigeria.

(a) November 2004

	α - BHC	β - BHC	γ - BHC	Heptachlor	Aldrin	Chlordane	p, p' - DDE	o, p' - DDD	p, p' - DDD	Dieldrin	Endrin	Endosulfan	p, p' - DDT	Total burden per sampling site
Pond water 1	4.32 ± 0.02	ND	1.98 ± 0.10	6.22 ± 0.31	8.19 ± 0.40	ND	3.76 ± 0.13	8.90 ± 0.10	ND	4.59 ± 0.21	5.52 ± 0.30	6.84 ± 0.17	3.97 ± 0.14	54.29 ± 1.88
Pond water 2	ND	ND	ND	ND	ND	ND	17.92 ± 0.50	ND	4.61 ± 0.56	25.07 ± 1.31	ND	ND	ND	47.60 ± 2.37
Pond water 3	4.05 ± 0.09	ND	1.67 ± 0.32	7.68 ± 0.30	11.09 ± 0.71	ND	27.14 ± 0.73	5.98 ± 0.08	3.77 ± 0.31	7.59 ± 0.07	5.26 ± 0.31	6.39 ± 0.14	8.98 ± 0.80	89.50 ± 3.86
Mean $\pm$ s.d.	2.79 ± 2.42	ND	1.22 ± 1.07	4.63 ± 4.08	6.43 ± 5.75	ND	16.27 ± 11.78	4.96 ± 4.54	2.79 ± 2.46	12.42 ± 11.06	3.59 ± 3.11	4.41 ± 3.83	4.32 ± 4.50	63.83 ± 22.51
Range	ND- 4.32	ND	ND- 1.98	ND- 7.68	ND- 11.09	ND	3.76- 27.14	ND- 8.90	ND- 4.61	4.59- 25.07	ND- 5.52	ND- 6.84	ND- 8.98	47.60- 89.60

Value = mean of triplicate analysis $\pm$ s.d.

(b) December 2004

	α - BHC	β - BHC	γ - BHC	Heptachlor	Aldrin	Chlordane	p, p' - DDE	o, p' - DDD	p, p' - DDD	Dieldrin	Endrin	Endosulfan	p, p' - DDT	Total burden per sampling site
Pond water 1	18.47 ± 0.52	3.24 ± 0.03	14.65 ± 0.27	11.87 ± 0.24	20.83 ± 0.30	6.00 ± 0.03	42.82 ± 2.94	13.35 ± 0.13	20.30 ± 0.83	13.39 ± 0.61	18.33 ± 0.60	12.29 ± 0.31	37.20 ± 0.47	232.74 ± 7.28
Pond water 2	8.12 ± 0.01	ND	ND	ND	2.08 ± 0.17	4.27 ± 0.07	11.94 ± 0.01	7.39 ± 0.32	5.80 ± 0.40	13.63 ± 0.30	3.06 ± 0.02	10.81 ± 0.43	14.68 ± 0.20	81.78 ± 1.93
Pond water 3	ND	ND	ND	ND	ND	ND	ND	ND	5.53 ± 0.27	16.11 ± 0.11	ND	ND	7.61 ± 0.09	29.25 ± 0.47
Mean $\pm$ s.d.	8.86 ± 9.26	1.08 ± 1.87	4.88 ± 8.46	3.96 ± 6.85	7.64 ± 11.47	3.42 ± 3.09	18.25 ± 22.10	6.91 ± 6.69	10.54 ± 8.45	14.38 ± 1.51	7.13 ± 9.82	7.70 ± 6.71	19.83 ± 16.04	114.59 ± 105.64
Range	ND - 18.47	ND - 3.24	ND - 14.65	ND - 11.87	ND - 20.83	ND - 6.00	ND - 42.82	ND - 13.35	5.53- 20.30	13.39- 16.11	ND - 18.33	ND - 12.29	7.61- 37.20	29.25- 232.74

Value = mean of triplicate analysis $\pm$ s.d.

(c) January 2005

	α - BHC	β - BHC	γ - BHC	Heptachlor	Aldrin	Chlordane	p, p' - DDE	o, p' - DDD	p, p' - DDD	Dieldrin	Endrin	Endosulfan	p, p' - DDT	Total burden per sampling site
Pond water 1	5.82 ± 0.90	ND	8.33 ± 0.15	3.39 ± 0.20	4.73 ± 0.31	2.43 ± 0.30	3.35 ± 0.33	8.46 ± 0.45	5.30 ± 0.44	7.65 ± 0.27	6.75 ± 0.50	12.39 ± 0.75	9.42 ± 0.83	79.02 ± 5.43
Pond water 2	5.56 ± 0.04	ND	15.95 ± 0.30	5.75 ± 0.33	14.58 ± 0.66	5.17 ± 0.05	17.23 ± 2.63	15.31 ± 0.36	9.95 ± 1.07	18.77 ± 0.11	16.69 ± 0.46	19.98 ± 0.91	5.19 ± 0.42	150.13 ± 7.34
Pond water 3	3.18 ± 0.19	ND	32.89 ± 0.75	14.89 ± 0.36	13.62 ± 0.39	24.63 ± 0.14	ND	23.43 ± 0.52	12.73 ± 0.02	25.42 ± 1.73	22.91 ± 0.75	27.83 ± 1.06	8.98 ± 0.60	210.51 ± 6.51
Mean ± s.d.	4.85 ± 1.45	ND	19.06 ± 12.57	8.01 ± 6.07	10.98 ± 5.43	10.74 ± 12.10	6.86 ± 9.14	15.73 ± 7.49	9.33 ± 3.75	17.28 ± 8.98	15.45 ± 8.15	20.07 ± 7.72	7.86 ± 2.33	146.22 ± 65.82
Range	3.18- 5.82	ND	8.33- 32.89	3.39- 14.89	4.73- 14.58	2.43- 24.63	ND- 17.23	8.46- 23.43	5.30- 12.73	7.65- 25.42	6.75- 22.91	12.39- 27.83	5.19- 9.42	79.02- 210.51

(d) February 2005

	α - BHC	β - BHC	γ - BHC	Heptachlor	Aldrin	Chlordane	p, p' - DDE	o, p' - DDD	p, p' - DDD	Dieldrin	Endrin	Endosulfan	p, p' - DDT	Total burden per sampling site
Pond water 1	7.16 ± 0.63	ND	14.49 ± 0.53	16.23 ± 0.36	9.53 ± 0.29	23.79 ± 0.25	23.52 ± 0.22	17.01 ± 0.67	8.21 ± 0.45	15.10 ± 0.36	18.68 ± 0.17	10.91 ± 0.09	25.30 ± 0.42	190.43 ± 4.44
Pond water 2	14.79± 0.48	ND	6.58 ± 0.04	4.85 ± 0.39	23.75 ± 0.61	18.65 ± 0.07	21.6 ± 0.44	12.06 ± 0.41	10.51 ± 0.70	31.99 ± 0.67	34.30 ± 0.52	17.77 ± 1.01	26.73 ± 0.37	223.58 ± 5.71
Pond water 3	ND	ND	5.19 ± 0.41	ND	14.14 ± 0.36	3.07 ± 0.14	ND	10.67 ± 1.10	2.41 ± 0.60	27.60 ± 0.06	ND	9.01 ± 0.04	ND	72.09 ± 2.71
Mean ± s.d.	7.32 ± 7.40	ND	8.75 ± 5.02	7.03 ± 8.33	15.81 ± 7.26	15.17 ± 10.79	15.04 ± 13.06	13.25 ± 3.33	7.04 ± 4.17	24.90 ± 8.76	17.66 ± 17.17	12.56 ± 4.61	17.34 ± 15.04	162.03 ± 79.64
Range	ND- 14.79	ND	5.19- 14.49	ND- 16.23	9.53- 23.75	3.07- 23.79	ND- 23.52	10.67- 17.01	2.41- 10.51	15.10- 31.99	ND- 34.30	9.01- 17.77	ND- 26.73	72.09- 223.58
Dry Season mean ± s.d.	5.96 ± 5.73	0.27 ± 0.90	8.48 ± 9.75	5.91 ± 5.82	10.21 ± 7.70	7.33 ± 9.40	14.11 ± 13.44	10.21 ± 6.74	7.43 ± 5.42	17.24 ± 8.71	10.96 ± 11.02	11.19 ± 7.96	12.34 ± 7.43	121.67 ± 43.31
Seasonal range	ND- 18.47	ND- 3.24	ND- 32.89	ND- 16.23	ND- 23.75	ND- 24.63	ND- 42.82	ND- 23.43	ND- 20.30	4.59- 31.99	ND- 34.30	ND- 27.83	ND- 37.20	29.25- 232.74

Table 4. Matrix of Correlation Coefficients of OCPs in Pond Water from Oke-Osun Farm Settlement, Osogbo, Nigeria.

	α - BHC	β - BHC	γ - BHC	Heptachlor	Aldrin	Chlordane	p, p' - DDE	o, p' - DDD	p, p' - DDD	Dieldrin	Endrin	Endosulfan	p, p' - DDT
α -BHC	1.00												
β -BHC	0.48*	1.00											
γ -BHC	0.76*	0.28	1.00										
Heptachlor	0.77*	0.69*	0.70*	1.00									
Aldrin	0.69*	0.66*	0.39	0.81*	1.00								
Chlordane	0.65*	0.57*	0.75*	0.64*	0.41*	1.00							
p,p'-DDE	0.78*	0.34	0.48*	0.74*	0.84*	0.26	1.00						
o,p'-DDD	0.73*	0.70*	0.62*	0.97*	0.88*	0.54*	0.77*	1.00					
p,p'-DDD	0.82*	0.37	0.67*	0.77*	0.83*	0.40*	0.93*	0.81*	1.00				
Dieldrin	0.76*	0.73*	0.58*	0.80*	0.81*	0.62*	0.69*	0.83*	0.74*	1.00			
Endrin	0.89*	0.47*	0.74*	0.82*	0.79*	0.67*	0.80*	0.81*	0.85*	0.83*	1.00		
Endosulfan	0.84*	0.58*	0.66*	0.85*	0.85*	0.53*	0.84*	0.87*	0.87*	0.90*	0.91*	1.00	
p,p'-DDT	0.87*	0.48*	0.68*	0.89*	0.80*	0.50*	0.88*	0.89*	0.91*	0.77*	0.89*	0.85*	1.00

*Values are significant at 0.05 level (N = 24).

Table 5. Monthly Mean Levels of OCPs ($\mu\text{g/kg}$) in Fish from the Pond Water of Oke-Osun Farm Settlement, Osogbo, Nigeria.

	α - BHC	β - BHC	γ - BHC	Heptachlor	Aldrin	Chlordane	p, p' - DDE	o, p' - DDD	p, p' - DDD	Dieldrin	Endrin	Endosulfan	p, p' - DDT	Total Body Burden
May 2004	16.34 ± 0.12	13.52 $\pm$ 0.11	2.05 $\pm$ 0.06	ND	43.47 ± 0.01	3.81 $\pm$ 0.07	24.29 ± 0.15	20.23 ± 0.05	40.77 ± 0.06	20.80 ± 0.01	0.52 ± 0.10	0.57 ± 0.10	70.68 ± 0.21	257.05 ± 0.94
June 2004	10.56 ± 0.01	41.02 $\pm$ 0.14	1.96 $\pm$ 0.22	1.22 $\pm$ 0.07	40.39 ± 0.11	ND	30.94 ± 0.03	ND	50.72 ± 0.06	40.36 ± 0.05	ND	1.22 ± 0.08	40.78 ± 0.21	259.39 ± 0.77
July 2004	20.54 ± 0.19	15.00 $\pm$ 0.04	0.69 $\pm$ 0.02	1.93 $\pm$ 0.17	37.31 ± 0.15	ND	49.86 ± 0.09	ND	42.84 ± 0.21	43.33 ± 0.49	ND	6.13 ± 0.15	66.34 ± 0.03	283.97 ± 1.54
August 2004	14.03 ± 0.11	33.94 $\pm$ 0.10	1.12 $\pm$ 0.08	2.27 $\pm$ 0.14	51.92 ± 0.21	0.29 $\pm$ 0.06	43.58 ± 0.12	14.56 ± 0.15	33.23 ± 0.17	51.97 ± 0.29	0.68 ± 0.05	8.29 ± 0.13	81.78 ± 0.13	337.67 ± 1.74
Rainy season mean $\pm$ s.d.	15.37 ± 4.19	25.87 $\pm$ 13.73	1.46 $\pm$ 0.66	1.36 $\pm$ 1.00	43.27 ± 6.29	1.03 $\pm$ 1.86	37.16 ± 11.65	8.70 ± 10.31	41.89 ± 7.19	39.12 ± 13.17	0.30 ± 0.35	4.05 ± 3.76	64.90 ± 17.34	284.47 ± 37.52
November 2004	32.85 ± 0.46	25.25 $\pm$ 0.15	1.91 $\pm$ 0.21	0.96 $\pm$ 0.18	35.53 ± 0.14	4.32 $\pm$ 0.21	48.09 ± 0.13	23.00 ± 0.08	20.68 ± 0.25	43.74 ± 0.43	2.70 ± 0.26	3.09 ± 0.39	90.60 ± 0.31	332.72 ± 3.20
December 2004	41.43 ± 0.12	36.15 $\pm$ 0.33	4.66 $\pm$ 0.17	2.64 $\pm$ 0.42	44.88 ± 0.16	4.54 $\pm$ 0.09	51.04 ± 0.02	42.65 ± 0.12	31.78 ± 0.10	63.97 ± 0.19	4.38 ± 0.53	2.92 ± 0.17	121.43 ± 0.22	452.47 ± 2.64
January 2005	ND	ND	ND	ND	ND	ND	ND	44.94 ± 0.16	ND	ND	ND	5.97 ± 0.19	96.13 ± 0.97	147.04 ± 1.32
February 2005	43.68 ± 0.33	39.12 $\pm$ 0.37	12.54 $\pm$ 0.12	3.94 $\pm$ 0.21	35.50 ± 0.23	2.97 $\pm$ 2.09	63.84 ± 0.44	52.93 ± 0.24	23.15 ± 0.24	35.20 ± 0.15	13.39 ± 0.64	3.07 ± 0.36	88.70 ± 0.54	418.03 ± 4.32
Dry season mean $\pm$ s.d.	29.49 ± 20.21	25.13 $\pm$ 17.78	4.73 $\pm$ 5.52	1.89 $\pm$ 1.75	28.98 ± 19.82	2.96 $\pm$ 0.75	40.74 ± 28.01	40.88 ± 12.71	18.90 ± 13.47	35.73 ± 26.70	5.12 ± 5.80	3.76 ± 1.47	99.22 ± 15.14	337.52 ± 135.57
Range	ND-43.68	ND-39.12	ND-12.54	ND-3.94	ND-51.92	ND-4.54	ND-63.84	ND-52.93	ND-50.72	ND-63.97	ND-13.39	0.57-8.29	40.78-121.43	147.04-452.27

Table 6. Bioconcentration factor (BCF) of OCPs in fish from the pond water of Oke-Osun Farm Settlement, Osogbo, Nigeria.

OCPs	α -BHC	β -BHC	γ -BHC	Heptachlor	Aldrin	Chlordane	p, p'-DDE	o, p'-DDD	p, p'-DDD	Dieldrin	Endrin	Endosulfan	p, p'-DDT
Rainy season BCF	1.10	1.72	0.06	0.06	1.76	0.08	1.02	0.22	2.00	1.42	0.01	0.12	1.79
Dry season BCF	4.95	93.07	0.56	0.32	2.84	0.40	2.89	4.00	2.54	2.07	0.47	0.34	8.04

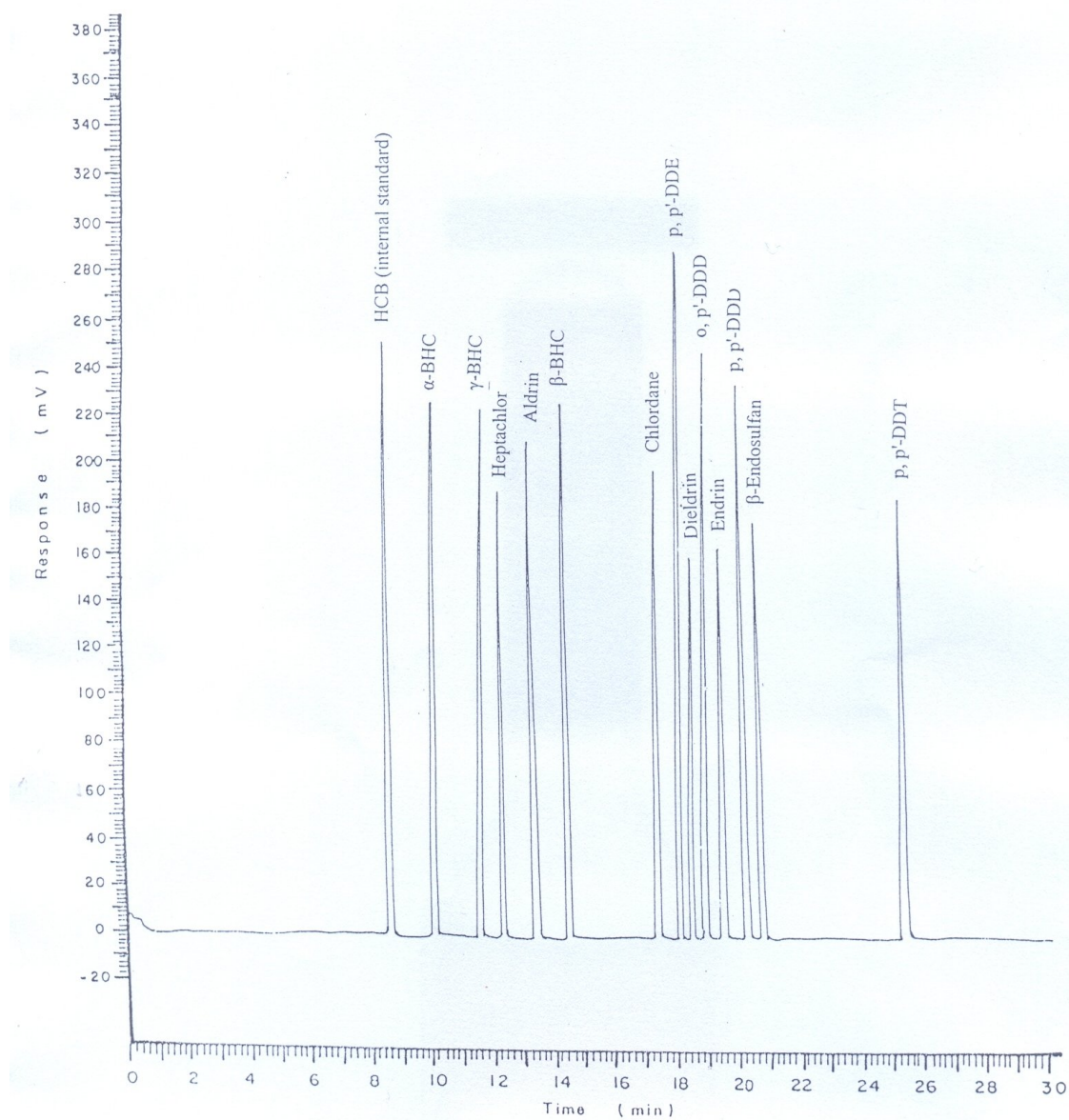


Figure 2. Representative chromatogram of the organochlorine pesticides (OCPs) standards.

Table 7. Matrix of Correlation Coefficients of OCPs in Fish from Oke-Osun Farm Settlement, Osogbo, Nigeria.

	α - BHC	β - BHC	γ - BHC	Heptachlor	Aldrin	Chlordane	p, p'- DDE	o, p'- DDD	p, p'- DDD	Dieldrin	Endrin	Endosulfan	p, p'- DDT
α - BHC	1.00												
β - BHC	0.49	1.00											
γ - BHC	0.50	0.68	1.00										
Heptachlor	0.67	0.34	0.77*	1.00									
Aldrin	0.56	0.86*	0.64	0.46	1.00								
Chlordane	0.23	0.73*	0.42	0.14	0.91*	1.00							
p, p'- DDE	0.62	0.79*	0.89*	0.64	0.58	0.30	1.00						
o, p'- DDD	0.48	-0.07	0.05	0.11	0.08	-0.02	0.07	1.00					
p, p'- DDD	0.64	0.23	0.47	0.84*	0.24	-0.11	0.54	0.04	1.00				
Dieldrin	0.60	0.71*	0.95*	0.81*	0.64	0.34	0.91*	0.12	0.59	1.00			
Endrin	0.56	0.71*	0.98*	0.76*	0.70*	0.45	0.89*	0.20	0.46	0.97*	1.00		
Endosulfan	0.32	-0.41	-0.29	0.17	-0.36	-0.56	-0.15	0.59	0.46	-0.08	-0.19	1.00	
p, p'- DDT	0.46	0.56	0.40	0.12	0.50	0.34	0.45	0.53	-0.14	0.51	0.54	-0.01	1.00

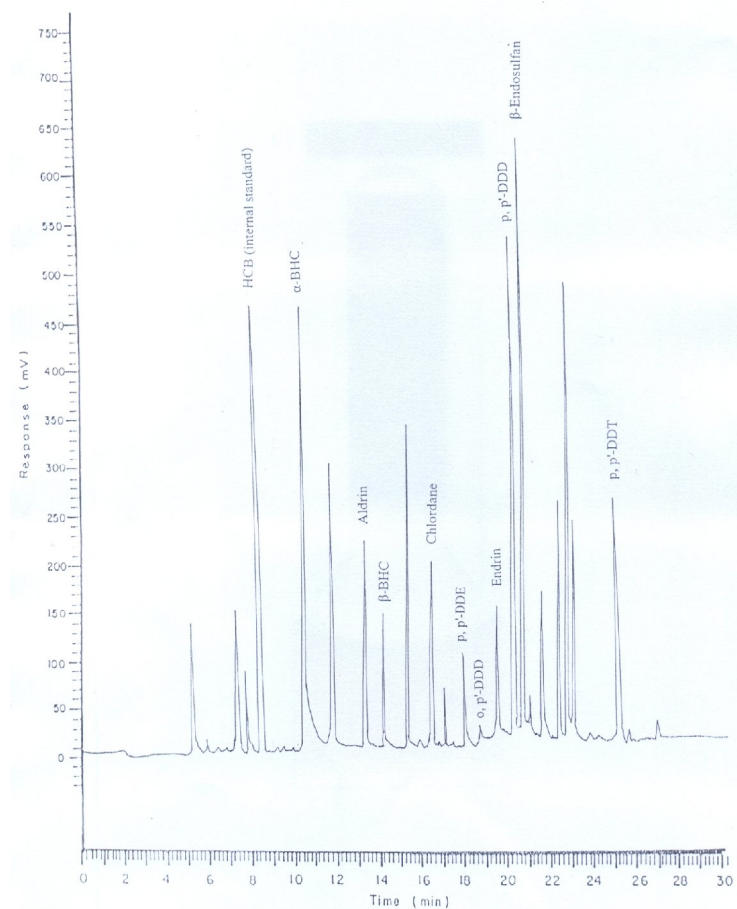


Figure 3. Representative chromatogram of the organochlorine pesticides (OCPs) in pond water.

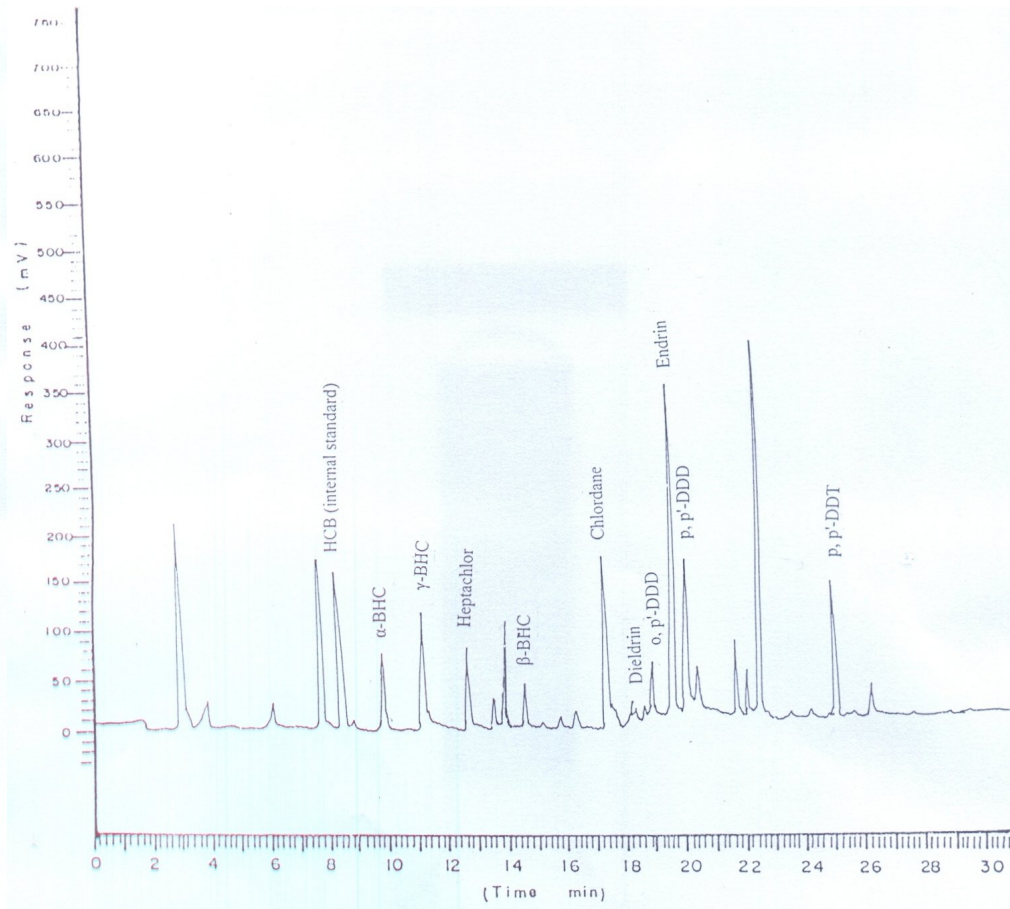


Figure 4. Representative chromatogram of the organochlorine pesticides (OCPs) in fish.

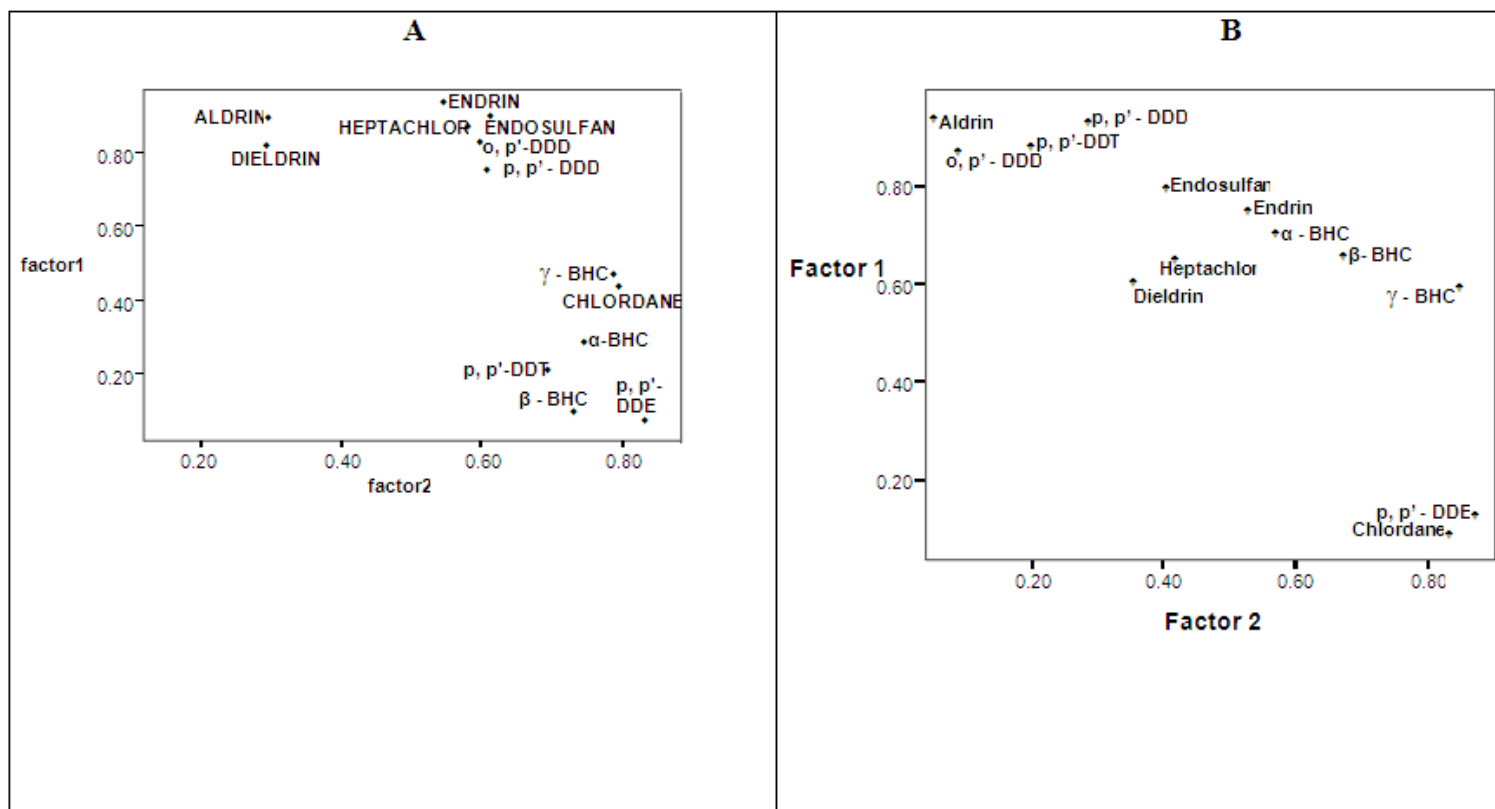


Figure 5. Cluster analyses for OCPs in pond water (A) and Fish sample (B) from Oke Osun farm settlement, Osogbo, Nigeria.

Levels of OCPs in pond water during the rainy season are presented in Tables 2 (a-d). While the monthly mean levels in $\mu\text{g/L}$ ranged from chlordane 2.63 (3.50) in May to p, p'-DDE 85.12 (26.35) in June, the seasonal mean had a range between chlordane 12.42 (12.75) and o, p'-DDD 39.60 (36.61) in the pond water. Agunloye (1984) and Tongo (1985) studied the occurrence and levels ($\mu\text{g/L}$) of chlorinated hydrocarbons in water of rivers and lakes in Southern Nigeria and obtained for lindane (ND - 167), aldrin (ND - 190), endosulfan (ND - 750), HCB (ND - 9.2) and heptachlor (ND - 96). Ogunlowo (1991) studied the OCPs levels in 9 rivers in Ondo state and obtained a range of ND to 2150 ng/L for lindane, heptachlor, endrin, aldrin and dieldrin. The results obtained in the present study showed that the presence of these pesticides in the pond water occurred at levels comparable to or in some cases higher than those of the earlier workers mentioned above who used similar methods and instrumentation for the analysis of OCPs. The OCPs: α -BHC, p, p'-DDE, o, p'-DDD, dieldrin, endrin, endosulfan and p, p'-DDT occurred in 100% of the pond water samples; γ -BHC, heptachlor, chlordane and p,p'-DDD occurred in 91.7% of the pond water samples while aldrin and β -BHC respectively occurred in 83.3% and 66.7% of the pond water samples studied.

Tables 3 (a - d) represents the dry season levels of OCPs in the pond water. The lowest monthly mean level in $\mu\text{g/L}$, ND, was recorded for β -BHC in November, January and February and chlordane in November while the highest monthly mean level of 24.90 (8.76) $\mu\text{g/L}$ was recorded for dieldrin in February. For the dry season mean level, the OCPs levels ranged between β - BHC 0.27 (0.90) and dieldrin 17.24 (8.71) $\mu\text{g/L}$. The results showed higher values in comparison to the values of α - and β - BHC (1 - 302 ng/L), lindane (7 - 297 ng/L), aldrin (ND - 19 ng/L), heptachlor (4 - 202 ng/L) and total DDT (ND - 1266 ng/L) reported by Nwankwoala and Osibanjo (1992) for surface waters in Ibadan. The values obtained in this study, however, were quite higher than the values obtained in one of the earliest studies on River Nile (El-Sebae and Abu-Elamayen, 1979) in which the levels of lindane, heptachlor, o,p'-DDT and p,p'-DDT ranged between 100 and 950 ng/L in all the water samples. The concentration levels of OCPs in the pond water suggested the use of the pesticides in the recent past despite the fact that some of the OCPs detected such as DDT had been outlawed since 1990 (FEPA, 1991).

The matrix of correlation coefficients of the OCPs in the pond water at 0.05 levels in Table 4

showed positive significance of 92.2% for all the correlated items. The pesticides with significant positive correlations likely shared common sources and were probably affected by related factors in the aquatic environment. Table 5 contains a list of the mean levels of OCPs (in $\mu\text{g/kg}$) in fish samples on a monthly basis. The rainy season monthly mean values of OCPs ($\mu\text{g/kg}$) in fish ranged from endrin 0.30 ± 0.35 to p, p'-DDT 64.90 ± 17.34 with a total mean body burden of 284.47 ± 37.52 $\mu\text{g/kg}$. On the other hand, during the dry season, the monthly mean values of OCPs ($\mu\text{g/kg}$) in fish ranged between 1.89 ± 1.75 of heptachlor to 99.22 ± 15.14 of p, p'-DDT with a total OCPs body burden of 337.52 ± 135.57 $\mu\text{g/kg}$. α -BHC, endosulfan and p, p'-DDT were found in all (100%) of the fish samples; β -BHC, γ -BHC, aldrin, p, p'-DDE, p, p'-DDD and dieldrin in 87.5% of the samples; heptachlor and o, p'-DDD in 75.0% of the samples; and chlordane and endrin in 62.5% of the samples.

The levels of OCPs detected in the fish samples of the present study, were in most cases relatively higher than the values obtained by Amakwe (1984) in freshwater fish samples collected from various locations in Oyo and Ogun states of Nigeria in which lindane (7 - 106.0); p, p'-DDE (2.0 - 30.0); p, p'-DDD (2.0 - 60.0); p, p'-DDT (3.0 - 18); total DDT (3.3 - 161); heptachlor (1.0 - 300); endosulfan (3 to 904); and α - HCH (0.2 - 5.0) were detected at ng/kg levels. However, the results of the present study for levels of OCPs in fish compared well with those of Fayomi (1987): α - HCH (1.0); aldrin (ND - 14.9); p,p'-DDE (ND - 4.2); DDD (ND - 8) $\mu\text{g/kg}$; Osibanjo and Bamgbose (1990): HCB (0.04 - 9.48); lindane (ND - 5.30); endosulfan (ND - 4.95); DDT (0.15 - 18.6); aldrin (ND - 54.60) $\mu\text{g/kg}$; and those of Manirakiza *et al.* (2002): HCB (5.4 - 81.5), heptachlor (ND - 13.1), aldrin (ND - 0.2), p, p'-DDE (6.7-197.5), o, p'-DDD (ND - 12.1), p, p'-DDT (ND - 31.6), dieldrin (ND - 7.1), endrin (ND - 14.4) and endosulfans (1.5 - 49.7) $\mu\text{g/kg}$. Figures 3 and 4 are representative chromatograms for OCPs detected in the raw pond water and fish samples respectively. The bioconcentration factors (BCF) of OCPs for fish in the two seasons are listed in Table 6. The values ranged from 0.01 (endrin) to 2.00 (p, p'-DDD) in rainy season and from 0.32 (heptachlor) to 93.07 (β - BHC) in dry season. Although the levels of OCPs in the pond water were higher during the rainy season, the BCF was higher in all the cases considered during the dry season. This, by implication, means that the fish samples were more contaminated with OCPs during the dry season than in the rainy season. The higher dry season levels of

OCPs in fish (Table 5) generally might be due to their feeding more on insect larvae, coarse vegetable matters and sediment-associated particles that had accumulated the OCPs overtime. The mean seasonal levels of OCPs in the fish were higher, in most cases, than the recommended WHO Maximum Residue Levels (MRLs) in food items. For example, γ -BHC (lindane) and dieldrin with WHO MRLs of 6.0 $\mu\text{g/kg}$ (Darko and Acquah, 2007) occurred in this study at 25.02 and 27.47 $\mu\text{g/kg}$ levels respectively during the rainy season and at 8.48 and 17.24 $\mu\text{g/kg}$ levels respectively during the dry season. However, the levels of DDT in the fish samples were generally lower than the 500 $\mu\text{g/kg}$ WHO MRLs (Darko and Acquah, 2007).

The matrix of correlation coefficients of OCPs in fish samples (Table 7) showed significant positive correlations at 0.05 level for β - BHC and aldrin, chlordane, p, p' - DDE, dieldrin and endrin; γ - BHC and heptachlor, p, p' - DDE, dieldrin and endrin; heptachlor and p, p' - DDD, dieldrin and endrin; heptachlor and p, p' - DDD, dieldrin and endrin; p, p' - DDE and dieldrin and Endrin; and dieldrin and endrin.

Cluster analysis, a method for dividing a group of objects into classes so that similar objects are in the same class (Miller and Miller, 2000), was applied in this study to see the possible association of the OCPs. Three clusters of OCPs were found (Figure 5 A) in the pond water: aldrin, o,p'-DDD, p,p'-DDT and p,p'-DDD; endosulfan, endrin, α -BHC, β -BHC, γ -BHC, heptachlor and dieldrin; and p,p'-DDE and chlordane, which shows that each of the three classes of compounds are associated. Their presence in the samples may be due to factors such as transportation of applied OCPs by surface run-off from the nearby agricultural soils into the pond, formation of the metabolites of OCPs and careless handling and disposal of materials contaminated with the OCPs e.g. washing of empty OCPs containers into the pond water after application by the farmers. For the fish, feeding directly on contaminated food items within the pond or drinking of contaminated water and metabolite formation within and outside the body tissue of fish are considered to be the likely factors responsible for the presence of the three clusters of OCPs observed (Figure 5 B). The observed clusters in fish are: aldrin and dieldrin; endrin, heptachlor, endosulfan, o,p'-DDD, and p,p'-DDD; γ -BHC, chlordane, p,p'-DDT, α -BHC, β -BHC and p,p'-DDE. The occurrence of OCPs in water and fish from the pond of Oke Osun Farm settlement is

consistent with the agricultural activities of the study area due to pesticide usage by the farmers for the control of weeds and pests. Some of the pesticides found in the matrices like chlordane, endosulfan, DDE and DDT have been implicated to have endocrine disrupting properties which may greatly impact on the bio-diversity of the aquatic ecosystem.

Conclusion

In conclusion, this study has shown that pond water and fish samples from Oke Osun farm settlement are contaminated with OCPs in varied degrees in both dry and wet seasons.

Higher levels of OCPs were recorded in the pond water during the rainy season with a mean range of $12.42 \pm 12.75 \mu\text{g/L}$ chlordane – $39.60 \pm 36.61 \mu\text{g/L}$ o,p'-DDD than the dry season with a mean range of $0.27 \pm 0.90 \mu\text{g/L}$ β -BHC – $17.24 \pm 8.71 \mu\text{g/L}$ dieldrin. Conversely, however, higher levels of OCPs were found in the fish samples in the dry season than during the rainy season due to possible greater bioaccumulation tendency in the fish species during the dry season. Generally, the mean seasonal levels of OCPs in the fish were higher, in most cases, than the recommended WHO Maximum Residue Levels (MRLs) in food items and should give cause for concern. Occurrence of the OPCs in fish and water from the pond is consistent with the agricultural activities of the study area due to pesticide usage by the farmers. Further work on pesticide residues in blood serum of the farmers and municipal water sources of the study area is recommended.

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References

- Adeyemi, D., G. Ukpo, C. Anyakora and J. P. Unyimadu. 2008. Organochlorine pesticide residues in fish samples from Lagos lagoon, Nigeria. *Am. J. Enviro. Sci.* 4(6):649–653.
- Adeyeye, A. and O. Osibanjo. 1990. Residues of organochlorine pesticides in fruits, vegetables and tubers from Nigerian markets. *Sci. Total Env.* 231:227-333.
- Agunloye, T. O. 1984. A survey of chlorinated hydrocarbons in rivers of southern Nigeria.

- MSc. Thesis. Department of Chemistry, University of Ibadan, Nigeria.
- Ajayi, S. O and O. Osibanjo. 1981. Pollution studies on Nigerian rivers: water quality of some Nigerian rivers. *Env. Pollut.* 2:87-95.
- Akinnifesi, T. A., O. I. Asubiojo and A. A. Amusan. 2006. Effects of fungicide residue on the physico-chemical characteristics of soils of a major cocoa-producing area of Nigeria. *Sci. Total Environ.* 366:876-879.
- Allen, D. L. and D. L. Otis. 1998. Relationship between deer mouse population parameters and dieldrin contamination in the rocky mountain arsenal national wildlife refuge. *Can. J. Zool.* 76:243-250.
- Amakwe, C. 1984. Organochlorine pesticide residues in Nigerian freshwater fish. BSc. Dissertation. Department of Chemistry, University of Ibadan, Nigeria.
- Arias-Estevez, M., E. Lopez-Periago, E. Martı́nez-Carballo, J. Simal-Gańdara, J. C. Mejuto and L. Garcı́a-Rı́o. 2008. The mobility and degradation of pesticides in soils and the pollution of groundwater resources. *Agric. Ecosystems Env.* 123:247-260.
- Cabral J., R. Hall, L. Rossi, S. A. Bronczyk, and P. Shubik. 1982. Effects of long-term intake of DDT on rats. *Tumori.* 68:11-17.
- Clement, J. and A. Okey. 1974. Reproduction in female rats born to DDT - treated parents. *Bull. Env. Contam. Toxicol.* 12:373-377.
- Close, D. H. 1996. Biomonitoring at Rocky Mountain Arsenal using the Plains Pocket Gopher (*Geomys bursarius*). MS. Thesis, Colorado State University, Fort Collins, Co. 109 pp.
- Codex, Alimentarium Commission (CODEX) 2004. Report of the twenty-seven session, Geneva, Switzerland; 28 June-July 3.
- Cohn, J. P. A. 1999. Makeover for Rodey Mountain Arsenal: Transforming a Superfund Site into a National Wildlife Refuge. *Biosci.* 49:273-277.
- Creekmore, T. E., D. G. Whittaker, R. R. Roy, J. C. Franson and D. L. Baker. 1999. Health status and relative exposure of mule deer and white-tailed deer to soil contaminants at the rocky mountain. *Arsenal Environ. Toxicol. Chem.* 18:272-278.
- Darko, G. and S. O. Acquaaah. 2007. Levels of Organochlorine Pesticides in Meat. *Int. J. Environ. Sci. Tech.* 4(4):521 - 524.
- El-Sebae, A. H. and M. A. Abu-Elamayem. 1979. A survey to determine potential pollution of the Mediterranean by pesticides from the Egyptian region. *J. Etud. Pollut CIESM* (4):149-53.
- Eriksson, P., T. Archer, and A. Fredrikson. 1990. Altered behaviour in adult mice exposed to a single low dose of DDT and its fatty acid conjugate as neonates. *Brain Res.* 514:141-142.
- Fabro, S., J. A. McLachlan and N. M. Dames. 1984. Chemical exposure of embryos during the preimplantation stages of pregnancy: Mortality rate and intrauterine development. *Am. J. Obstetrics Gynecol.* 148:929-38.
- Fatoki, O. S. and F. Vernon. 1990. Phthalate Esters in Rivers of the Greater Manchester Area, U. K. *Sci. Total Env.* 95:227-232.
- Fatoki, O. S. and R. O. Awofolu. 2003. Methods for selstive determination of persistent organochlorine pesticide residues in water and sediments by capillary gas chromatography and electron capture detection. *Chromatog. A* 983(1-2):225-236.
- Fayomi, S. F. 1987. Determination of organochlorine pesticides and polychlorinated biphenyls (PCBs) in Nigerian fresh water fishes from Cross River State of Nigeria. BSc. Dissertation, Department of Chemistry, University of Ibadan, Nigeria.
- FEPA, 1991. Federal Environmental Protection Agency Guidelines and Standards for Environmental Pollution Control in Nigeria, FEPA, Lagos.
- Gonzalez-Lopez, N., R. Ria-otero, B. Cancho-Grande, J. Simal-Gandara and B. soto-Gonzalez. 2005. Occurrence of Organochlorine Pesticides in Stream Sediments from an Industrial Area. *Arch. Environ. Contam. Toxicol.* 48:296-302.
- Lopez-Perez, G.C., M. Arias-Estevez, E. Lopez-Periago, B. Soto-Gonzalaz, B. Cancho-Grande and J. Simal-Gandara. 2006. Dynamics of Pesticides in Potato Crops. *J. Agric. Food Chem.* 54:1797-1803
- Hamelink, J. L., D. R., Buckler, F. L. Mayer, D. V. Palawski and H. O. Sanders. 1986. Toxicity of

- fluridone to aquatic invertibrates and fish, *Env. Toxicol. Chem.* 5:87-94.
- Hannon, M. R., Y. A. Greichus, R. L. Applegate and A. C. Fox. 1970. Ecological Distribution of Pesticides in Lake Poinsett, S. Dakota, *Trans. Ann. Fish Sec.* 99(3):496.
- Ize-Iyamu, O. K., I. O. Asia and P. A. Egwakhide. 2007. Concentrations of residues from organochlorine pesticide in water and fish from some rivers in Edo State, Nigeria. *Int. J. Physical Sci.* 2(9):237-241.
- Johnson, R. L., P. J. Gehring, R. J. Kociba and B. A. Schwetz. 1973. Chlorinated Dibenzodioxins and Pentachlorophenol. *Environ. Health Prospect.* 5:171-175.
- Jonsson, Jr. H. T., J. E. Keil, R. G. Gaddy, C. B. Loadholt, G. R. Hennigar and E. M. Walker. 1976. Prolonged ingestion of commercial DDT and PCB, effects on progesterone levels and reproduction in the mature female rat. *Arch. Env. Cont. Toxicol.* 3: 470 - 490.
- Kaakeh, W., M. Maraqa, M. Hasan, M. El Marzouqi. 2004. Management of Pesticides in the United Arab Emirates. *Emir. J. Agric. Sci.* 16(1):15-31.
- Kaphila, B. S., R. Takroo, S. Mehtrota, U. Nigam and T. D. Seth. 1990. Organochlorine pesticide residues in different Indian cereals, pulses, spices, vegetables, fruits, milk, butter, deshi, ghee and edible oils, *J. Assoc. Off. Anal. Chem.* 73:509-512.
- Leiker, T.T. J, J. E. Madsen, J. R. Deacon and W. T. Foreman. 1996. Methods of analysis by the U.S. Geological Survey National Water Quality Laboratory- Determination of chlorinated pesticides in aquatic tissue by capillary-column gas chromatography with electron-capture detection: U.S. Geological Survey, Open-File Report 94 -710.
- López-Blanco, C., S. Gómez-Álvarez, M. Rey-Garrote, B. Cancho-Grande and J. Simal-Gándara. 2005. Determination of carbamates and organophosphorus pesticides by SDME-GC in natural water. *Anal. Bioanal. Chem.* 383:557-561.
- López-Blanco, C., S. Gómez-Álvarez, M. Rey-Garrote, B. Cancho-Grande, J. Simal-Gándara. 2006. Determination of pesticides by solid phase extraction followed by gas chromatography with nitrogen-phosphorous detection in natural water and comparison with solvent drop microextraction. *Anal. Bioanal. Chem.* 384:1002-1006.
- Luke, M. A., H. T. Matsumoto, T. Cairns and H. K. Hundley. 1988. Levels and incidence of pesticide residues in various foods analysed by the Luke multiresidue methodology for fiscal years 1982 – 1986, *J. Assoc. Off. Anal. Chem.* 71:415 – 433.
- Lundberg, C. 1974. Effects of DDT on cytochrome P - 450 and oestrogen-dependent functions in mice. *Env. Physiol. Biochem.* 4: 200 - 204.
- MAFF, 1989. Report of the working party on pesticide residues, 1985-1988, *Food Surveillance Paper No. 25*, London: HMSO. 189:71.
- Manirakiza, P., O. Akinbamijo, A. Covaci, S. A. Adediran, I. Cisse, S. T. Fall and P. Schepens. 2002. Persistent chlorinated pesticides in fish and cattle fat and their implications for human serum concentrations from Sene-Gambian region. *J. Environ. Monit.* 4:609-617.
- Merry, R. H., K. G. Tiller and A. M. Alston. 1983. Accumulation of copper, lead and arsenic in some Australia orchard soils. *Aust. J. Soil Res.* 21:549-61.
- Miller, J. N. and J. C. Miller. 2000. *Statistics and Chemometrics for Analytical Chemistry*, 4th Edition, Pearson Education Limited, pp. 271.
- Muino, M. A. F., M. T. Sancho, J. S. Gandara, J. M. C. Vidal, J. F. Huidobro and J. S. Lozano. 1995. Organochlorine pesticide residues in Galician (NW Spain) honeys. *Apidologie* 26:33-38.
- Nwankwoala, A. U. and O. Osibanjo. 1992. Baseline levels of selected organochlorine pesticides in surface waters in Ibadan (Nigeria) by electron capture gas chromatography. *Sci. Total Env.* 119:179-90.
- Ogunfowokan, A. O., O. I. Asubiojo and O. S. Fatoki. 2003. Isolation and determination of polycyclic aromatic hydrocarbons in surface runoff and sediment. *Water, Air Soil Poll.* 147: 245 – 261.
- Ogunfowokan, A. O., N. Torto, A. A. Adenuga and E. K. Okoh. 2006. Survey of levels of phthalate ester plasticizers in a sewage lagoon effluent and a receiving stream. *Env. Monitor. Assess.* 118: 457 – 480.

- Ogunlowo, S. O. 1991. Priority chemical pollutants in some rivers along the cocoa growing area of Ondo State. MSc. Thesis, Department of Chemistry, University of Ibadan, Nigeria.
- O'Shea, T. J., A. L. Everette and L. E. Ellison. 2001. Cyclodiene Insecticide, DDE, DDT, Arsenic and Mercury Contamination of Big Brown Bats (*Eptesicus fuscus*) Foraging at a Colorado Superfund Site. Arch. Environ. Contam. Toxicol. 40:112-120.
- Osibanjo, O. and O. Bamgbose. 1990. Chlorinated hydrocarbons in marine fish and shellfish of Nigeria. Mar. Pollut. Bull. 21:581-586.
- Rosa, M. Gonzalez-Rodriguez, R. Rial-Otero, B. Cancho-Grande, J. Simal-Ga'ndara. 2008. Determination of 23 pesticide residues in leafy vegetables using gas chromatography-ion trap mass spectrometry and analyte protectants. Journal of Chromatography A. 1196-1197:100-109.
- Ruiz-Gil, L., R. Romero-Gonzalez, A. G. Frenich and J. L. M. Vidal. 2008. Determination of pesticides in water samples by solid phase extraction and gas chromatography tandem mass spectrometry. J. Sep. Sci. 31:151-161.
- Skaare, J. U., N. H. Markussen, G. Norheim, S. Haugen and G. Holt. 1990. Levels of polychlorinated biphenyls, organochlorine pesticides, mercury, cadmium, copper selenium arsenic and zinc in the harbor seal, *Phoca vitulina*, in Norwegian waters. Environ. Pollut. 66:309-324.
- Sosan, M. B., A. E. Akingbohunge, I. A. O Ojo and M. A. Durosinmi. 2008. Insecticide residue in the blood serum and domestic water source of cacao farmers in southwestern Nigeria. Chemosphere 72:781-784.
- Suzuki, M., Y. Yamato and T. Watanabe. 1977. Analysis of environmental samples for organochlorine insecticides and related compounds by high resolution electron capture gas chromatography with capillary columns. Environ. Sci. Technol. 11:1109-1113.
- Tongo, A. A. 1985. Baseline study of levels of organochlorine pesticides in Nigerian rivers and their sediments. MSc. Thesis. Department of Chemistry, University of Ibadan, Nigeria.
- WHO, 1979. WHO/UNEP Project on Assessment of Human Exposure to Pollutants Through Biological Monitoring. Report of a Planning Meeting on Quality Control, Geneva, 26 February - 9 March.