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Effect of *Mentha piperita* essential oil against *Vibrio* spp. isolated from local cheeses

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ABSTRACT

The aim of this study, *Vibrio* spp. was detecting in cheese manufacture and effect of essential oil of *Mentha piperita* on this bacteria. A 126 isolates of *Vibrio* spp. were isolated from 30 samples of two types of local cheeses. The samples were collected from 14 markets in Basrah city. 8 species from *Vibrio* genes was obtained and defined by microscopic and biochemical tests. *Vibrio parahaemclyticus* and *Vibrio cholera* were the highest percentage among other isolates. It was 33% and 25 % respectively. Essential oil of *Mentha piperita* was extracted from leaves. It was 2% (v:w) which used for *Vibrio* spp. isolates inhibition. *Vibrio logei* was most sensitive against 15 µl of Mentha *piperita* essential oil. The MIC of *Vibrio* spp. was.0035 ml excepted *V. cholera* was 0.0041 ml and V. *harveyi*, *V. logei* were 0.0027 mL.

Key word: *Vibrio* spp., *Mentha piperita*, essential oil, local cheese.

INTRODUCTION

Vibrio genus is belongs to Vibrionaceae family. *Vibrio* are a genus of Gram-negative bacteria, possessing a curved-rod shape (comma shape), facultative anaerobes that test positive for oxidase and do not form spores. Several species of which can cause foodborne infection, usually associated with eating undercooked seafood. Typically found in salt water (Machado & Gram, 2015).

The *Vibrio* spp. isolation from Egyptian soft Domiati cheese which content 5.4- 9.5% NaCl and this bacteria was identified by molecular methods (El-Baradei *et al*., 2007). Fourteen different species included *Vibrio* spp. were isolated from surface four cheeses (Mounier *et al*., 2005). Forty types of food samples from markets of Dhaka city including meat, fish, vegetables, fruits, street food, bakery shop food, fast food, sweets and dairy products. They were used *Vibrio* spp. isolation (Mrityunjoy *et al*., 2013).

An essential oil is a concentrated hydrophobic liquid containing volatile aroma compounds from plants. Essential oils are also known as volatile oils, ethereal oils, aetherolea, or simply as the oil of the plant from which they were extracted, such as oil of clove. Essential oils are generally extracted by distillation, often by using steam. Other processes include expression, solvent extraction, absolute oil extraction, resin tapping, and cold pressing (Baser & Buchbauer, 2010).

Essential oils have antimicrobial and antioxidant activity which used in medical, pharmacy and food keeping (Pirbalouti *et al*., 2013; Zengin & Baysal, 2014; Niamah & Alali, 2016). The Essential oil extract of *Mentha* species used as antimicrobial, antioxidant and antimutagenic (Mimica-Dukić *et al*., 2010; Mickiene *et al*., 2011). This essential oil content more bioactive compounds. The Linalool is major compound found in essential oil of Mentha (Silva *et al*., 2015). The aim of this study was to isolate *Vibrio* species from local cheeses found in market of Basrah city/ Iraq and study the effect of these essential oils from Iraqi *Mentha piperita* plant on this bacteria

MATERIAL AND METHODS

Cheese sampling

Two types' local cheeses were collected from 13 markets of Basrah city. The samples divided into two groups. One group was included 17 samples of white soft Iraqi cheese, another group was included 13 samples of braids cheese. 50 grams of cheese samples were transferred into biotechnology Lab./ Agriculture college / Basrah university, under sterile conditions.

Bacterial isolation

Eleven grams of cheese sample transferred to conical flask contents 99 ml of alkaline peptone water at pH 8.6 and incubated at 37℃ for 6 hours (Lesmana *et al*., 1985), thereafter 1ml of last dilations transferred to petri dish, poured Thiosulfate citrate bile salt agar (TCBS) media (LAB company, UK.) and incubated at 37℃ for 24-48 hours (Barrow & Feltham, 2003).

Vibrio spp. identification

All isolates were identified to be *Vibrio* spp. depending on microscopic examinations and

biochemical tests which included gram staining, spore forming, motility, oxidase test, Voges-Proskauer test, growth without NaCl, growth with (1, 3, 6, 12)% NaCl, myo-inositol, D-mannitol, L-arabinose, cellabiose and sucrose fermentation, ammonia production from arginine, acid and gas production from glucose, nitrate reduction, indole and citrate utilization (Holt, 1994).

Extraction of *Mentha piperita* essential oil

Essential oil was extracted from *Mentha piperita* leaves using Clevenger apparatus. 250 g of leaves mixed with 500 ml of distilled water was transferred into oil distillation for 1-3 hours at 95℃. The essential oil was then collected and determined by calibrated tube. It was kept in the freezer (Niamah & Alali, 2016).

Antibacterial activity essay

The antibacterial activity of essential oil extract from *Mentha piperita* leaves was determined by Agar diffusion method. 1 ml of *Vibiro* spp. was streaked by L- shape on Mueller-Hinton agar (Hi-media, India) and worked on 3 wells (6 mm) in agar. 5, 10 and 15 µl of essential oil extract were transferred to wells and Petri dishes kept in the refrigerator for 2 hours and incubated at 37℃ for 24-48 hours, effective inhibitory was estimated by measuring diameters of clear zones (Valgas *et al*., 2007).

Determination of minimal inhibitory concentration (MIC)

The MIC of essential oil extract from *Mentha piperita* leaves was determined by (Mann & Markham, 1998). The essential oil of *Mentha piperita* was added into molten Iso- sensitest agar (Oxoid, UK) with 0·25% (v/v) Tween 20 at 45-50℃. The range of essential oil concentrations was from 0.001 ml to 0.005 ml (v/v) %. 0.1 ml (10^6-10^8 cfu/ mL) of Vibrio spp. transferred plates and incubated at 37°C for 18-24 hours. The MIC was determined as the lowest concentration of oil to result in no growth of *Vibrio* spp. bacteria.

RESULTS AND DISCUSSION

Bacteria isolation

Vibrio spp. were found in all samples except four samples from braids cheese. The numbers of *Vibrio* spp. were high in white cheese than with braids cheese because of the braids cheese was produced by acidic method (Abd El Razig *et al*., 2002) and *Vibrio* spp. growth was weak in acidic media and the starter cheese do on anther bacteria inhibition (Widyastuti *et al*., 2014). The starter no add into with soft Iraqi cheese (Hanna & Nader, 1996). *Vibrio* spp. transferred to cheeses by way washing water, which is used after the industry and during the sales process.

Identification of *Vibrio spp.*

A 126 isolates from 152 isolates were selective after microscopic tests. Green colonies and yellow colonies were selected from TCBS cultures. The isolates were curved or straight form, Gram staining, non-spore forming and motile. The biochemical tests shown in table 2. 33(21.71%) isolates as *V. parahaemclyticus*, 25 (16.44%) isolates as *V. cholera*, 15 (09.86%) isolates as *V. vulnificus*, 12 (07.89%) isolates as *V. alginolyticus*, 12 (07.89%) isolates as *V. mimicus*, 11(07.23%) isolates as *V. damsela*,8 (05.26%) isolates as *V. campbellii*, 6 (03.94%) isolates as *V. harveyi*, 4 (02.63%) isolates as *V. logei* and 26 (17.10%) non *Vibrio* isolates (Farmer & Hickman-Brenner, 2006). The colony appearance on selective media was followed by conventional biochemical tests, for detection of *Vibrio* spp isolates. The phenotypic similarities of the eight species observed in the results of biochemical tests (Noguerola & Blanch, 2007). Carbohydrates fermentation and growth with NaCl were important tests to differentiate of Vibrio spp (Paydar, 2013). Asserts that the tests which have been applied in this study were able to efficiently differentiate these species. Thus, for detection of the species of the isolates, the conventional biochemical tests showed good method. However, the overall findings of these tests indicated that they are able to be used for detection of *Vibrio* spp.

Bacteria inhibition

The yield of *Mentha piperita* essential oil was 2 % (v:w). The table 3 show effect of essential oil extract from *Mentha piperita* leaves against *Vibrio* spp. isolates from cheese samples. All isolates were inhibited by essential oil and inhibition zones were different between *Vibrio* spp. isolates when increased concentration essential oil of *Mentha piperita* led increase diameters of inhibition. *V. harveyi* and *V. logei* were larger inhibition among anther isolates. The inhibition zones of this bacteria were (19.29 and 20.33) mm at 15 µl of *Mentha piperita* essential oils. The MIC was 0.0035 ml of isolates excepted *V. cholera* was 0.0041 ml and V. *harveyi*, *V. logei* were 0.0027 mL. The essential oil of *Mentha piperita* was contented more compounds as inhibitors of G^+ and G^- bacteria (Soković *et al*., 2010; Mahboubi & Kazempour, 2014). It don't have selective antimicrobial activity. The antimicrobial activity of

Table 1. Numbers of *Vibrio* spp. isolated from local cheese samples

Sample	Type of cheese	Name of the sampling site	Count (CFU/ g) of *Vibrio* spp. on TCBS
1	white soft	Old Basra	3×10^4
2	braids	Old Basra	33×10^2
3	white soft	Ashar	96×10^4
4	white soft	Ashar	42×10^5
5	white soft	Ashar	1×10^5
6	braids	Ashar	22×10^2
7	braids	Al-Qibla	77×10^3
8	white soft	Abil Khaseeb	95×10^4
9	white soft	Abil Khaseeb	44×10^4
10	white soft	Al Jumhouriya	52×10^4
11	white soft	Al Jumhouriya	99×10^3
12	white soft	Hay Alhussain	72×10^4
13	white soft	Hay Alhussain	66×10^4
14	braids	Al Hartha	45×10^2
15	braids	Al Hartha	56×10^2
16	white soft	Al Hartha	33×10^5
17	white soft	Hitteen	31×10^5
18	braids	Hitteen	67×10^2
19	white soft	Al Madeena	93×10^4
20	white soft	Al Madeena	25×10^5
21	braids	Al Madeena	Nail
22	braids	Al Madeena	Nail
23	braids	Al Nashwa	Nail
24	white soft	Al Zubair	26×10^5
25	braids	Al Zubair	1×10^2
26	braids	Al Zubair	53×10^2
27	white soft	Al Zubair	1×10^5
28	white soft	Um Qasr	55×10^4
29	braids	Al Meethag	32×10^3
30	braids	Al Ez	Nail

Table 2. Microscopic and biochemical tests of *Vibrio* spp. isolates

	Test	*V. parahaemclyticus* (n=33)	*V. cholera* (n=25)	*V. vulnificus* (n=15)	*V. alginolyticus* (n=12)	*V. mimicus* (n=12)	*V. damsela* (n=11)	*V. campbellii* (n=8)	*V. harveyi* (n=6)	*V. logei* (n=4)
	TCBS agar	G	Y	G	Y	G	G	G	Y	G
	Gram staining	-	-	-	-	-	-	-	-	-
	Spore forming	-	-	-	-	-	-	-	-	-
	Motility	+	+	+	+	+	+	+	+	+
	Oxidase	+	+	+	+	+	+	+	+	+
	Voges-Proskauer	-	±	-	+	-	+	-	-	-
Growth in	0% NaCl	-	-	-	-	+	-	-	-	-
Growth in	1% NaCl	+	+	+	+	+	+	+	+	-
Growth in	3% NaCl	+	+	+	+	+	+	+	+	+
Growth in	6% NaCl	+	-	+	+	-	+	+	+	-
Growth in	12% NaCl	-	-	-	+	-	-	-	-	-
Fermentation	Myo-inositol	-	-	-	-	-	-	-	-	-
Fermentation	D-mannitol	+	+	+	+	+	-	+	+	+
Fermentation	L-arabinose	+	-	-	-	-	-	-	-	-
Fermentation	Cellabiose	+	-	+	+	+	+	+	+	+
Fermentation	Sucrose	-	+	-	+	-	-	-	+	-
	Arginine dehydratase	-	-	-	-	-	+	-	-	-
	Gas from glucose	-	-	-	-	-	+	-	-	-
	Acid from glucose	+	+	+	+	+	+	+	+	+
	Nitrate reduction	+	+	+	+	+	+	+	+	+
	Indole	+	+	+	+	+	-	+	+	-
	Citrate utilization	-	+	±	-	+	-	-	-	-

*Symbols: n= N0. of isolates

G green**, Y** yellow**, +** positive**, -** negative**, ±** 50-70% positive

Table 3. Inhibition zones (mm) by concentrations and MIC of *Mentha piperita* essential oils

Vibrio spp. isolates	Concentrations of essential oils *Mentha piperita*			MIC (mL)
	5 µL	10 µL	15 µL	
V. parahaemclyticus (n=33)	11.55±0.30	15.13±0.43	18.20±0.36	0.0035
V. cholera (n=25)	12.18±0.25	14.77±0.48	17.08±0.22	0.0041
V. vulnificus (n=15)	11.85±0.66	16.19±0.79	18.20±0.15	0.0035
V. alginolyticus (n=12)	12.00±0.55	14.11±0.33	17.86±0.75	0.0035
V. mimicus (n=12)	12.56±0.90	15.88±0.44	18.26±0.56	0.0035
V. damsela (n=11)	11.11±0.35	14.63±0.22	17.20±0.44	0.0035
V. campbellii (n=8)	12.08±0.40	14.77±0.66	18.75±0.33	0.0035
V. harveyi (n=6)	13.54±0.21	18.13±0.49	19.29±0.56	0.0027
V. logei (n=4)	13.95±0.11	16.00±0.63	20.33±0.61	0.0027

*__Symbols: n=__numbers of isolates; each value is expressed as mean ± SD (n = 3)

essential oil of *Mentha piperita* came back to found monoterpene hydrocarbons compounds. Although these compounds are not abundant in the essential oil, and it was important activity. It is necessary to indicate that the other compounds can contribute to the improvement of this activity (Mkaddem *et al*., 2009). Many researches were reported sensitive of *Vibrio* spp. against essential oil of *Mentha piperita* (Yano *et al*., 2006; Snoussi *et al*., 2015).

CONCLUSION

All species of *Vibrio* isolation are pathogenic bacteria except *V. campbellii*, *V. harveyi* and *V. logei*. This bacteria found in two type local chesses. It transfers into cheese by water washing during the industry, storage and sales process. The cheeses sour by starters cultures bacteria content low numbers of *Vibrio* spp. The essential oil extract from *Mentha piperita* leaves have antibacterial activity against all *Vibrio* spp. isolation from chesses samples. Add essential oil of *Mentha piperita* to cheeses production is reducing the viability cells of microbes and inhibiting some species.

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Identification and quantification of anabolic steroids in imported frozen beef muscles in sulaimani market using HPLC

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ABSTRACT

In Kurdistan region of Iraq, little information about steroid hormone residues in animal products is available. The work was developed for determination of eight natural and synthetic residues of steroid hormones in five trademarks (Kilcoy,Veal Topsides, Pure south, P.Van Horten & Zn, and Creek stone Farms) of beef muscles imported from five countries. The determined values were compared with the national limits to conclude either is in legal values. The imported frozen beef muscle samples (n=75) were collected from 10th January to 10th of May 2015. Liquid-liquid phase extraction was used, and then amino-propyl cartridges and dual silica used for clean-up. The analyses were quantified by High Performance Liquid Chromatography using a phenyl column coupled to an electrospray ionization UV spectrometer (LC–ESI-UV). The results presented that all meat samples were treated with the eight steroid; whereas, progesterone and estradiol benzoate were in safe level (0.41 ± 0.5 µg/kg and 0.64 ± 0.36 µg/kg respectively) in all meat samples. The rest steroids residues were higher than the national limits except medroxy progesterone which was (0.49 µg/kg) in safe level only in samples of Kilcoy. There was no significant difference among the steroids levels regarding the five trademarks except diethylstilbestrol level which was significantly different (P value = 0.046) between P.Van Horten & Zn and Creek Stone Farms and medroxy progesterone (P value = 0.03) between kilcoy and P.Van Horten & Zn.

Keywords: Growth Promoting Hormones; Frozen beef muscle, Liquid-Liquid Extraction, HPLC, Sulaimani markets.

INTRODUCTION

Anabolic steroids are a group of synthetic hormones which could promote the storage of proteins and the growth of tissues (Kicman, 2008). Indeed, some hormones are able to create young animals obtain weight faster and help to decrease the weighting time and the amount of feed consumed by an animal pre slaughtering in meat processing area(FDA, 2013). In the 1970s, anabolic steroids were widely used to increase growth rate and develop feed conversion ability animals (Ayyar, 2011). Whereas, further studies found that they had toxic or carcinogenic properties; hence, the European Commission (EC) and the Food and Drugs Administration (FDA) prohibited their usage (Ricke, 2012). To control not allowed treatments, it was necessary to establish sensory analytical methods. Therefore, a many different of procedures has been described for the determination of anabolic residue in tissues (Sofos, 2005). Recently, the EC recommended several methods for the detection of hormonally active compounds, including high performance liquid chromatography- (HPLC) (Corradini and Phillips, 2011) and gas chromatography-mass spectrometry (GC-MS) (Horie and Nakazawa, 2000), and immunoassay techniques such as enzyme linked immunosorbent assay (ELISA) which has developed for several compounds nowadays (Peng *et al.*, 2008). The steroid hormones of estrone, zeranol, diethylstilbestrol, trembolone, medroxy progesterone, melengestrole acetate, and progesterone and estradiol benzoate have been licensed as growth

promoters for farm animals in several meat-exporting countries. By contrast, it has been noticed that high level of the hormones injected to animals caused to be higher level of hormone residues and leads several health issues in humans (Kerth, 2013). By contrast, many investigation have reported their safety for animals and consumers, little is known about their fate after excretion by the animal; as well as, some steroids remain more than 3 months after implantation (te Pasetal, 2004). The best standard technique for steroid analysis has been liquid and gas chromatography yet (Yan *et al.*, 2009). However, HPLC-UV is a sensitive, robust and suitable technique for the assay of hormones (Warriss, 2000), it is necessary to have analytical methods to extract meat samples used in HPLC, as well as the complexity to look after admixture in tissue samples. Hence, it is acceptable method and has the minimum required performance limits (MRPLs). Understandably, the required concentrations and action limits respectively authorised by CD 96/23/EC, the Community Reference Laboratories (CRLs) and the National Reference Laboratory (WIV) (Corradini, 2010). So, the aim of this work was to apply a sensitive, applicable and reliable multi-procedure for the identification and confirmation of eight famous anabolic steroids in bovine muscle imported in to Kurdistan region to confirm that how far are they safe for human consumption.

Materials and Methods

Samples and sampling plan.

The information in (Table 1) elucidate the trade name, country of origin and company name for various samples.

Chemicals and Reagents

Anabolic hormone standards and solvents were provided for extraction and purification steps for HPLC application. Pure standards of synthetic hormones used in the study including internal standards were all purchased from Sigma (Sigma–Aldrich, Steinhem, Germany). Ethanol (HPLC grade) and tris (hydroxymethyl) aminomethane were provided from Merck (Darmstadt, Germany), hexane, β- glucuronidase, acetonitrile, dichloromethane, glacial acetic acid and sodium acetate were from Sigma (Sigma–Aldrich, Steinhem, Germany). Ultrapure water was produced with a Pure Lab system (Sation 9000, Spain). In addition, 2% ammonium/water solution was prepared by adding 8ml ammonium 25% in 92 ml of water. Oasis HLB (60 mg, 3 mL) cartridges were obtained from Waters (Milford, MA, USA) and Amino Supelclean NH_2 cartridges from Supelco (Bellenfonte, IL, USA).

Table 1. Sample collection regarding trade name, countries origin and companies

		Sampling		
No.	**Trade Name**	**Producer Country**	**No. of Sample**	**Collecting Period**
1	Kilcoy	Australia	3×5	11/1/2015 to 23/5/ 2015
2	Pure south	New Zealand	3×5	14/1/2015 to 20/5 2015
3	Veal Topsides	Switzerland	3×5	23/1/2015 to 27/5/2015
4	P.Van Horten & Zn	Holand	3×5	15/1/2015 to 27/5/2015
5	Creek stone Farms	America	3×5	15/1/2015 to 20/5/2015

* Samples for each company collected from three different markets and 5 samples in each market for each type.

The stock standard solutions (1 mg/ml) were prepared in ethanol and stored at −20ºC in the dark. Formic acid (99%), acetic acid (99%) and trifuoroacetic acid (99%) were from Acros Organics (New Jersey, USA). Anhydrous sodium sulphate and ammonium formic were all in analytical grade (Beijing, China). To avoid contamination, all the glassware was sterilised for 4 h at 400ºC prior to use. In addition, procedural blanks were conducted for each batch of samples to ensure minimal contamination. Spiking and calibration mixtures at various concentration levels were obtained by combing aliquots of stock solutions and internal standards with mobile phase and stored at 4ºC.

Instrumentation

HPLC analysis was carried out using a Shimadzu HPLC system equipped with two LC-20AT Solvent Delivery Units, a SUS20A gradient controller, and a SPD-20A UV-VIS Detector (Shimadzu, Kyoto, Japan). An N-2000 Chromatographic workstation (Zheda Zhineng Co. Ltd., Hangzhou, China) was used as a data acquisition system. The analytical column was purchased from RStech Co., Korea (3 µm particle

size 50 mm×4.6 mm I.D, C18, 5.0 µm). The mobile phase was (A) 0.1% Ammonium formate (NH4HCO) in ultra-pure water. Solvents V/Methanol: Acetonitrile HPLC grade, and linear gradients0% B to 100%B. The detection wavelength of the detector was set at 242 nm. And the flow rate 1.4 ml/min at 35ºC.

Standard Solution Preparation

According to (Liu *et al*, 2011), primary stock solutions were prepared in ethanol at concentration of 1000 ng/ μL^{-1} and the working solutions were prepared by 100× and 1000× dilution in ethanol/water (50/50). Solutions were stored in dark glass container at −20ºC. Sodium acetate buffer 0.2 M (pH 5.4) was prepared by dissolving 54.5 g sodium acetate in 600 mL 0.2 M glacial acetic acid. Then, the solution was diluted to 2 L with ultrapure water and the NH_2 (100 mg, 1 mL) cartridges were provided for the purification. Frozen meat samples (75) were collected from local markets and saved in fridge temperature. All the samples were homogenized by a disintegrator separately.

Liquid-Liquid Extraction Samples

To follow (Huopalahti and Henion, 1996), the liquid-liquid extraction was carried out by using 20g of each minced meat separately. All samples were homogenized with 100 ml ethanol. The homogenate were then vortex-mixed with 10 ml of 0.03M sodium acetate. The pH of the solution was brought to 4.5 with glacial acetic acid. The enzymatic hydrolysis was initiated by adding 100µl of β-glucuronidase. After 8 hours of enzymatic hydrolysis at 37ºC, 20 ml of acetonitrile was added followed by mixing on a vortex mixer for 30 seconds. The homogenate was then centrifuged (5000 rpm for 20 min) and the supernatant (30ml) were then transferred to a clean test tube. Hexane (8mL) and dichloromethane (2 mL) were added and mixed by rotation for 3 minutes. Samples were then centrifuged (2000 rpm for 2 min) and extracted with 25 mL acetonitrile and transferred to a 20 ml scintillation vial and evaporated to dryness then re-dissolved in 1 ml of dichloromethane and the aqueous extracting phase was filtered through a single-use 0.22µm nylon syringe filter (Aldrich) pass through disposable filter 2.5um prior to analysis on HPLC system under optimum separation condition, the concentre of residual hormone were quantitatively determined by comparison the resulted peak areas of authentic standard with the peak area of samples under the same separation condition.

Sample Injection

The list in (Table 2 represents the sequence of the eluted material of the standard (1µg/mL).

Table 2. Sequence of the eluted material of the standard (1µg/mL)

Hormones	Retention time	Peak area	Concentration (µg/mL)
Estrone	0.553	42003	10
Zeranol	1.64	28684	10
Diethylstilbestrol	2.53	15531	10
Trembolone	3.89	14678	10
Medroxy progesterone	4.70	17622	10
Melengestrole acetate	5.64	19788	10
Progesterone	6.56	21383	10
Estradiol benzoate	7.46	24298	10

* The residue concentrations from each injection (10µl) obtained from the peaks calculations and modified to µg/kg: [Meat sample] = peak area of samples/peak area of standrd×1×10

* 1= [Meat sample] concentration of standard/µg/ml modified to µg/kg (ppb)

* 10= dilution factor

Statistical Analysis

The data were calculated and statistically processed using the statistical package SPSS 18.0 (SPSS, Chicago, IL, USA). The level of statistical significance was set at 0.05 for *t*-test and ANOVA.

RESULTS AND DISCUSSION

The extraction efficiency by liquid-liquid extraction was directly related to the extraction solvent used for tissue sample. As glacial acetic acid (0.1 mol/L), 0.1 mol/L ethanol, sodium acetate, dichloromethane and hexane are the common for extracting steroids from animal tissues were investigated to evaluate their efficiency for extracting steroids from animal tissue. The results showed that the liquid-liquid extraction is advisable and applicable method of extraction and high efficiency with less matrix interferences was obtained from glacial acetic acid and ethanol extraction system. Therefore, acid and alcohol were employed as the extraction solvent in this work.

Table 3. Hormone concentration (µg/kg) resulted in HPLC-UV chromatograms

Trade Name	*Kilcoy	*Pure South	*Veal Topsides	*P.Van Horten & Zn	*CreekStone Farms
Hormones					
Estrone	**Australia**	**Newzland**	**Switzerland**	**Holand**	**America**
Zeranol	0.36	0.309	0.476	0.373	0.318
Diethylstilbestrol	0.483	0.4075	0.405	0.623	0.405
Trenbolone	0.582	0.871	0.749	1.893	0.543
Medroxyprogestrone	0.851	0.504	0.818	0.838	0.44
Melengestrol Acetate	0.492	0.63	0.81	1.104	0.706
Progestrone	0.581	0.834	0.622	0.973	0.764
Estradiol benzoate	0.531	0.61	0.5015	0.855	0.742
	0.645	0.368	0.289	0.425	0.364

*Kilcoy, *Pure South,*Veal Topsides, *P.Van Horten & *Zn and Creek Stone Farms, are the trade name of the meat samples which also refer the name of meat produced companies which are *Australia, *Newzland, *Switzerland, *Holand and *America respectively.

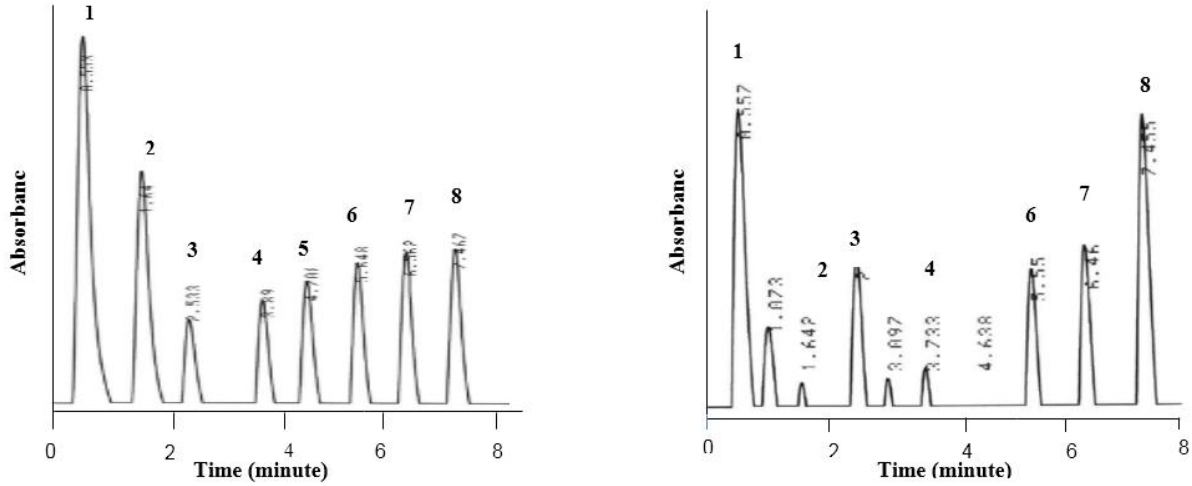

Figure 1.Chromatogram of high performance liquid chromatography-Ultra Violet (HPLC-UV) of standards and frozen meat samples of Kilcoy solutions of hormonal residues by using Mobile phase (A) 0.1% Ammonium Formate in de-ionized water. Solvents V/Methanol: Acetonitrile HPLC grade, and linear gradients0% B to 100%B. 1-Estrone, 2-Zeranol, 3-Diethylstilbestrol, 4- Trembolone, 5-Medroxy progesterone, 6- Melengestrole acetate, 7- Progestrone, Estradiol benzoate

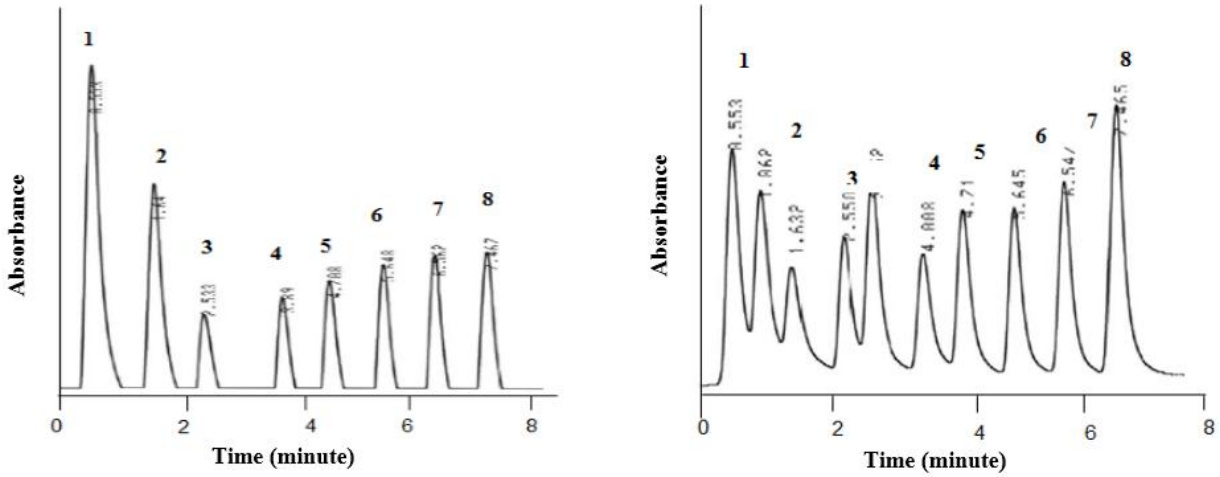

Figure 2. Chromatogrm of high performance liquid chromatography-Ulra Violet (HPLC-UV) of standards and frozen meat sample of Pure South solutions for hormonal residues by using Mobile phase (A) 0.1% Ammonium Formate in de-ionized water. Solvents V/Methanol: Acetonitrile HPLC grade, and linear gradients0% B to 100%B. 1-Estrone, 2-Zeranol, 3-Diethylstilbestrol, 4- Trembolone, 5-Medroxy progesterone, 6- Melengestrole acetate, 7- Progestrone, Estradiol benzoate

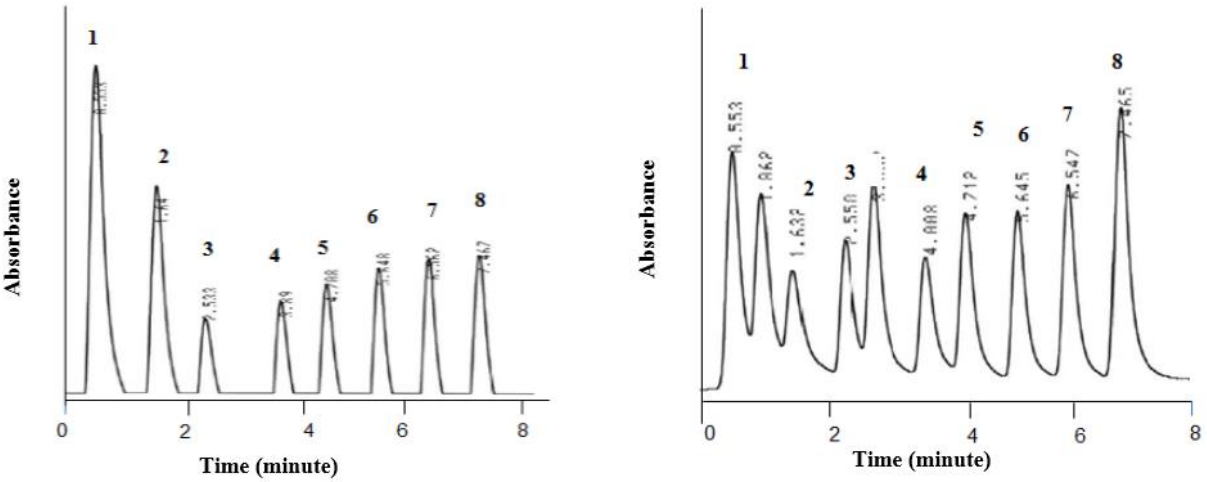

Figure 3. Chromatogram of high performance liquid chromatography- Ultra Violet (HPLC-UV) of standards and frozen meat sample of Pure south solutions for hormonal residues by using Mobile phase(A) 0.1% Ammonium Formate in de-ionized water. Solvents V/Methanol: Acetonitrile HPLC grade, and linear gradients0% B to 100%B. 1-Estrone, 2- Zeranol, 3-Diethylstilbestrol, 4- Trembolone, 5-Medroxy progesterone, 6- Melengestrole acetate, 7-Progestrone, Estradiol benzoate

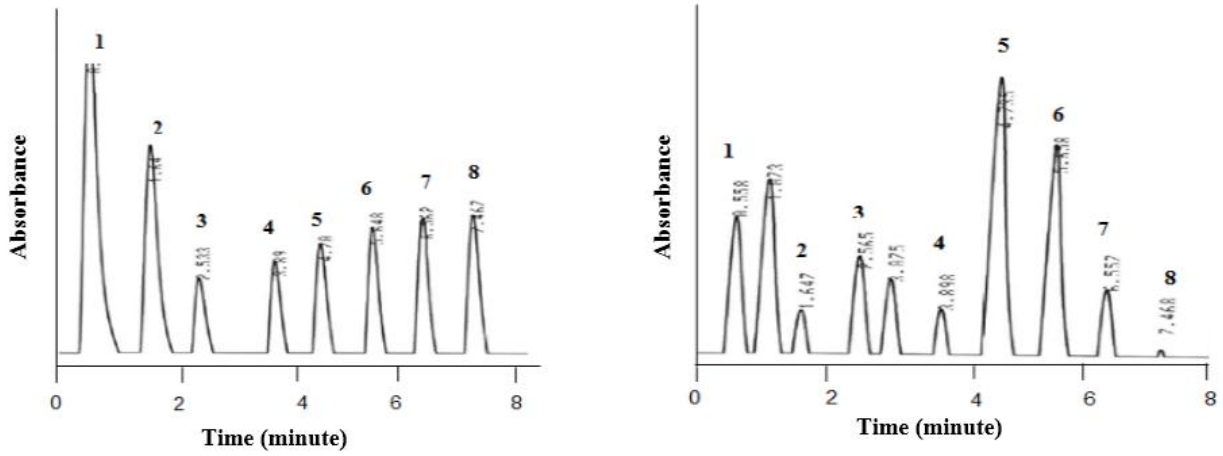

Figure 4. Chromatogram of high performance liquid chromatography-Ultra Violet (HPLC- UV) of standards and frozen meat sample of P.Van Horten & Zn solutions of hormonal residues by using Mobile phase (A) 0.1% Ammonium Formate in de-ionized water. Solvents V/Methanol: Acetonitrile HPLC grade, and linear gradients0% B to 100% B. 1-Estrone, 2- Zeranol, 3-Diethylstilbestrol, 4- Trembolone, 5-Medroxy progesterone, 6- Melengestrole acetate, 7- Progestrone, Estradiol benzoate

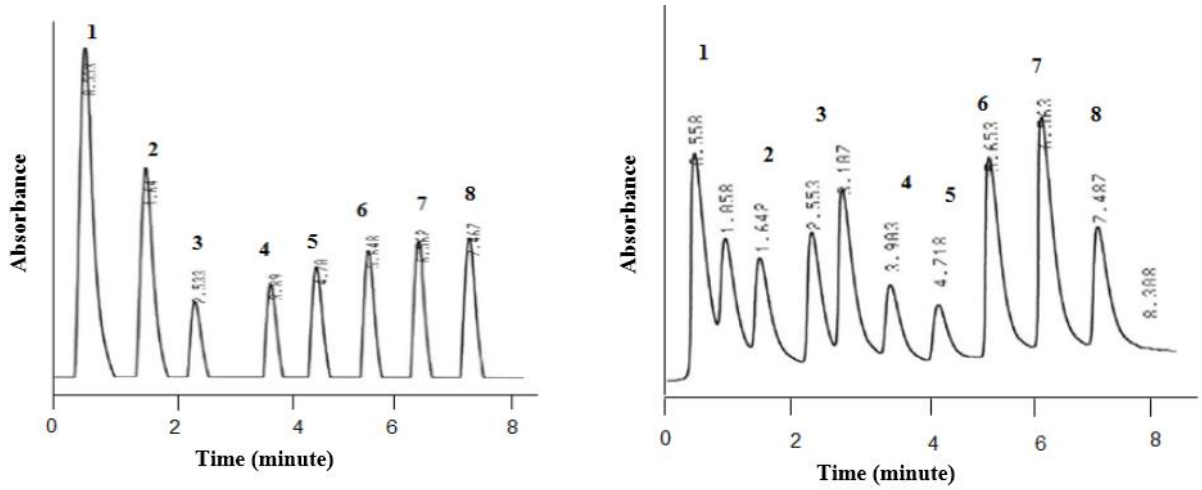

Figure 5. Chromatogram of high performance liquid chromatography-Ultra Violet (HPLC-UV) of standards and frozen meat sample of Creek Stone Farms solutions of hormonal residues by using Mobile phase (A) 0.1% Ammonium Formate in de-ionized water. Solvents V/Methanol: Acetonitrile HPLC grade, and linear gradients0% B to 100%B. 1-Estrone, 2- Zeranol, 3-Diethylstilbestrol, 4- Trembolone, 5-Medroxy progesterone, 6- Melengestrole acetate, 7-Progestrone, Estradiol benzoate.

Meat and meat products play an important role in human nutrition; therefore, it should be safe and does not contain factors or substances harmful for human health (Clark and Henderson, 2003). However, the anabolic agents used for various purposes in animal husbandry for slaughter, could leave residues and hence cause consumers health issue (Brusca *et al*. 2014). The European Economic Community (EEC) banned the use of anabolic compounds as growth accelerators in food animals, while the United States Food and Drug Administration (USFDA) permitted the limited use of some hormones with natural origins (such as estradiol) and some synthetic hormones (such as zeranol and trenbolone) (Nielen *et al*, 2007). According to (WHO, 2006) beef muscle have to be lack of estrone residues; whereas, the results in this study presented the high values of estrone in which no of the samples from the five countries of Australia, Newzland, Switzerland, Holand and America were in

Table 4. The evaluation of the average values of the anabolic residues (µg/ kg) of the meat samples from statistical aspects

Hormones / Trade Name	Estrone	Zeranol	Diethyl stilbestrol	Trenbolone	Medroxy progestrone	Melengestrol Acetate	Progesterone	Estradiol benzoate
Kilcoy	0.360 ±0.25	0.483±0.23	0.582ab±0.26	0.851±0.40	0.492a±0.28	0.581±0.18	0.531±0.34	0.645±0.34
Pure South	0.309±0.12	0.407±0.13	0.871ab±0.23	0.504±0.31	0.630ab±0.40	0.834±0.30	0.610±0.30	0.368±0.50
Veal Topsides	0.476±0.51	0.405±0.38	0.749ab±0.41	0.818±0.90	0.810ab±0.29	0.622±0.31	0.501±0.36	0.289±0.69
P.Van Horten & Zn	0.373±0.13	0.623±0.26	1.893b±1.23	0.838±0.49	1.104b±0.47	0.973±0.38	0.855±0.37	0.425±0.48
Creek Stone Farms	0.318±0.29	0.405±0.75	0.543a±0.43	0.440±0.25	0.706ab±0.27	0.764±0.21	0.742±0.45	0.364±0.49
Total	0.3672 ±0.26	0.4647 ±0.35	0.9276*±0.51	0.6902 ±0.47	0.7484*±0.34	0.7548±0.27	0.6479 ±0.36	0.4182± 0.50

a, b, c : The differences between the groups which have different letters under the same subgroup columns are statistically significant ($P < 0.05$).

* : The difference of hormone residue level in meat samples statistically important ($p< 0.05$).

Table 5. Maximum hormones residue limits (MHRLs), present in beef muscles from non- treated and treated cattle (compilation of data reported by (EFSA, 2007), (JECFA, 2005) and (Codex, 2007) which established for beef muscles).

Hormones substances	Inter-national limits	Reference	[Hormones] resulted in HPLC-UV				
			A	B	C	D	E
Estrone	*a.0.0 pg/g *b. 0	(WHO,2006)	0.360 ±0.259	0.309±0.126	0.476±0.517	0.373±0.138	0.318±0.298
Zeranol	*a. 0.01-0.73 ppb *b. 0–0.5µg/kg	(FSIS,2002)	0.483±0.236	0.407±0.130	0.405±0.383	0.623±0.263	0.405±0.754
Diethylstilboestrol	*a.0.15 µg/kg *b.<10µg/kg	(FDA 2013)	0.582±0.268	0.871±0.231	0.749±0.413	1.893±1.238	0.543±0.431
Trenbolone	*a. 0- 0.02 µg/kg *b. 0.18-0.28 ppb	(JECFA/Codex,2007)	0.851±0.401	0.504±0.317	0.818±0.909	0.838±0.493	0.440±0.253
Medroxy progesterone	*a.0.23 *b.0.53	(Codex, 2007)	0.492±0.280	0.630±0.402	0.810±0.297	1.104±0.474	0.706±0.272
Melengestrol Acetate	*a. - *b. 0–0.03 µg/kg	(FSIS,2002) (FSIS,2002)	0.581±0.188	0.834±0.301	0.622±0.315	0.973±0.384	0.764±0.212
Progesterone	*a. 0.23- 0.77 ppb *b. 3 µg/kg	(JECFA/Codex,2007) (FDA 2013)	0.531±0.342	0.610±0.309	0.501±0.367	0.855±0.379	0.742±0.457
Estradiol benzoate	*a. 0-30µg/kg *b. 5 ppb	(EFSA, 2007)	0.645±0.347	0.368±0.502	0.289±0.691	0.425±0.489	0.364±0.494

*a: Hormone residue in Non-treated animals * b: Hormone residue in Treated animals with hormones.

A=Kilcoy B= Pure South C= Veal Topsides D= P.Van Horten & Zn E= Creek Stone Farms

safe level of estrone regarding the Kilcoy, Pure South, Veal Topsides, P.Van Horten & Zn, Creek Stone Farms companies (respectively) meat products (see table 3 and 4). This indicates that acceptable tolerance limits mentioned above should be immediately taken into account for usage of estrone in animal husbandry and its control in meat and meat products for mentioned companies have to be focused.

Moreover, the use of zeranol was also banned by the European Community in 1985 (Jodlbauer *et al*., 2000); whereas, very small (0–0.5µg/kg) amount allowed to be available in treated animal in 2007 (EFSA, 2007). On the other hand, the zeranol level was much higher in the samples of all the mentioned five countries than the international limits and there was no significant difference (P value= 0.56) between the countries regarding zeranol level. This is also indicated that the frozen beef samples of Kilcoy, Pure South, Veal Topside, P.Van Horten & Zn and Creek Stone Farms are not in allowed in terms of zeranol value (see table 5). Furthermore, diethylstilbestrol which is a carcinogenic and is not metabolized by the organism, has been banned in animal husbandry and not permitted to be present in food stuffs (EC, 1999).Consequently, the results presented that the slaughtered cattle has been treated with diethylstilboestrol and the residues in all the companies samples were much more than the international limits (<10µg/kg) (FDA, 2013).

Trenbolone was also detected in higher (0.18-0.28 ppb) in comparison with the national limits (0.69ppb) (JECFA/Codex, 2007) in all the five groups of samples. Medroxy progesterone and melengestrol acetate levels were also not in safe level (0.7484ppb and 0.7548ppb respectively) except in Kilcoy which was (0.49ppb)(see table 4), the rest four countries have extra containing medroxy progesterone and melengestrol acetate in their products. According to table 3, the cattle muscles of all the 5 trademarks were treated with progesterone and estradiol benzoate; whereas, the level of both hormones were (0.6479 and 0.4182 respectively) in safe level. Steroids can remain parts per billion even 2 or 3 months after implantation; understandably, it can be stated that the hormones were higher than the international limits, have been implanted before slaughtering closely (Nazil *et al*, 2005). In addition, the Post Hoc tests of ANOVA resulted that there is no significant difference of each estrone, zeranol trenbolone, melengestrol acetate, progesterone and estradiol among the five trademarks as the P values were, 0.649, 0.567, 0.40, 0.80, 0.26 and 0.29 respectively. There were also not significant differences between Kilcoy, Pure South and Veal Topsides regarding Diethylstilbestrol concentration, but also the difference was only between P.Van Horten & Zn and Creek Stone Farms(P value=0.046). In addition, P.Van Horten & Zn again was significantly different from Kilkoy in Medroxy progesterone level (P value=0.03); whereas, no significant difference seen in Medroxy progesterone concentration among the rest companies. According to (Shao *et al*, 2005), the conducting recovery tests before the study will be useful for a correct test result. Understandably, the results of this work are highlighted as they give some information about the hormone residues and comparing with some international standards to improve the meat industry and animal husbandry.

CONCLUSION

This study presents that the anabolic steroids might be available in almost samples with different levels regarding each company and country, which indicates the importance of analyzing the residue level of the anabolic agents in different imported meat, since the anabolic agents may pose a potential risk to public health. Estrone, zeranol, diethylstilbestrol, Trembolone, medroxy progesterone, melengestrole acetate, progesterone, estradiol benzoate are still implanting into cattle to promote growth rate; however, progesterone, estradiol benzoate residues were in safe level in this work. While the samples of Kilcoy, Pure South, Veal Topsides, P.Van Horten & Zn and Creek Stone Farms could be harmful and have to be banned.

Acknowledgements

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Evaluation of microbiological quality of raw milk, homemade *Ergo* and homemade *Ayib* in North Shoa District, Amhara, Ethiopia

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ABSTRACT

An entire of 60 food samples were investigated according to the standard methods in order to check the bacteriological quality of traditional milk and milk products to reduce food borne disease. The microbial load of raw milk revealed that total aerobic mesophilic count (TAMC), total spore count (ASC), coliforms, *Staphylococci* and yeasts and molds were in the range of 6 to 9.5, 3.4 to 9.4, 2.1 to 4.7, 2.4 to 5.4, and 3.2 to 6.1 log CFU/mL correspondingly. In respect to the microbial load of *ergo;* TAMC, ASC, coliforms, *Staphylococci* and yeasts and molds were in the range of 5 to 9.1, 3.9 to 8.6, 1.9 to 4.5, 2.1 to 4.7 and 0 to 7.9 log cfu/ml respectively. The corresponding microbial counts in homemade *ayib* range from 5 to 8, 0 to 3, 2.4 to 4.8, 2.7 to 4.3 and 3.1 to 4 log CFU/g respectively. No psychrotrophic bacteria were detected in none of the food samples. In conclusion, all samples collected from the five North Shoa districts in the context of the present study had significant number of TAMC, ASC, coliforms, *Staphylococci* and yeasts and molds. Therefore, appropriate hygienic actions should be applied from production to consumption chain.

Keywords: *Ayib*, *Ergo*, Milk, North showa, Spoilage microbes

INTRODUCTION

In Ethiopia, pasteurization and quality control measures is rarely applied for milks produced at home level. The subsistence farmers in the rural areas without dairy facilities are contributing for about 98% of the annual milk production. Furthermore dairy processes in the country are mostly restricted to smallholder level and its hygienic quality is low. (Alganesh *et al*., 2009).

Milk is a medium that may favor the growth of numerous microbial species (Ahmed *et al*., 2010). Raw milk consumption, in the occurrence of pathogenic microorganisms may be of public health importance. Milk may be contaminated with pathogenic and/or spoilage bacteria at various points: during the process of milking; in the instant of milk pass away of the teat, by milking equipment, milking personnel or during transportation and storage (Ahmed *et al*., 2010). Milk from a healthy udder comprises very little microorganisms but it is contaminated with spoilage and pathogenic microbes after the time it leaves the teat of the cow till it is used for further processing. The presence of microorganisms in milk suggests the method of milk handling and the milk quality. Milk produced from hearty animal under well-equipped sanitary condition may not contain more than 5 x 10^5 bacteria/ml (O'Connor, 1994).

The raw milk is further transformed in to a traditional fermented products like *ergo* and *ayib* in Ethiopia. *Ergo* is a traditional Ethiopian fermented milk product, which is frequently manufactured at domestic level (Anteneh *et al*., 2011). *Ayib* is a traditional Ethiopian cottage cheese produced from fermented milk after the removal of fat by churning. It is an acidic product (Mogessie, 2006). In rural societies, fermented milk products are used for various purposes (Akabanda, 2010). Fermented milk products can be used for consumption as food and beverage and its market value and shelf life are extended over that of raw milk. It is also used as cosmetics by rural people (Akabanda, 2010). Such products have been considered, based both on research findings and popular wisdom as more nutritious and health promoting than fresh milk (Akabanda, 2010).

Currently, the microbial food safety is associated with public health concern. Various epidemiological reports have indicates, unpasteurized milk and milk products as the main cause for food-borne illness. Milk can be spoiled by pathogens from faecal source or by direct excretion from the udder into it (El-ziney and Al-turki, 2010). The issue for dairy

products with respect to food-borne diseases is of great concern throughout the globe and this is especially true in developing countries where production of milk and various milk products takes place in unhygienic circumstances (Mogessie, 1990).

The presence of pathogenic microbes in milk has been a matter of public health concern. The consumption of raw milk and dairy products prepared from raw milk is related with numerous health risks. The frequent occurrence of milk borne typhoid and scarlet fever confirmed the necessity of pasteurization in milk processing (Rayser, 2001). The detection of coliform bacteria and pathogens in milk shows the spoilage of milk by possible entry of the bacteria either from the udder, milk utensils or water supply used (Bonfoh *et al*., 2003). Insufficient pasteurization or post-pasteurization contaminations of milk have been also linked to several food borne disease outbreaks (ICMSF, 1998). The presence of $>10^5$ cfu/ml microbial count in milk is a sign for unhygienic milk production hygiene, whereas the production of milk with bacterial count less than 10^5 cfu/ml is an indication for good sanitary practice (Ombui *et al*., 1995). The objective of this study was inspecting the microbiological quality of raw milk, home-made ergo and home-made ayib.

MATERIAL AND METHODS

Description of the study area

The study was conducted between January to August 2013 in five districts of North Shoa Zone of Amhara Regional State. The Districts included: Debre Berhan, Deneba, Debresina, Shewarobit and Ankober.

Sample collection and processing

A total of 60 samples of which 25 samples of homemade *ergo* and 25 samples of raw milk and 10 samples of *ayib* were collected, transported and analyzed according to standard methods described by Richardson (1985). Raw milk and *ergo* samples were collected from Shoarobit, Debresina, Ankober, Deneba and Debrebirhan while *ayib* samples were collected only from Ankober and Debrebirhan. Samples were collected aseptically in 500ml sterile glass bottle and transported to Debre berhan University Biology department laboratory, kept aseptically under refrigeration (4ºC) and analyzed within 12-24 hours of sampling. Then the samples were serially diluted by taking 25 ml (25 g) and adding to 225 ml of sterile peptone water in a clean500 ml sterile flask, shaken to make 10^{-1} dilution, and then serially diluted. Appropriate dilutions were surface plated on appropriate agar media for enumerating different groups of spoilage and pathogenic microorganisms (Aal and Awad, 2008).

Total aerobic mesophilic count (TAMC)

The total aerobic mesophilic count estimates the number of viable aerobic bacteria per gram or milliliter of a product. A portion of the diluted sample was spread over the surface of plate count agar medium (OXOID, Hampshire, UK) and incubated at a temperature of 32^0C for 48 hrs (Abebe *et al*., 2012).

Aerobic mesophilic spore count

The samples were heat-shocked at 80°C for 10 min to destroy vegetative cells. After being cooled in an ice bath, the samples were immediately plated on plate count agar and incubated at 32 °C for 48h (Al-Kadamany *et al*., 2003).

Enumeration of psychotropic microorganisms

From each previously prepared serial dilution of the samples 0.1 ml was inoculated on to duplicates of plate count agar medium. Both inoculated and control plates were incubated at 7°C for 10 days. Total psychrotrophic count/ml or gm of examined samples was calculated and recorded (Al-Kadamany *et al*., 2003).

Coliform count

From each previously prepared serial dilution of the samples, portions of 0.1 ml of appropriate dilutions were inoculated in duplicate on Violet Red-Bile Agar (VRBA); Petri dishes were incubated at 32°C for 24 h and counts were made on typical dark red colonies normally measuring at least 0.5 mm in diameter on un-crowded plates (Richardson, 1985).

Staphylococci count

Portions of 0.1 ml from the previously prepared dilutions of the examined samples were transferred and evenly spread on the surface of mannitol salt agar medium (OXOID, Hampshire, UK) plates. Inoculated plates were incubated at 37°C for 48 h. and *Staphylococci* counts per ml or g of tested product were calculated and recorded (Aal and Awad, 2008).

Enumeration of molds and yeasts

The enumeration of molds and yeasts were performed by inoculating 0.1ml portions of the samples' suspension on potato dextrose agar (PDA) acidified (PH 3.5) by 10% lactic acid. Then, the petridishs were incubated at 25^0C for five days and colonies were counted (Abdalla and Nabi-Ahmed, 2010).

Microbial identification

After determination of microbial load of the samples, five colonies of different groups of microbes were picked randomly from countable agar plates and purified by repeated plating on agar media. The isolates were characterized using the following tests (Aneja, 2005).

Catalase test

A drop of 3% solution of hydrogen peroxide (H_2O_2) was placed on a clean microscope slide, and a pure colony from a 24 h culture on plate count agar was transferred and mixed using a sterile wire loop. Formation of gas bubbles was considered as a positive test for catalase producing microbes (Harley, 2002).

Gram reaction (KOH test)

The KOH test was conducted according to Gregerson (1978). A pure colony form a 24-h culture was picked from plate count agar and put on a clean slide and stirred with two drops of 3% KOH for approximately 2 min. Gram-negative bacteria were raised up with inoculating needle and form 0.5 to 2cm length; whereas the Gram positives did not raise up with inoculating needle.

Cell shape and cell arrangement

From agar media, a single pure colony was picked and transferred and smeared on a clean microscope slide with a drop of sterile water (Wet mount) and covered with a cover slip. The preparation was observed under light microscope using an oil immersion objective. The observed cell shapes and cell arrangements were recorded (Harley, 2002).

Statistical Analysis

Mean and standard error of the mean were analysed using SPSS (version16.0, SPSS Inc, Chicago, IL, USA, 2007).

RESULTS AND DISCUSSION

Microbial load of raw milk

The study was aimed to evaluate the microbiological quality of raw milk collected from five sites in and around Debre berhan. The microbial load of raw milk revealed that a total aerobic mesophilic count, aerobic spore count, coliforms, *Staphylococci*, and yeasts and molds count in the range of 6 to 9.5, 3.4 to 9.4, 2.1 to 4.7, 2.4 to 5.4 and 3.2 to 6.1 log CFU/mL respectively (Table 1).

This range for total aerobic mesophilic count is much greater than the acceptable value for America $<3x10^5$ CFU/mL (Ombui *et al*., 1995), for Ethiopian $2x10^6$ CFU/mL (Kiiyukia, 2003) and for Kenya $<2x10^6$ CFU/mL (Ombui et al., 1995). Similarly the coliform count recorded in the present study is greater than the general standard count set for raw milk which is $1.5x10^2$ CFU/mL (Ombui *et al*., 1995) except for the milk samples collected from Debre berihan. The present study results are in agreement with the study done by Abebe *et al.* (2012) who report a mean total aerobic mesophilic count and coliform count of 9.82 log CFU/mL and 4.3 log CFU/mL respectively, in raw whole cow's milk in Gurage zone. In addition to this, a study on microbiological quality of Ethiopian milk and milk products showed that the mean total bacterial counts ranges from 8.3 to 10 log CFU/mL in milk collected from Debre Zeit, Adama and Jimma and the mean coliform counts is greater than 4 log CFU/mL of in milk sampled from all study sites (Zelalem, 2009).

The overall mean TAMC reported in the instant study was higher than the maximum acceptable limits given for raw milk intended for processing (10^5 CFU/mL) and direct human consumption (5×10^4 CFU/mL) (Bodman and Rice, 1996). This higher level of milk contamination may be due to initial contamination contributed from udder surface, cleaning water poor quality, utensils as well as materials used for filtration of milk. However, major cause of high TAMC is attributed to poor hygienic practices during milking. Moreover, the milk residues on the surface of equipment also provide nutrients for the growth and diversification of microorganism with special reference to bacteria that can subsequently contaminate milk during milking.

The detection and count of *Staphylococcus* in this study is in agreement with the standard level described by health and protection agency which is $\leq 10^4$ CFU/mL (Health Protection Agency, 2009) and slightly greater than the standard level of Ethiopian Health and Nutrition Research Institute (EHNRI) which is 10^3 CFU/gM according to Kiiyukia (2003). In agreement with the present study, the microbial quality of milk collected from distribution containers in Hawassa harbor on average 7.13 log CFU/mL of yeasts and molds (Haile *et al*., 2012). Similarly, according to the results of a study assessing the microbiological safety of raw milk in Khartoum state, the *Staphylococci* and yeast count is 6.0 and 5.8 log CFU/mL respectively (Ali, 2010). The present results were also higher than the study conducted on microbial quality of raw milk in Slovenia that shows the mean coagulase positive bacterial count of 1.9 log CFU/mL and yeast and mold count of 2.3 log CFU/mL except for psychrotrophs count of 3.5 log CFU/mL which is greater than this study (Torkar and Teger, 2008). In contradiction to this study, the mean count of psychrotrophs, aerobic spore count

and yeasts and molds are 3.8 log CFU/mL, log 2.1cfu/ml and 1.9 log CFU/mL in camel milk from Saudi Arabia (El-ziney and Al-turki, 2010). The result on microbiological safety of raw milk in khartoum state indicates, spore forming count of 2 log CFU/mL (Ali, 2010). In other study a single colony of spore formers are not observed on plates, in all the four samples of UHT milk during storage, at room temperature for 12 weeks (Hassan et al., 2009).

Microbial load of *Ergo*

The present study were showed that the average count of total aerobic mesophilic count, Aerobic spore count, psychrotrophs, coliforms, *Staphylococci* and yeast and mold in homemade *ergo* ranges from 5 log CFU/mL to 9.1 log CFU/mL, 3.9 log CFU/mL to 8.6 log CFU/mL, 0 log CFU/mL, 1.9 to 4.5 log CFU/mL, 2.1 to 4.7 log CFU/mL and 0 to 7.9 log CFU/mL respectively (Table 2). The mean aerobic mesophilic counts of homemade *ergo* in the present study are comparable with the study done by Mbaeyi and Anyanwu (2010); Abdalla and Nabi-Ahmed (2010) and Mogessie (1995). The mean counts of coliforms in homemade *ergo* in this study (around 4 log CFU/mL) is comparable to the study performed by Farzana *et al.* (2009); Kumbhar *et al*. (2009) and Mogessie (1995) who reports a coliform count of 5 to 6 log CFU/mL. Similar to the present study, Uzeh *et al.* (2006) also report the mean total plate count of 8.55 log CFU/mL and coliform count of 7.63 log CFU/mL in *nono* and the mean total plate count of 8.67 log cfu/ml and coliform count 7.38 log cfu/ml in *wara*. Likewise, the study on Sudanese fermented milk (Rob) reveals an aerobic mesophilic count of 5.53 to 6.57 log CFU/mL (Abdelgadira *et al*., 2001). Similarly, Zelalem (2012) also reports a mean coliform count of 4.51 log CFU/mL in traditional Ethiopian fermented milk '*ergo'*. In other study, Gulzar *et al*. (2013) reports a mean count of coliform in branded and unbranded yoghurt that ranges from 0.9 to 2.01 log CFU/mL.

This high number of aerobic mesophilic counts is not necessarily a health risk but it indicates an overall lack of hygiene (Ray, 2004) and the high count of coliform reflects highly poor hygienic conditions and improper sanitation during making *ergo* (Mogessie, 1995). In the present study the mean *Staphylococcus* counts in homemade *ergo* were in the range of 2.1 to 4.7 log cfu/ml. Abdella and Nabi-Ahmed (2010) also report that the mean *Staphylococci* count in the Sudanese fermented dairy product 'mish' ranges from 6.11 to 7.49 log CFU/mL. Similarly, a study conducted on different milk products in Abuja, Nigeria revealed a *Staphylococci* count ranging from 3.9 to 4.0 log CFU/mL (Okpalugo *et al*., 2008). Correspondingly, El-Malt *et al*. (2013) also reports an average count of *Staphylococci* in yogurt in the range of 2.97 to 3.93 log CFU/mL.

The average yeasts and molds count in homemade *ergo* were ranged from 0.0 to 7.9 log CFU/mL in the present study. Likewise, the mean fungi count of 7.11 and 7.12 log CFU/mL are recorded in *nono* and *wara* respectively (Uzeh *et al*., 2006). In agreement with this study, a yeast count ranging from 6.38 to 7.64 log CFU/mL is noticed in Sudanese fermented milk (Rob) (Abdelgadira *et al*., 2001). Correspondingly, Abdella and Nabi-Ahmed (2010) report the mean yeast and mold count that ranges from 4.97 to 5.01 log CFU/mL in Sudanese fermented dairy product 'mish'. Akbanda *et al*. (2010), also report a yeast count ranging from 1.22 to 6.63 log CFU/mL in Ghanaian traditional fermented milk, *Nunu* at different fermentation time. In the same way a study conducted in Ethiopian *ergo* indicates a yeast and mold count of 8.38 log CFU/mL (Zelalem, 2012). Contrarily, the study conducted on fruit-flavored yoghurt shows a yeast and mold count that ranges from 2.10 to 2.89 log CFU/mL (Tarakçi and küçüköner, 2003).

Microbial load of *Ayib*

The microbial load of homemade *ayib* were showed that the average count of Total aerobic mesophilic count, Aerobic spore count, psychrotrophs, coliforms, *Staphylococci* and yeast and molds ranges from 5 to 8 log CFU/g , 0 to 3 log CFU/g, 0 log CFU/g , 2.4 to 4.8log CFU/g, 2.7 to 4.3 log CFU/g and 3.1 to 4 log CFU/g respectively (Table 3). The microbial count in the present study is much higher than the maximum limit set by different quality standard authority. The mean total aerobic mesophilic counts in the present study were higher than the acceptable limit (4.7log CFU/g) for cooked cheese in the markets (Al-Khatib and Al-Mitwalli, 2009). The mean coliform counts in this study were also greater than the acceptable level of coliforms (1 log CFU/g) in Ethiopian cottage cheese (Kiiyukia, 2003). The mean *Staphylococcus* count in homemade *ayib* was beyond the standard limit set for fresh cheese (0 log CFU/g) (Council Directives 92/46 EEC. 1992).

The mean count of TAMC in this study was comparable with 7.41log CFU/g average total bacterial count in traditional Ethiopian cottage Cheese 'Ayib' (Zelalem, 2012). Similarly, the total viable bacterial counts in *Metata ayib* are ranging from 5.40 log cfu/g to 7.84 log CFU /g (Eyassu, 2013). Correspondingly, Tekletsadik and Tsige (2011) are also reported aerobic mesophilic bacteria of 6.13 log CFU/g of *ayib* in Jimma. Oladipo and Jadesimi (2013) also report the total microbial count

Table 1. Mean count of different microorganisms in various raw milk samples

Microorganism type	Microbial counts (log_{10} CFU/mL) in raw milk samples				
	Site of sample collection				
	Shoarobit	**Debresina**	**Deneba**	**Ankober**	**Debre berhan**
TAMC	8.5±.50	9.5±.41	6.0±.01	9.0±.01	6.0±.01
ASC	6.5±.48	3.4±.18	6. ±.00	9.4±.12	6.8±.03
Psychrotrophs	0.00	0.00	0.00	0.00	0.00
Coliforms	4.6±.15	4.3±.01	4.6±.12	4.7±.50	2.1±.08
Staphylococci	5.4±.19	2.4±2.4	5.3±.16	2.4±2.4	4.1±.07
Yeasts and molds	6.1±2.8	5.2±.38	4.3±.30	6.0±.01	3.2±.08

TAMC=Total aerobic mesophilic count, ASC=Aerobic spore count, CFU=colony forming unit

Table 2. Mean count of different microorganisms in various homemade *ergo* samples

Microorganisms type	Microbial counts (log_{10} CFU/mL) in homemade *ergo* samples				
	Site of sample collection				
	Shoarobit	Debresina	Deneba	Ankober	Debrebirhan
TAMC	5.6±.06	8.6±.08	8.6±.08	9.1±.35	5.0±.01
ASC	4.3±.17	3.9±3.9	3.9±.39	8.6±.18	5.6±.15
Psychrotrophs	0.00	0.00	0.00	0.00	0.00
Coliforms	4.5±.15	4.4±.01	4.4±.01	4.5±.15	1.9±.17
Staphylococci	4.5±.20	4.7±.08	4.7±.08	2.8±.01	2.1±.02
Yeast and Mold	0.00	7.9±.14	7.9±.14	4.8±.14	3.4±.35

TAMC=Total aerobic mesophilic count, ASC=Aerobic spore count, CFU=colony forming unit

Table 3. Mean count of different microorganisms in various homemade *ayib* samples

Types of microorganisms	Microbial counts (log_{10} cfu/gm) in homemade *ayib* samples	
	Site of sample collection	
	Ankober	Debrebirhan
TAMC	8.0±.01	5.0±.02
ASC	0.00	3.0±.01
Psychrotrophs	0.00	0.00
Coliforms	4.8±.07	2.4±.09
Staphylococci	4.3±.25	2.7±.08
Yeasts and Mold	4.0±.00	3.1±.03

TAMC=Total aerobic mesophilic count, ASC:=Aerobic spore count, cfu=colony forming unit

Table 4 Morphological and biochemical characteristics of certain microbes from agar media

Source of Microbes	Gram reaction	Catalase test	Cell shape
MSA	+	+	Coccus
VRBA	_	+	Rod shape

MSA-mannitol salt agar, VRBA- Violet Red Bile Agar

that ranges from 4.48 log CFU/g to 7.94 log CFU/g in West African soft cheese 'wara'. Thabet *et al*. (2013) also reveals a mesophilic bacterial count that range from 5.08 to 7.98 log CFU/g of cheese in Yemen. The average total bacterial counts in soft white cheese are 7.38 log CFU/g (Alsawaf and Alnaemi, 2011). In other study Vural *et al*. (2010) is reported an average total aerobic mesophilic bacterial count of 8.2 log CFU/g in orgu cheese in Turkey.

The coliform count in the present study is in agreement with 4.42 log cfu/g coliform count in traditional Ethiopian cottage Cheese 'Ayib' (Zelalem, 2012). In other study the enterobacteriaceae count of 5.96 log CFU/g in *ayib* in Jimma are comparable with the present study (Tekletsadik and Tsige, 2011). Correspondingly, the coliform counts of cheese in Yemen are range from 4.04 to 5.22 log CFU/g (Thabet *et al*., 2013) and the average coliform bacteria of orgu cheese in Turkey are 4.55 log cfu/g (Vural *et al*., 2010). However, the coliform counts in fresh cheese from Serbia (8.33 log CFU/g) are higher than the present study (Mikulec, *et al*., 2012). On the other hand, coliforms are not detected in the Metata Ayib (Eyassu, 2013).

In this study, the mean count of *staphylococci* in homemade *ayib* was range from 2.7 to 4.3 log CFU/g. The *staphylococci* counts in *ayib* from Jimma (5.4 log CFU/g) are equivalent with *staphylococci* count in the present study (Tekletsadik and Tsige, 2011). Correspondingly, the *Staphylococci* counts of cheese in Yemen are range from 4.16 to 5.83 log CFU/g (Thabet *et al*., 2013). Likewise, the numbers of coagulase-positive *Staphylococc*i in soft cheese are 5.86 log CFU/g (Torkar and Teger, 2006). Similarly, microbiological studies on orgu cheese in Turkey are showed a mean *Staphylococci* count of 5.15log CFU/g (Vural *et al*., 2010). In different from the

present study, *Staphylococci* aer not detected from fresh soft cheese in Serbia (Mikulec *et al*., 2012).

The average count of yeast and mold in *ayib* which was 3.1 to 4 log CFU/gM in the present study are lower than 8.26 log CFU/gM 'Ayib' (Zelalem, 2012). Similarly, Eyasu (2013) also report, yeast and mold count ranging from 2.08 to 3.79 log CFU/gM in Metata Ayib. The mean counts of molds (5.84 log CFU/gM) and yeast (5.5 log CFU/gM) from ayib samples in Jimma are higher than the yeast and mold count in this study (Tekletsadik and Tsige, 2011). Similarly, the yeast counts from fresh soft cheese in Serbia are 5.48 log CFU/gM (Mikulec *et al*., 2012), and the mean count of yeast and mold of orgu cheese in Turkey are 4.22 log CFU/gM (Vural *et al*., 2010).

Gram reaction, catalase test and cell shape were tested only for microbes grew on mannitol salt agar and violet red bile agar. The microbes from mannitol salt agar were gram positive, catalase positive and coccus in cell morphology whereas microbes that had been taken from violet red bile agar were gram negative, catalase positive and rod shape in cell morphology. In this study it was observed that all samples of raw milk, *ergo* and *ayib*, collected from the five North Shoa districts, were detected with significant levels of TAMC, ASC, coliforms, *Staphylococci* and yeasts and molds. Relatively, lower microbial populations were observed in the food samples collected from Debre Berhan compared to the other four sampling sites. This study showed that the microbiological quality of the dairy products manufactured in the study area was poor. Hence, adequate sanitary measures should be taken throughout the food chain, from production to consumption. These measures should include proper animal handling, personnel hygiene, use of hygienic milking and processing equipment's, improving the sanitary condition of milk and milk handling environment.

CONCLUSIONS

In this study it was observed that all samples of raw milk, *ergo* and *ayib*, collected from the five North Shoa districts, were detected with significant levels of TAMC, ASC, coliforms, *Staphylococci* and yeasts and molds. Relatively, lower microbial populations were observed in the food samples collected from Debre Birhan compared to the other four sampling sites. This study showed that the microbiological quality of the dairy products manufactured in the study area was poor. Hence, adequate sanitary measures should be taken throughout the food chain, from production to consumption. These measures should include proper animal handling, personnel hygiene, use of hygienic milking and processing equipment's, improving the sanitary condition of milk and milk handling environment.

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Conflict of interest

The authors declare that there is no conflict of interest among them.

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Impact of the dehydration process on guava by using of the parabolic trough solar concentrator

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ABSTRACT

Parabolic Trough Solar Concentrator (PTSC) was developed for the purposes of dehydration of agricultural products *i.e.* guava. The guavas were cut into two pieces and dip in the solution of 1% potassium Meta-Bi-Sulphate and dried with the temperature of 65 0C at 25% humidity level; drying time was 19 hours. The result showed that total solar intensity in the month of October was noted 598 Kj, followed by 538 and 457 Kj for the November and December respectively. From the result, it was noted that the moisture content was reduced by up to 11% in the month of October due to the highly solar intensity. Similarly, the drying rate of the product was noted in 0.033gH2O. dm. cm-2.hr-1 higher than November and December 0.032 gH2O. dm. cm-2.hr-1 and 0.029 gH2O. dm. cm-2.hr-1 respectively. However, the results showed that the efficiency of PTSC for the month of October was noted 30% higher than the month of November and December 25% and 23% and moisture content were reduced up to 11%, 13% and 15% respectively. Therefore, it was recommended that PTSC was good for drying of different fruits and vegetables up to 9 hours a day time.

Key words: Solar Energy, Parabolic trough solar Concentrator, Guavas, Efficiency, Moisture lost and Drying Rate

INTRODUCTION

Guava (Psidium guajava L) belongs to the family (Myrtaceae), which is 1.6 inches long and 4.7 inch round or oval, cultivated in many tropical and sub-tropical countries, is a common fruit in Pakistan. Trees of guava can survive with the temperature of 25 F (−4 °C) for short periods of time in cold-hardy places. It contains 82%, 0.7% and 11% of water, protein and carbohydrate respectively, while desirable amounts of vitamin A, B and C are present (Ali et al, 2009). The total area of guava cultivation in Pakistan is about 58.5 thousand hectares with the production of 468.3 thousand tones. Trees of Guava grow in the heavy clay, light sand, gravel bars or limestone with a pH range of 4.5 to 9.4 (Urooba *et al.*, 2008).

Pakistan has high production of guava but there are 35-40% losses occurred with the spoilage. Insect attacked and also with the improper storage facilities. For future using the Guava fruits was dried in a solar dryer, to decrease the insect attacks and spoilage of fruits. Drying is the most efficient way to dehydrate the fruits and remove all the moisture. These solar dryers are the very fast drying method which quickly dehydrates the product within 24 hours with the high temperature (Hanif *et al*, 2013). Kaleta & Gornicki, 2010 reported that in solar dehydrator the fruits were dried more quickly with the maximum temperature and less humidity level. The solar collector was decrease the drying time of fruits with the increasing of temperature, while the temperature of the dryer higher than 60 °C has a negative impact on the dried products (Kang *et al.*, 2006). The total solar energy received from the sun in Pakistan through the year was about 3.85 x 1024 W.yr-1, while in Pakistan, Peshawar (Khyber Pakhtunkhwa) which is suited with the latitude of 34o and receiving maximum solar intensity to 650 Kj. m-2. hr-1 (Fahim *et al*, 2013).

Parabolic trough solar concentrator with the drying chamber was used to dry the product with the higher temperature and low humidity rate. It was improving the solar drying method which could facilitate early crop harvest, long term storage of fruits and quality of products (Saeid and Parsa, 2014). Efficiency is the important parameter and the key factors of solar collector. The increasing efficiency of a parabolic

trough solar concentrator was the flow rate of the collector fluid flowing inside the collector and the ambient temperature. Efficiency depends on the optimum combination of temperature and flow rate and verified building of solar collector (Azmi et al, 2012). Santos, 2005 studied that increasing the temperature of the collector, increased the efficiency. To use a parabolic trough solar concentrator efficiently for agricultural purposes, like drying of fruits such as Guava with the objective of drying time of guava fruits, determination of moisture content and also check the efficiency of PTSC.

MATERIALS AND METHODS

The experiment was conducted in the Department of Agricultural Mechanization, Agriculture University Peshawar, and Khyber Pakhtunkhwa Pakistan having latitude of (34/71). For receiving maximum solar radiation intensity the collector was focused on the sun rays with the latitude of the area where the experiment performed.

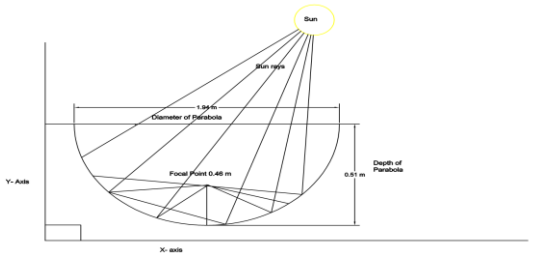

Fig 1. Dimension of Parabola

Description of the Parabolic Trough Solar Concentrator

The figure 2. Show the assembling of Parabolic Trough Solar Concentrator that consists of two units as parabolic trough solar reflector and drying chamber

Parabolic Trough Solar Reflector

Parabolic trough solar reflector was made of steel sheet which is easily available in the local market. It is used as a reflector, which reflects of sun rays on the absorber area (collection of heat) with the diameter of 0.05m as showed in Figure 2 (Fahim et al, 2013). The absorber received ambient air from inlet valve and heated with reflected rays from the collector and this heated air delivered to the drying chamber which is connected to the outlet valve of the absorber.

Before constructing the structure of parabola from steel sheet, we focus on the desired focal length and area which are using by the following equation (Fahim *et al.,* 2013) as showed in the figure 1.

Focal length= (Diameter of parabolic trough)/16* Depth of parabolic trough

Drying chamber

The drying chamber was constructed from steel box which was fully insulated inside from polystyrene foam. There were exhaust fan in the drying box which was used for the sucking hot air from the absorber pipe and the outlet pipe (0.02m Diameter) at the toper of the drying chamber, to remove humid air from the drying unit (Hanif et al., 2013). The moisture loss (%) was determined by the Guava in each hour of drying time was dried up to less than 10%, which is secure from moisture content, mold, insects and bacterial attack was determined by using equation (Ahmed, 2011). Where Mc is the moisture content (%), Wi is the initial mass and Wf is the final mass of the product.

$$Mc=(Wi-Wf))/Wi \ X100 \qquad (2)$$

Temperature and humidity of PTSC were recorded every hour with the digital hygrometer (Mahmood et al., 2005). Drying rate of the product is defined as the quantity of water evaporated per gram of dry matter per unit area in unit time. It was determined by using the formula developed by Ehiem *et al.* (2009). Where Dr is the drying rate (gH2O. g-1dm .cm-2.hr-1), Wi is the initial mass, Wf is the final mass in (g), Dm is dry matter in (g), Ap is cross sectional area in (cm2) and Dt is the time of drying product (hr).

$$Mc=(Wi-Wf))/(Dm \ X \ Ap \ X \ Dt) \qquad (3)$$

Solar energy

Solar Energy (kJ. m-2. hr-1) is the energy which falls on the collector per unit time from sun, is recorded with the help of mechanical Pyranometer and also drying rate of the product were determined in unit Dr (gH2O.g-1dm.cm-2.hr-1). The solar intensity which falls on the reflector and absorber area was determined by the given formula (Hanif *et al.,* 2013). Where, "Si" is the solar irradiance (KJ.Sec-1), Pc is Chart Constant 367.78 Kj.m-2.hr-1 and Cv are the chart value.

$$Si= Pc \ x \ Cv \qquad (4)$$

Efficiency of parabolic trough solar concentrator was the ratio of heat available to the reflector in the form of solar radiation intensity was determined by the below formula (Ahmed, 2011).

Eff=(Heat available to collector)/(solar radiation)x 100

Experimental procedure

For experiment good quality of Guava fruits were used. All the guavas were cut into halves and then blanched for 3 minutes in hot water at 75 oC and then put these blanched guavas into 1% Potassium Meta-BiSulphate solution for two minutes. The initial moisture content was determined by oven drying method. Put these guava in the trays of drying chamber of parabolic trough collector having 75% perforation and dried up to 9% remained moisture content. Solar irradiation was measured with the help of solar power Meter (SAM206) and humidity, temperature of the PTSC were recorded every hour during drying.

RESULTS AND DISCUSSION

The figure 3 shows the energy available to the collector during the drying period was recorded in the graph/chart with the help of mechanical Pyranometer from the time (6:00 am to 6:00 pm). The figure shows that the intensity of solar energy was higher from 11:00 am to 01:00 pm have maximum heat of the collector which is 598 Kj in the month of October, during this time the efficiency of the PTSC was recorded high similarly found in Aušra et al. (2015). In the month of November, the solar energy was highest at the same time which is 538 Kj, having highest efficiency of the PTSC by Fahim et al. (2013). Similarly in the month of December the energy is low then the other two months, due to the cloudy days and also rain which were recorded 457 Kj. From the graph, it is clearly shown that the solar irradiance was higher at these months and have better for the drying of agricultural products such as guava in the PTSC. The graph shows that drying time of the product starts from 9:00 am till 4:00 pm having better heat of the collector because the site receives a brilliant amount of solar radiation. The result was found similarly by Tonui *et al.* (2014). Therefore, total of 9 hours was available for the drying of guava under a temperature range of 45-75°C with the 25% less humidity was found by the Hanif *et al.* (2013).

The results in Figure 4, showed the efficiency PTSC for the three months of the year, 2014. The efficiency of PTSC was noted 30%, 25% and 23% for the month of October, November and December respectively. Data were analyzed and found the efficiency of PTSC with the significant interval difference ($P < 0.073$). From the figure, R2 value showed that there is inter relationship between the months of the year. . The efficiency of the PTSC was higher in the month of December due to the greater intensity of sun radiation, while in the month of November the efficiency of PTSC was maximum for the month of October due to sunny days with greater sun irradiation. The result was similar with the finding of Fahim *et al.,* (2103); Hanif *et al.,* (2013) and Tonui *et al.,* (2014).

Figure 5, showed the moisture content of Guava for the three months of the year, 2014. The moisture content was reduced slowly to the increasing temperature of the collector. The graph shows that the moisture of the product was decreased from 11% in the month of October are similar with the result of Dhanushkodi *et al.* (2104). Similarly in the month of November the moisture content was reduced to 13% for the increasing temperature of the collector. Therefore in the month of December the moisture content was reduced to 15% due to greater sun radiation and increasing temperature of the collector which showed in the figure 5. The results are similar to the result found by Fadhel *et al.* (2014). The drying rate of the product guava was reduced in the month of October 0.033 gH2O. dm. cm-2.hr-1 given in the figure 6. Guava showed a fast drying rate in November 0.032 gH2O. dm. cm-2.hr-1 with the finding of Fadhel et al. (2014) and Saeid & Pooya, (2014). Similarly the drying rate in the month of December, 2014 was 0.029 gH2O. dm. cm-2. hr-1 due to the increasing temperature and efficiency of the PTSC. The result was dissimilar with the finding of Azmi et al. (2012).

In the figure.7 shows the relation between temperature and humidity of the collector drying box. The temperature of the absorber was increased due to the clear days and greater highest sun radiation of PTSC from 9:00 am to 4:00 pm in reaching to 135 ^{o}C with the agreement with the finding of Mohsin et al. (2011). The temperature in the drying box was exceeded 45 ^{o}C at 10:00 am and reached maximum 65 ^{o}C at 12:00 noon which showing that the efficient drying time was 7 hrs i.e. 9:00 am to 4:00 pm. The ambient and drying temperature of the collector was determined by digital thermo-hygrometer, which was found water content less than 15 %. The result was similar with the finding of Azmi *et al.* (2012).

Fig 2. Isometric view of Parabolic Trough Solar Concentrator

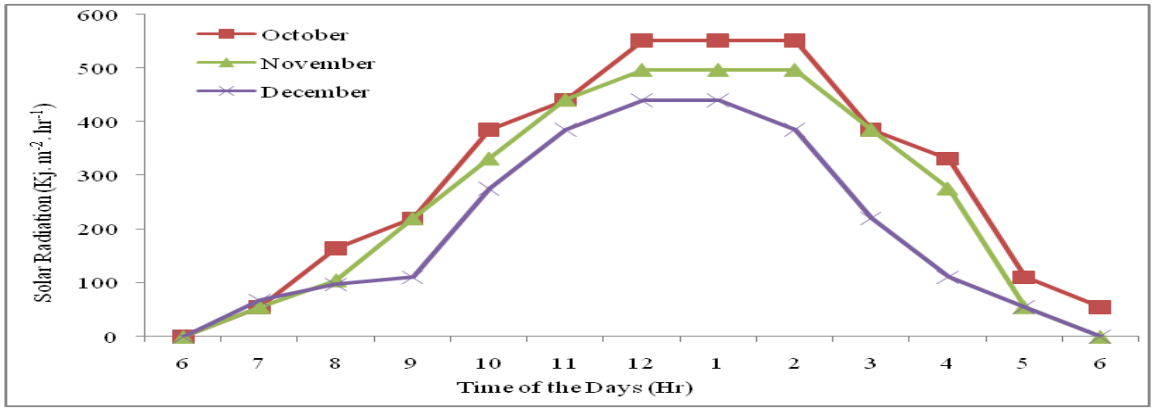

Fig 3. Solar radiation recorded for three months from October to December, 2014

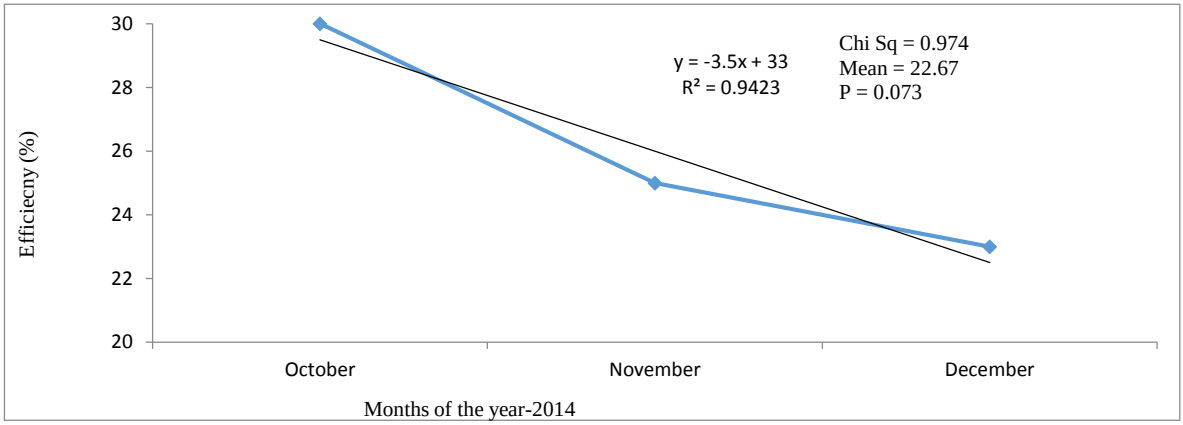

Fig 4. Efficiency recorded for three months from October to December, 2014

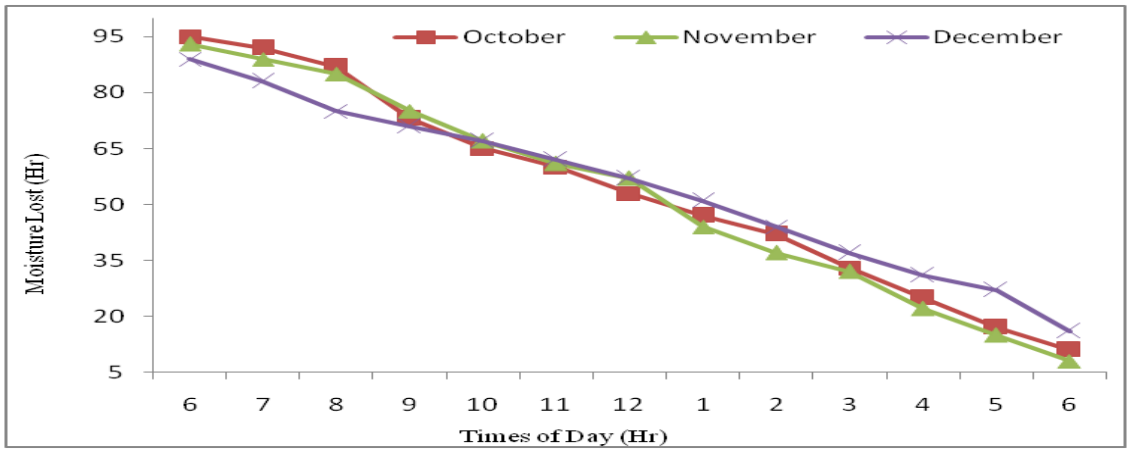

Fig 5. Moisture content for three months from October to December, 2014

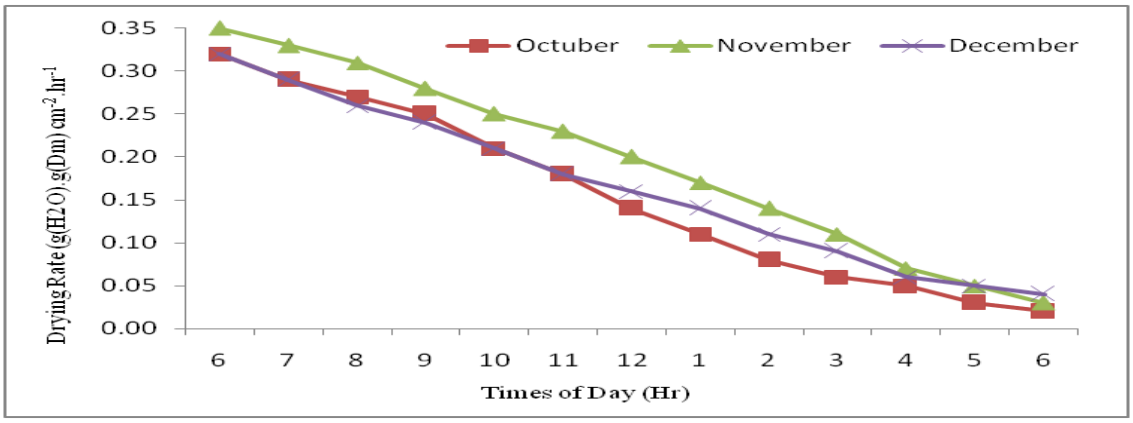

Fig 6. Drying Rate for three months from October to December, 2014

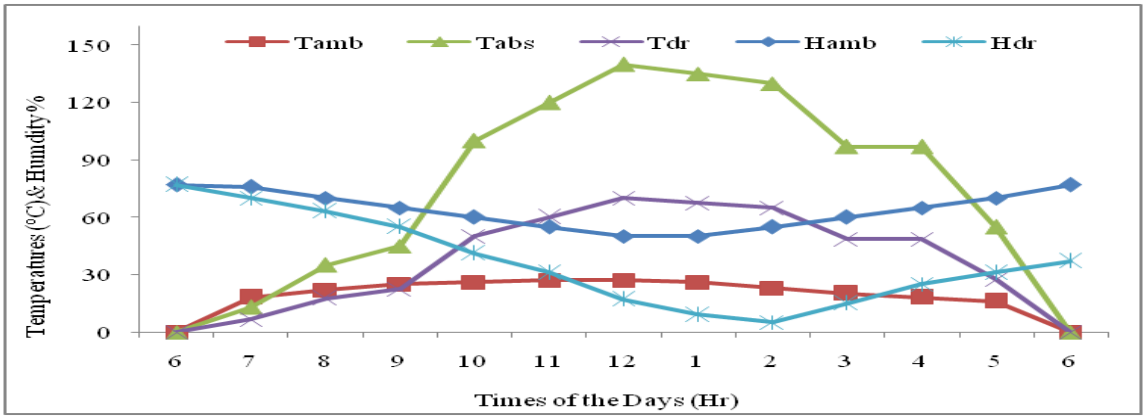

Fig 7. Temperature & Humidity recorded for the months from October to December, 2014

CONCLUSION

Based on the study findings, it was concluded that, increasing the temperature of PTSC was significantly increased the efficiency. From the result, it was noted that PTSC was reduced the drying time period of the products to be dried. From the result, it was recommended that PTSC was better to operate drying agricultural product i.e. guava in the month of October and November. It was noted from the experiment that PTSC worked efficiently for 9 hours in a day time. From the result, it was studied that the efficiency of PTSC for the month of October was higher than the other two months and moisture content was reduced up to 11%. The drying rate of the product was noted in 0.033gH2O. dm. cm-2.hr-1, 0.032 gH2O. dm. cm-2.hr-1 and 0.029 gH2O. dm. cm-2.hr-1 for the month of October, November and December respectively. Therefore, it was recommended that PTSC was good for drying of different fruits and vegetables in day time from 8 am to 4 PM during October, November and December.

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Human health risk assessment of Zinc, Copper, Lead and Cadmium in the myonematic tissues of eight fish species sold in major markets in Lagos Metropolis, Nigeria

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ABSTRACT

The study was undertaken to assess the human health risk of some heavy metals namely Zn, Cu, Pb and Cd in the myonematic tissue of selected fish species sold in some major fish markets in Lagos metropolis, Nigeria, with a view of evaluating the safety of such fish for human consumption. Analysis for the aforesaid metals was carried out using Atomic Adsorption Spectrophotometric technique. The mean concentration of Zn in fish species ranged from 11.54mg/kg in *Pomadasys peroteti* to 26.42mg/kg in *Hydrocynus brevis* while the mean concentration of Cu in fish species ranged from 6.11mg/kg in *Pomadasys peroteti* to 15.00mg/kg in *Ethmalosa fimbriata.* the mean concentration of Pb ranged from 0.00mg/kg in *Hydrocynus brevis* to 0.04mg/kg in *Mugil cephalus* while the mean concentration of Cd ranged from 0.00mg/kg in *Mugil cephalus* to 0.04 mg/kg in *Galeoides decadactylus*. The estimated daily intake (EDI) values in mg/person/day for heavy metals, ranged from below quantification limit (BQL) for Cd and Pb to 0.63 for Zn, `while the estimated annual intake (EAI) values in mg/person/year, ranged from BQL for Pb and Cd to 229.95 for Zn. The toxic/hazard quotient (TQ) values for the metals ranged from 0.28 for Cu to 0.52 for Zn while the margin of exposure (MOE) values, ranged from 1.91 for Zn to 3.75 for Cu. The theoretical maximum daily intake (TMDI) for heavy metals was 2404 mg/person/day. The mean concentrations of the aforementioned metals in all fish species fell below the limits established by the Food and Agricultural Organization of the United Nations (FAO) for metals in food/fish. Thus, the consuming public would not immediately experience risk of hazardous metals via consumption of fish. It was advocated that in order to keep the levels of these metals within tolerable limits, Health Inspection Officers from the Ministry of Health and Environmental Monitoring Officers from the Ministry of Environment, be mandated to jointly monitor the activities of fish mongers and to carry out periodic spot checking of fishery products.

Key words: Heavy metals, Fish, Toxic/hazard quotient, Lagos, Nigeria.

INTRODUCTION

Around the world, interest has been generated in environmental research, planning and management, owing to the accelerated expansion of human activities which has produced negative consequences on the environment. In this realm, environmental pollution with regard to heavy metals, has received significant attention globally (Tabari *et al*,. 2010). Heavy metals are typical pollutants in urban environments and are of particular concern due to their persistence and toxicity to both wildlife and man (Moore *et al.*, 2011). The aquatic ecosystem in which fish thrives is frequently the ultimate recipient of heavy metal contamination (Obasohan *et al.*, 2006). In natural water bodies, heavy metals are potentially accumulated in finfish and shellfish and subsequently transferred to man via the food chain (Wangboje *et al.*, 2013). In third world contries, waste water treatment is not given the necessary priority it deserves hence industrial and domestic waste water containing heavy metals are discharged into receiving water bodies without treatment, resulting in pollution of rivers, loss of aquatic life and uptake of contaminated water by plants and animals (Dan'azumi and Bichi, 2010). Heavy metals in aquatic bodies could be derived from both natural and anthropogenic sources and thereafter become transported as dissolved species in water or as an integral part of suspended sediments (Wogu and Okaka, 2011). Nigeria has witnessed the progressive deterioration in its environmental quality due to an upsurge of domestic, agricultural and industrial activities that have a direct link to population growth,

with the associated generation of more waste products (Tawari-Fufeyin, 2015). Furthermore, the country is the most industrialized in West Africa with about 70% of manufacturing industries including electric power generating stations, petroleum refineries, pulp and paper mills located in the coastal region (Calamari and Naeve, 1994). The contamination of fishery products by heavy metals portends a potential health hazard to man who may eventually consume such products (Oronsaye *et al.*, 2010). In Nigeria, scientific studies abound on the levels of heavy metals in diverse aquatic bodies, resulting from anthropogenic impact. For example, Rivers (Horsfall and Spiff, 2002; Akan *et al.*, 2011; Butu and Iguisi, 2013; Olele *et al.*, 2013; Wangboje and Ikhuabe, 2015), Lagoons (Olowu *et al.*, 2010; Adedeji and Okocha, 2011; Jimoh *et al.*, 2011), Creeks (Ohimain *et al.*, 2008; Oribhabor and Ogbeibu, 2009;Wangboje *et al.*, 2014) , Dams (Osasona and Ipinmoroti, 2008; Wangboje and Oronsaye, 2013; Wangboje and Ekundayo, 2013), Streams (Obasohan, 2008; Ekpo *et al.*, 2013) and Aquifers (Erah *et al.*, 2002; Medjor *et al.*, 2012). There is however paucity of information regarding the heavy metal content in fish species that are sold in market places, against the backdrop that such fish could have been harvested from contaminated aquatic bodies, thus potentially exposing the consuming public to tainted fish. The cardinal objective of this study therefore, was to assess the human health risk regarding the presence of heavy metals in fish sold in some selected major fish markets in Lagos metropolis, in order to safeguard public health and to fill an existing gap in knowledge. The heavy metals of concern were Lead (Pb), Copper (Cu), Cadmium (Cd) and Zinc (Zn). Lead and Cd are non-essential elements that are of no use in the human body, while Cu and Zn are essential elements, needed for a host of metabolic and enzymatic functions.

MATERIALS AND METHODS

Description of study area

Lagos state (Fig. 1) is located in southwestern Nigeria on the West coast of Africa, within Latitudes 6°23'N and 6°41'N and Longitudes 2°42'E and 3°42'E. A quarter of its land mass consists of liquid surface comprising Lagoons, Creeks and Coastal river estuaries (Jimoh *et al.*, 2011). The state has four Lagoons (Lagos, Lekki, Ologe and Epe), that are rich sources of fisheries resources, including fin fish and shell fish. Climate-wise, the wet season runs from April to October while the dry season falls between November and March. Typically rainfall peaks in July and September with reduced rainfall in August. Air temperature ranges from 22ºC to 33ºC annually, while annual relative humidity is between 67% and 97% .Vegetation is typical of the tropical rainforest belt within which the state lies. The state is the most highly populated in the country and also the most industrialized. Industrialization and urbanization has brought in its wake, an upsurge in the production of waste products; coupled with the weak enforcement of environmental Laws and Regulations, pollution threats especially to the aquatic environment in Lagos is a harsh reality. The state's main agricultural products are fish, prawns, palm oil, cassava, maize, vegetables and fruits. The study was carried out at four major fish markets namely, Ikeja market, Layeni market (Suru-Alaba), Asoro market (Mile 2), and Mushin market. These locations were specifically selected for the study because of the teeming human population of the areas and the seemingly popularity of these markets. The study was conducted between the months of February and May, 2015. Fish samples were purchased from the aforesaid markets twice every month for the study.

Preparation of samples

In the laboratory, the fish samples were identified using taxonomic keys (Idodo-Umeh, 2003; Adesulu and Sydenham, 2007), while cross referencing was done with the fish-base website (https://www.fishbase.org). The identified species were, *Arius latiscutatus* (Gunther, 1864), *Clarias gariepinus* (Burchell, 1822), *Galeoides decadactylus* (Bloch, 1758), *Hydrocynus brevis* (Gunther, 1864), *Pomadasys peroteti* (Cuvier, 1830), *Tilapia zillii* (Gervais, 1848), *Ethmalosa fimbriata* (Bowdich, 1825) and *Mugil cephalus* (Linnaeus, 1758). The myonematic tissues were extracted with a stainless steel dissecting kit from the flanks of the fishes, while wearing disposable latex gloves. The extracted parts were oven-dried to constant weight at 80°C for 48 hours. Milling of dried portions was achieved using a porcelain mortar and pestle, prior to digestion.

Digestion procedure

One gram of milled fish tissue was carefully weighed into a 100 ml Erlenmeyer flask, after which 5 ml perchloric acid (70%) and 10 ml nitric acid (55%) were added. Digestion was done on a hotplate at 200° C, for about 4 hours or until the solution became clear (Van Loon, 1980). Filtration was achieved using acid-resistant filter paper (0.45 µm) and a vacuum pump. After filtration, the filtering system was rinsed with distilled water to remove all traces of metals. Samples were made to 50 ml with distilled water (Nussey *et al.*, 2000). Blanks were prepared using the same volume of mixed acids. An Atomic Absorption

Spectrophotometer, UNICAM 696 Series® equipped with solar software using air acetylene flame, was used to analyze digested samples for Pb, Cu, Cd and Zn. All values were expressed in mg/kg.

Estimation of daily intake (EDI) of heavy metals by man

The daily intake of metals was calculated in order to estimate the daily loading of metals into the body system of man via the consumption of fish (Wangboje *et al.*, 2014).

$$\text{EDI} = \frac{40\text{g/person/day} \times \text{HM (mg/kg)}}{1000\text{g/kg}}$$

Where: 40g/person/day = Estimated consumption of fishery products in Lagos state

(Williams, 2013).

HM = Mean concentration of heavy metal in fish species.

Estimation of annual intake (EAI) of heavy metals by man

Based on the calculated EDI values for individual metals, the EAI values are obtained by multiplying the EDI values by 365 days. Thus, EAI = EDI*365 days.

Margin of exposure (MOE) for heavy metals

Margin of exposure (MOE) was applied to assess the species-specific risk from consumption of contaminated fish with heavy metals as given by Watanabe *et al.* (2003). MOE is a ratio of two factors which assesses for a given population the dose at which a small but measurable adverse effect is first observed and the level of exposure to the substance considered. This is a ratio of the reference dose or health based criteria to the calculated or actual exposure. This ratio gives an indication of which metal is of significant ecological concern (Wangboje *et al.*, 2014).

$$\text{MOE} = \frac{\text{Reference Dose}}{\text{Calculated or Actual Exposure}}$$

Theoretical maximum daily intake (TMDI)

This represents a relationship between the maximum limit for metal in fish and the per capita/food regional consumption. It serves as a theoretical indication of the expected maximum daily intake of heavy metal residue via fish and fishery products (WHO, 1997).

$$\text{TMDI} = \sum ML^1 \times F^1$$

ML = Maximum limit for metal in fish

F = Per capita/Regional food consumption

Toxicity/Hazard quotient (TQ) for heavy metals

Toxicity Quotient (TQ) indicates the risk of human health problem by consuming heavy metal pollutants in fish. It gives an estimate of metal hazard on the population in the latter life with fish consumption. If the TQ value is greater than one this indicates possibility of potential risk on the population due to fish consumption. However, if TQ value is less than one then the population would not experience risk of hazardous metals (Du *et al.*, 2013; Wangboje and Ikhuabe, 2015).

$$\text{TQ} = \frac{\text{concentration of heavy metal in fish sample}}{\text{Health based Criteria}}$$

Statistical methods

Data obtained were analyzed using GENSTAT® computer software (Version 12.1 for Windows). Analysis of variance (ANOVA) was used to test for significant differences between means at 5% level of probability while significant means were separated with Duncan Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

Zinc (Zn)

As shown in Figure 2, the mean value of Zn in fish at the markets ranged from 11.81mg/kg recorded at Mushin market to 17.40mg/kg recorded at Mile 2 market. The rank profile of Zn in fish samples from the various markets was as follows: Mile 2 > Suru-Alaba > Ikeja > Mushin. In Figure 3, the monthly mean value of Zn ranged from 9.69mg/kg recorded in April to 22.25mg/kg recorded in March. Mean concentration of Zn in fish species (Table 1) ranged from 11.54mg/kg in *Pomadasys peroteti* to 26.42mg/kg in *Hydrocynus brevis*.

Copper (Cu)

As shown in Figure 2, the mean value of Cu recorded ranged from 7.93mg/kg at Ikeja market to 8.917mg/kg at Mile 2 market. The rank profile of Cu in fish samples from the various markets was as follows: Mile 2 > Mushin > Suru-Alaba > Ikeja. The monthly mean concentrations of Cu ranged from May (5.277mg/kg) to February (15.617mg/kg), as shown in Fig. 3. Mean concentration of Cu in fish species ranged from 6.11mg/kg in *Pomadasys peroteti* to 15.00mg/kg in *Ethmalosa fimbriata* (Table 1).

Lead (Pb)

The mean concentration of Pb in fish, as shown in Figure 2, ranged from 0.0233mg/kg at Ikeja and Mushin markets to 0.02667mg/kg at Suru-Alaba and Mile 2 markets. The rank profile of Pb in fish samples from the various markets was: Suru-Alaba, Mile 2 > Mushin >Ikeja. The monthly mean value of the metal ranged from 0.0000 mg/kg in March to 0.04667mg/kg in May (Fig. 3). As shown in Table 1, the mean concentration of Pb ranged from 0.00mg/kg in *Hydrocynus brevis* to 0.04 mg/kg in *Mugil cephalus*.

Cadmium (Cd)

As shown in Fig. 2, the mean concentration of Cd ranged from 0.02333mg/kg at Mile 2 market to 0.01667mg/kg at Ikeja market. The rank profile of Cd in fish species from the various markets was: Mile 2/Mushin > Ikeja/Suru-Alaba. The highest mean value of 0.03mg/kg and lowest mean value of 0.01mg/kg were recorded in April and February respectively (Fig. 3). In fish, mean concentration of Cd ranged from 0.00mg/kg in *Mugil cephalus* to 0.04 mg/kg in *Galeoides decadactylus* (Table 1).

The concentrations of Zn, Cu, Pb and Cd in this study, varied according to location, time and fish species. For example, in the case of Zn, the highest mean concentration of the metal was recorded at Mushin market, while time wise the highest mean concentration of the metal was recorded in the month of March. The highest mean concentration of the metal was observed in *H. brevis.* In the same format (Market, Month and fish species), for Cu, the observed trend was Mile 2 market, February and *E. fimbriata* while for Pb, it was Suru-Alaba, May and *M. cephalus.* For Cd, the observed trend was Ikeja market, April and *G. decadactylus.* The mean concentrations of Cu and Zn in fish were higher in the dry months of February and March while the mean concentrations of Cd and Pb were higher in the wet months of April and May. This observation is a possible indication of how seasonal variation may account for differences in the metal content of fish species. Statistically, significant differences ($P<0.05$), were observed in the mean concentrations of the same metal amongst fish species, an indication that the fish species may have been sourced from different aquatic bodies. It could also be an indication that the fish species accumulated metals to different levels, from their respective host aquatic media. It has been reported that anthropogenic sources of heavy metals derived from mining, smelting, agriculture, the petrochemical industry, printing, aquaculture, the electronic industry and municipal waste are ultimately discharged into aquatic media, where they can be bioaccumulated by aquatic organisms and can be biomagnified through the food chain, resulting in elevated levels in predatory organisms (Wang *et al.,* 2013).

Estimated daily intake (EDI) of heavy metals

The EDI values in mg/person/day for heavy metals, ranged from below quantification limit (BQL) for Cd and Pb to 0.63 for Zn (Figure 4).

Estimated annual intake (EAI) of heavy metals

The EAI values in mg/person/year for heavy metals, ranged from below quantification limit (BQL) for Pb and Cd to 229.95 for Zn (Figure 5). The estimated daily intake (EDI) and estimated annual intake (EAI) values for heavy metals in this study were dominated by Zn with values of 0.63 mg/person/day and 229.95 mg/person/year respectively. This finding is not surprising, as Zn clearly had the highest mean concentration in all the investigated fish species. Wangboje *et al.*, (2014), observed a similar finding for Zn, in the Mangrove oyster harvested from a Creek in the Niger Delta region of Nigeria. On the other hand, Cd and Pb had EDI and EAI values that were below quantification limits, primarily owing to the fact that their mean concentrations in fish, were close to sub-zero values.

Toxicity/Hazard Quotient (TQ) for heavy metals

The TQ values for the metals ranged from 0.28 for Cu to 0.52 for Zn, as shown in Figure 6.

Margin of exposure (MOE) for heavy metals

The MOE values for heavy metals, ranged from 1.91 for Zn to 3.75 for Cu, as shown in Fig. 7.

Theoretical maximum daily intake (TMDI) values for heavy metals

The calculated TMDI value for heavy metals was 2440 mg/person/day, as presented in Table 2.

The toxicity/hazard quotient (TQ) values for metals were all below unity, an indication that prospective consumers of fish would not immediately experience risk of hazardous metals. However, the margin of exposure (MOE) rating revealed that Cu (MOE= 3.75) is the metal that should be closely monitored as it may have the propensity to be of hazard in future. The theoretical maximum daily intake (TMDI) values for metals revealed that Zn and Cu had the highest individual TMDI values, accounting for 99.83% of the total TMDI while Cd and Pb accounted for the balance 0.17%. The logical implication of this finding is that based on the per capita consumption of fish in Lagos

Table 1. Mean concentrations of heavy metals (mg/kg) in specific experimental fish species

Species	Zn	Cu	Pb	Cd
Arius latiscutatus	16.62±0.01^{f}	7.97±0.02^{g}	0.023±0.01^{b}	0.017±0.01^{bc}
Clarias gariepinus	17.02±0.01^{e}	8.19±0.01^{f}	0.027±0.01^{b}	0.017±0.01^{bc}
Galeoides decadactylus	17.40±0.01^{d}	8.92±0.01^{d}	0.027±0.02^{b}	0.037±0.01^{a}
Hydrocynus brevis	26.42±0.02^{a}	10.14±0.01^{c}	0.000^{c}	0.013±0.01^{c}
Ethmalosa fimbriata	21.53±0.01^{c}	15.00±0.01^{a}	0.02±0.01^{b}	0.000^{d}
Mugil cephalus	22.44±0.01^{b}	14.26±0.01^{b}	0.04±0.01^{a}	0.000^{d}
Pomadasys peroteti	11.54±0.02^{h}	6.11±0.01^{h}	0.000^{c}	0.000^{d}
Tilapia zillii	11.81±0.01^{g}	8.50±0.02^{e}	0.023±0.01^{b}	0.023±0.02^{b}

Means with the similar superscripts are not significantly different (P >0.05). Vertical comparisons only

Table 2. Theoretical maximum daily intake (TMDI) values for heavy metals

Metals	Maximum limit (mg/kg) for metal in fish (FAO, 1983)	Per capita fish consumption (mg/person/day)	Individual TMDI values for metals
Zn	30	40	1200
Cu	30	40	1200
Cd	0.5	40	20
Pb	0.5	40	20
			∑ **2440**

Table 3. Comparison of heavy metal concentrations (mg/kg) in *Clarias gariepinus* with some other studies

Metal	*C. gariepinus* muscle tissue (This study)	*C. gariepinus* muscle tissue (Wangboje *et al.*, 2013)	*C. gariepinus* whole fish (Olele *et al.*, 2013).	*C. gariepinus* whole fish (Wangboje and Ikhuabe, 2015)	*C. gariepinus* cephalic tissue (Osasona and Ipinmoroti, 2008)
Zn	17.02	0.09	45.03	70.97	4.46-7.36
Cu	8.19	0.23	12.65	0.967	N/D
Pb	0.027	0.19	40.89	0.036	N/D
Cd	0.017	0.03	4.99	0.076	N/D

N/D = Not detected

Table 4. Comparison of heavy metal concentrations (mg/kg) in *Arius latiscutatus* with another study on *Arius heudelotii*

Metal	*Arius latiscutatus* muscle tissue (This study)	*Arius heudelotii* muscle tissue (Fonge *et al.*, 2011)
Zn	16.62	0.379
Cu	7.97	0.350
Pb	0.023	0.395
Cd	0.017	N/D

N/D = Not detected

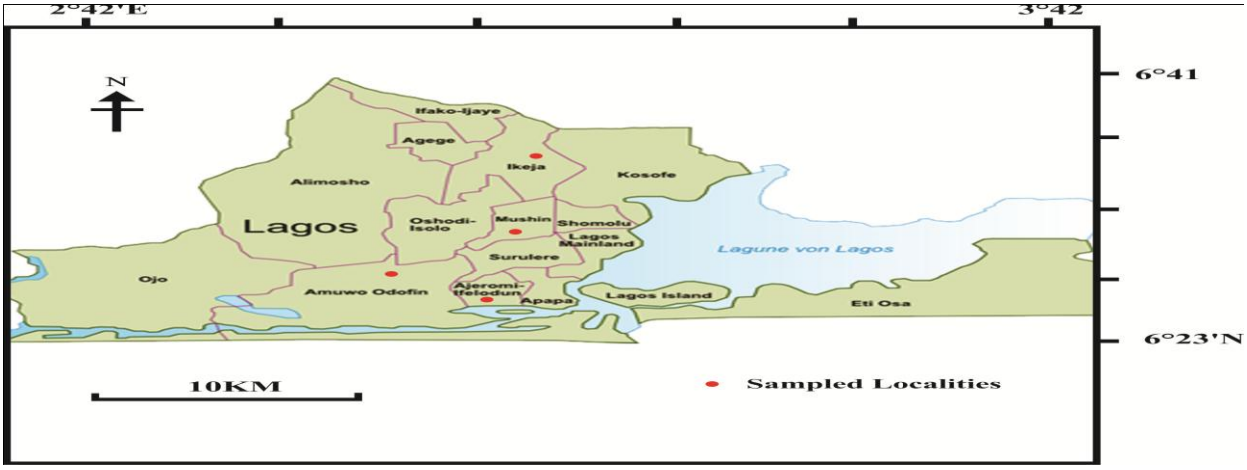

Fig 1. Map of Lagos State showing the sampled localities Source: Google Images (2015)

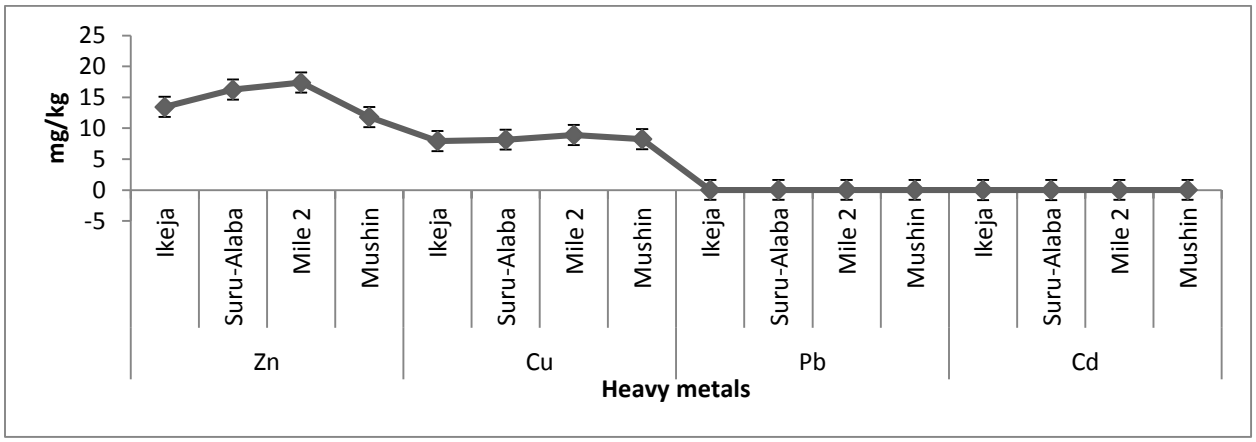

Fig. 2. Mean concentrations of heavy metals in fish from various markets

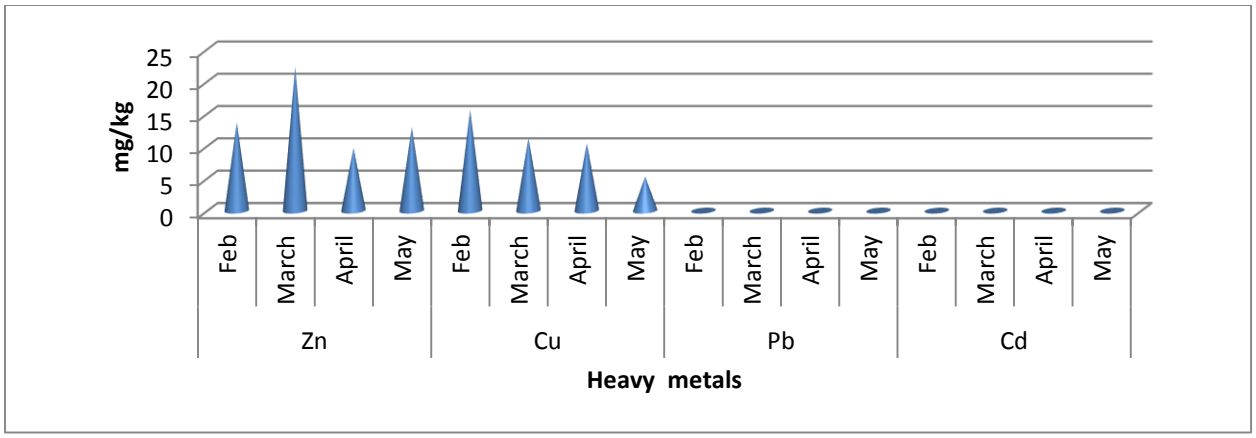

Fig. 3. Monthly mean concentrations of heavy metals in fish

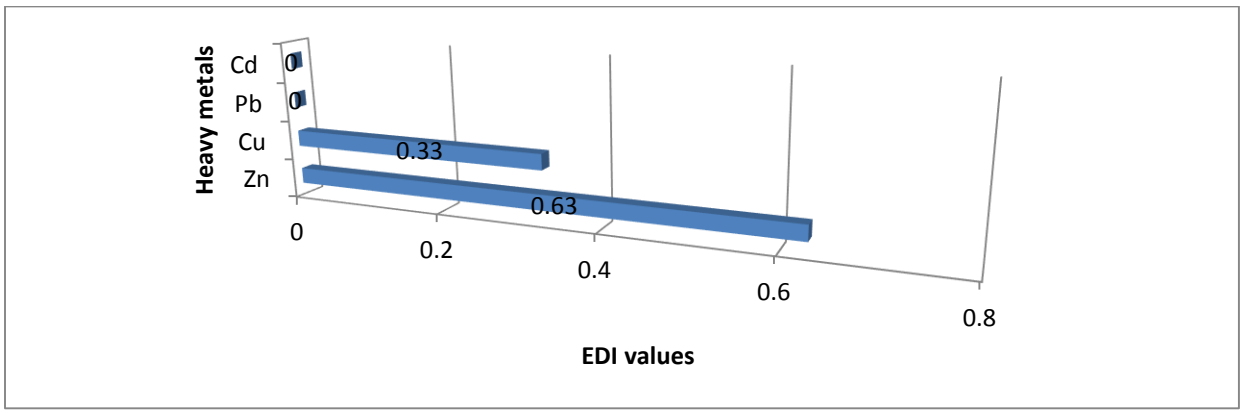

Fig. 4. Estimated daily intake (EDI) values for heavy metals

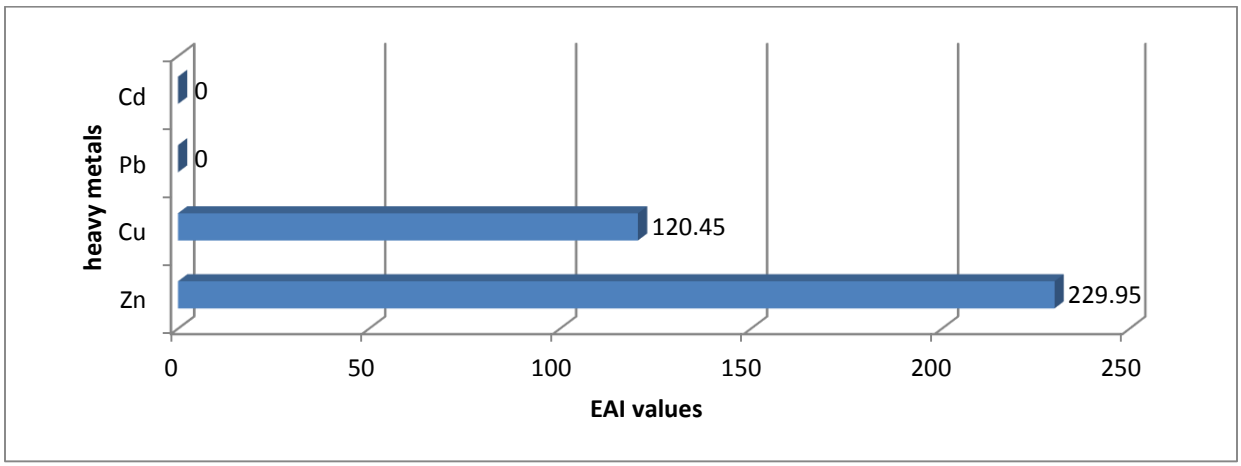

Fig. 5. Estimated annual intake (EAI) values for heavy metals

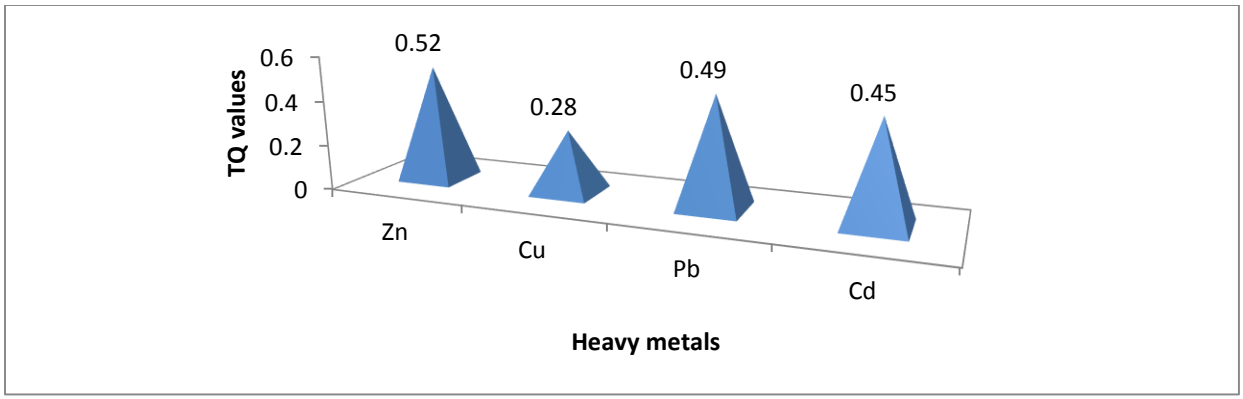

Fig. 6. Toxicity/Hazard quotient (TQ) values for heavy metals

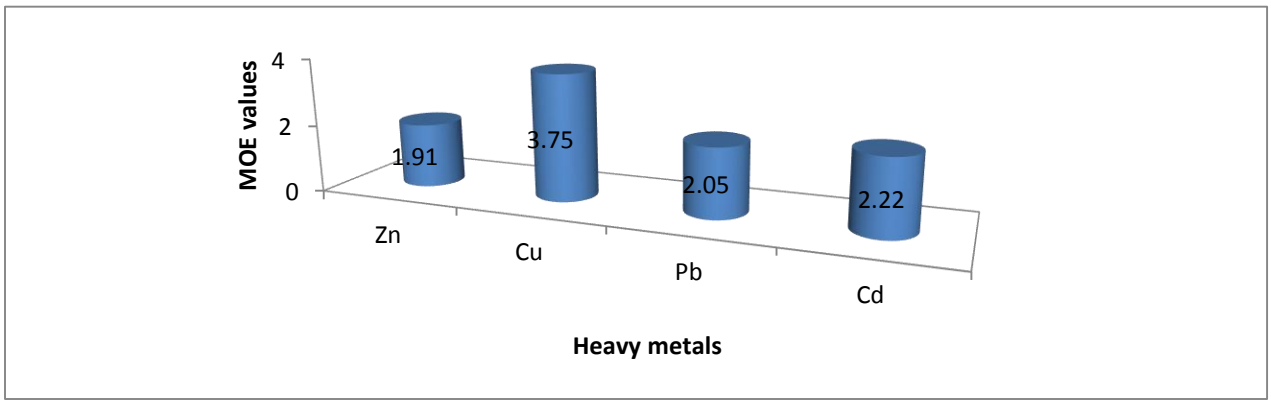

Fig. 7. Margin of exposure (MOE) for heavy metals

state, consumers would be getting predominantly more of Zn and Cu via the consumption of these fish species. According to the World Health organization (WHO, 1997), the TMDI values are particularly convenient for making a first estimate of metal residue intake via a food source.

It was observed that the mean concentrations of the investigated metals in all fish species fell below the maximum limits established by the Food and Agricultural Organization of the United Nations (FAO) for metals in food/fish. This observation indicates that such fish are thus safe for human consumption. This finding, further lends credence to the toxicity/hazard quotient (TQ) values for metals calculated in this study. In this study, *Clarias gariepinus*, was the species that was commonly encountered in the various markets visited. This observation may be connected to the fact that the species is widely used in Nigeria, for aquaculture purposes. The mean concentrations of Zn, Cu, Pb and Cd recorded in this particular fish species, compared well with findings from other studies (Table 3), although these cited investigations were not conducted on fish from market places. The mean concentrations of Zn and Cu in *Arius latiscutatus* in this study, far exceeded the mean concentrations of the same metals in *Arius heudelotii*, a sister Ariid fish species, harvested from the Doula Estuary, Cameroon (Table 4). Elnabris *et al.*, (2013), studied the heavy metal content in commercially important fish species in the Gaza strip (Palestine) and observed lower mean concentrations of Zn (12.78 mg/kg) and Cu (0.91 mg/kg) in *Mugil cephalus* compared to the levels of these metals in the same fish species in this study. Also, the workers reported lower mean concentrations of Zn (7.52 mg/kg) and Cu (0.64 mg/kg) in *Oreochromis niloticus* (Nile Tilapia) compared to the levels of these metals in a fellow Cichlid, *Tilapia zillii*, recorded in this study.

CONCLUSION

The study successfully determined the concentrations of Zn, Cu, Pb and Cd in eight different fish species sold in four major markets in Lagos. It was revealed that the mean concentrations of the aforementioned metals in all fish species fell below the limits established by the Food and Agricultural Organization of the United Nations (FAO) for metals in food/fish. As a result, the consuming public would not immediately experience risk of hazardous metals via consumption of fish. In order to ensure that fish sold in markets remain safe for human consumption, it is advocated that Health Inspection Officers from the Ministry of Health and Environmental Monitoring Officers from the Ministry of Environment, be mandated to jointly monitor the activities of fish mongers and to carry out periodic spot checking of fishery products. Since the present study covered four months, it is suggested that a longer sampling campaign be carried in follow up investigations in order to establish a trend in the concentrations of heavy metals in fish. It is also suggested that chemical elements not covered in this study be included in future studies in order to obtain a more holistic and robust metal profile.

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Conflict of interest

The authors declare no conflict of interest

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