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African Journal of Biotechnology

Full Length Research Paper

Effects of heat sources on the levels of polycyclic aromatic hydrocarbon in selected fish samples from Ogidingbe (Escravos Estuaries)

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The riverine areas of the Niger-Delta region of Nigeria are popularly known for their smoked fish from both fresh and salt water. To the people living in these areas, smoking is a method of preservation as the fish is known to undergo spoilage within 8 h of harvest from its natural habitat; though not without the potential health hazard associated with smoked foods. During smoking, polycyclic aromatic hydrocarbons (PAHs) and its carcinogenic derivatives are produced. Two species of locally consumed fish: *Clarias gariepinus* and *Micropogonias undulantes* were used for this study. The *M. undulantes* were collected from the creeks and estuaries of Ogidigbe and the *C. gariepinus* from a local pond, Ereyi farms, in Benin City. They were both analyzed for PAHs level in both the fresh and dried form. Comparison of the PAHs level was also done for various dried samples using four different drying methods. Extraction of the PAHs were carried out using solvents by ultrasonication, clean-up was done using solid phase extraction and thereafter analyzed for 16 US EPA PAHs using gas chromatography-flame ionization detector (GC-FID). The results show that drying with mangrove wood charcoal gave the highest PAHs level and it was for samples harvested from the Escravos Estuaries.

Key words: Benzo(a)pyrene, *Clarias gariepinus, Micropogonias undulantes,* polycyclic aromatic hydrocarbons (PAHs).

INTRODUCTION

Fresh fishes are known to contain oils and plenty of water and thus will undergo spoilage within certain hours of harvest from their natural habitat unless they are placed in refrigerators or preserved by other methods. In Nigeria, fish preservation is either by smoking or drying with charcoal or wood fire and in the process of preservation by drying, the water and oil content of the fish is reduced. Charcoal or firewood drying is frequently practised in Nigeria as a method of fish preservation either due to unavailability of means of refrigeration, non-availability of light, need for ease of transportation of preserved fish to the market without spoilage or because of the flavor the

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smoke or heat adds to the dried fish product. However, this is not without the health implication as these methods of preservation can have negative impact on the health of those consuming them. Processing fresh fish by subjecting them to heat treatment like drying, smoking, roasting, baking, and frying has reportedly impacted and subsequently increased the level of polycyclic aromatic hydrocarbons (PAHs) in them (Ishizaki et al., 2010; Farhadian et al., 2011). The flames used in the drying process contain PAHs that adhere to the surface of the dried fish product (Cross and Sinha, 2004).

The environment from which the fish is harvested is another source through which the fish can bioaccumulate these PAHs, most especially if they are in areas with a lot of oil exploration and exploitation activities. Years back in Delta State and most of the Niger-Delta areas of Nigeria, there has been numerous petroleum leaks from transport vessels, pipeline, incessant gas flaring and exploration wells. Oil spills from production well accidents and bunkery has also resulted in several spills (Farwell et al., 2009; Nwilo et al., 2000; Olagoke, 1996). Olaji et al. (2010) had reported some levels of PAH in cage reared fish in Deghele area of Delta State. Also, Chimezie and Herbert (2007) reported high levels of PAHs in the fish caught in the Niger Delta area. Thus, PAHs' contamination could be from the aquatic environment where they are harvested from.

Consumption of these contaminated locally harvested river and sea food species is a major route for human exposure to these toxic PAHs (Rotkin-Ellman et al., 2012; Xia et al., 2012). Nonetheless, it is difficult to estimate the contribution of each source of PAHs exposure in different individuals as they are also exposed to them in different ways depending on the nature of their job, environment, type of diet and method of food preparation or a combination of two or more of these factors. Thus, there is need for more data on the levels of PAHs in locally harvested fishes from these areas predisposed to oil exploration activities, in a bid to increase the database of PAHs levels in fishes and sea foods from the Niger-Delta rivers and creeks. It is also necessary to use a reliable, accurate and effective method to qualitatively and quantitatively describe the level of PAHs in these samples. The aim of this study was to determine the effect the source of fish and heat treatment would have on the level of PAHs in the fresh and dried fish samples.

MATERIALS AND METHODS

Sample collection

Escravos Estuary in Ogidigben area of Delta State and Ereyi Farms Escravos Estuary in Ogidigben area of Delta State and Ereyi Farms which contains catfish ponds located in Oredo Local Government Area of Edo State were used as the sampling site. These two locations are within the south-south region or Niger-Delta region of Nigeria. Two species of locally consumed fish samples, viz; *Clarias gariepinus* also known as catfish and *Micropogonias undulates* commonly called croaker fish were used in this study. Croaker fish samples were collected from Ogidigben, in Escravos Estuaries with the aid of the local fishermen while catfish were collected from Ereyi Farms local pond in Benin City. The fishermen go into the estuary with fishing boats and nets mostly at nights and early hours of the day to catch the fishes.

On arrival at the shores, the fish samples which are representative of the sampling area are sorted out. Three croaker fish (*M. undulantes)* samples were collected from the catch at every time of collection (bimonthly) and washed. The samples were kept in polythene bags, labelled properly, kept in coolers with ice and transported to the laboratory where they are stored in refrigerator prior to treatment. Catfish (*C. gariepinus*) were harvested from Ereyi Farms from which three were carefully chosen bimonthly for the study.

Sample preparation

The fresh fish samples from the two sampling sites were washed and re-rinsed with distilled water. They were weighed using top loading balance; the lengths were taken using metre rule and then cut into two parts longitudinally. One part was kept back in the refrigerator to be extracted fresh and the other smoked using African traditional processes of smoking using charcoal from mangrove wood, until the fish samples were properly dried. The time taken was noted, the fresh and smoked portions were separately homogenized using a blender and then stored in a refrigerator at 4°C prior to extraction and analysis. In the last month, the fish samples from the two sampling sites were divided into five parts. One was processed fresh while the other four parts were dried using oven, forest wood charcoal (Owewe), mangrove wood charcoal and wood shavings, respectively.

Fish drying

Drying with charcoal

A metal bowl lined with clay of about two inches thick was packed with charcoal and metal gauze placed over it. The charcoal was lit and allowed to burn to certain point before the fish cuts were placed on the gauze over the fire. It was then dried for a period of 4 to 6 h depending on the size or weight of the fish sample. Weighing was done at intervals before the fish was said to be completely dried. Drying was stopped when there was no more change in weight of the fish samples when weighed using electronic balance after interval of 20 min. The samples were allowed to cool, stored in polythene bags and properly labelled. This method of drying was carried out for 10 months of sampling period.

In the last sampling month, three other drying methods were used, namely: drying using electronic oven, drying using charcoal collected from tropical rain forest (owewe) and drying using wood shavings from saw mill. The wood shavings were the ones from white wood used for planks.

Drying with wood shavings

A drum of about 2 to 3 ft long with wire gauze or mesh over it was used. A small section is cut from the base where the wood shaving is added to refuel the oven as the fire is going down.

Gas chromatography operating procedure

HP gas chromatography system 6890 series was used with flame ionization detector. The basic chromatography parameters used for

Table 1. Average PAHs level for *C. gariepinus.*

Parameter	Pond FF	Pond DF	2Pond FF	2Pond DF	3Pond FF	3Pond DF	4Pond FF	4Pond DF	5Pond FF	5Pond DF
Naphthalene	0.005	0.004	0.005	0.014	0.001	0.024	0.007	0.016	0.008	0.023
Acenaphthalene	0.002	0.001	0.002	0.005	0.007	0.011	0.010	0.019	0.004	0.007
Acenaphthene	0.003	0.004	0.006	0.002	0.009	0.011	0.018	0.008	0.004	0.003
Florene	0.003	0.009	0.003	0.004	0.007	0.010	0.010	0.012	0.004	0.004
Phenathrene	0.007	0.005	0.007	0.005	0.005	0.005	0.009	0.010	0.004	0.005
Anthracene	0.003	0.008	0.006	0.010	0.015	0.011	0.001	0.010	0.004	0.003
Fluoranthene	0.003	0.144	0.006	0.004	0.014	0.009	0.000	0.000	0.002	0.004
Pyrene	0.004	0.018	0.006	0.007	0.005	0.008	0.001	0.000	0.006	0.004
Benzo(a)anthracene	0.003	0.001	0.005	0.002	0.001	0.003	0.012	0.028	0.003	0.003
Crysene	0.001	0.003	0.001	0.003	0.000	0.002	0.002	0.000	0.003	0.002
Benzo(b)fluoranthrene	0.000	0.000	0.000	0.002	0.001	0.001	0.000	0.003	0.003	0.001
Benzo(k)fluoranthrene	0.000	0.023	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000
Benzo(a)pyrene	0.000	0.000	0.000	0.003	0.000	0.009	0.000	0.002	0.000	0.001
Indeno(1,2,3) perylene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.011
Dibenzo(a,h)anthracene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Benzo(g,h,i) perylene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Total PAH (mg/kg)	0.035	0.221	0.047	0.061	0.067	0.105	0.069	0.108	0.045	0.072

Pond FF, Average PAHs concentrations (mg/kg) for samples in the first sampling month for fresh fish samples collected from pond. Pond DF, average. PAHs concentrations (mg/kg) for samples in the first sampling month for dried fish samples collected from pond. 2pond FF, average PAHs concentrations (mg/kg) for samples in the second sampling month for fresh fish samples collected from pond. 2pond DF, average PAHs concentrations (mg/kg) for samples in the second sampling month for dried fish samples collected from pond. 3pond FF, average PAHs concentrations (mg/kg) for samples in the third sampling month for fresh fish samples collected from pond. 3pond DF, average PAHs concentrations (mg/kg) for samples in the third sampling month for dried fish samples collected from pond. 4pond FF, average PAHs concentrations (mg/kg) for samples in the fourth sampling month for fresh fish samples collected from pond. 4pond DF, average PAHs concentrations (mg/kg) for samples in the fourth sampling month for dried fish samples collected from pond. 5pond FF, average PAHs concentrations (mg/kg) for samples in the fifth sampling month for fresh fish samples collected from pond. 5pond DF, average PAHs concentrations (mg/kg) for samples in the fifth sampling month for dried fish samples collected from pond.

the analysis of PAHs were as follows: Initial temperature of 100°C at a rate of 1:4°C min^{-1} with a detector temperature of 300°C and final temperature of 330°C.

Extraction and clean-up of samples

The fresh and dried fish samples were separately ground using a blender (Mikachi meat grinder). The blender was washed, rinsed and re-rinsed with distilled water after using it for each fish sample. 50 g of the ground fish sample was mixed with 25 g of sodium sulphate and 200 ml of 50/50 cyclohexane/acetone mixture in a tight fitted covered amber coloured bottle and 10 ml of the internal standard was added to each bottle. Each bottle containing the sample, solvents mixture, sodium sulphate and the internal standard mix were placed inside the ultrasonic bath (Astrabro ultrasonic cleaner) model 7E for 2 h. The bottles were brought out after every 10 min and shaken.

The extract (25 ml) was then collected using a pipette, filtered and concentrated using rotary evaporator to 5 ml. Each 5 ml concentrate was further concentrated to 1 ml using nitrogen gas. Clean up was done using solid phase extraction with SPE cartridges designed by Biotage and named Isolute*SI* with sorbent Si, sorbent mass 500 mg, 3 ml volume and cyclohexane was employed as the eluting solvent using 10 ml of the eluting solvent. The cleaned up sample was concentrated to 1 ml using nitrogen gas, stored in 1-ml vials and subjected to GC analysis using FID detector.

Statistical analysis

One-way analysis of variance was used to statistically test for the variability of PAHs in fish samples collected from the Escravos Estuaries and pond.

RESULTS AND DISCUSSION

Results obtained (Table 1) show that the average concentration of PAHs for fish samples (*C. gariepinus)* collected from the pond ranged from 0.035 to 0.069 mg/kg, while for dried fish sample, it was from 0.061 to 0.221 mg/kg. Similar trend was observed for fresh and dried fish (*M. undulantes*) collected from Escravos Estuaries ranging from 0.0014 to 0.073 mg/kg and 0.025 to 0.129 mg/kg. Results obtained from dried and fresh sample were not as high as those reported by Nnaji and Ekwe (2018) on smoked fish from Michael Okpara University of Agriculture, Umudike in Abia State of Nigeria. Concentration of benz[a]anthracene and benzo[a]pyrene obtained in fresh and dried fish for *C. gariepinus* ranged from 0.001 to 0.012 and 0.000 to0.000 mg/kg for fresh fish as well as 0.001 to 0.028 and 0.001 to 0.009 mg/kg for dried fish from pond; whereas

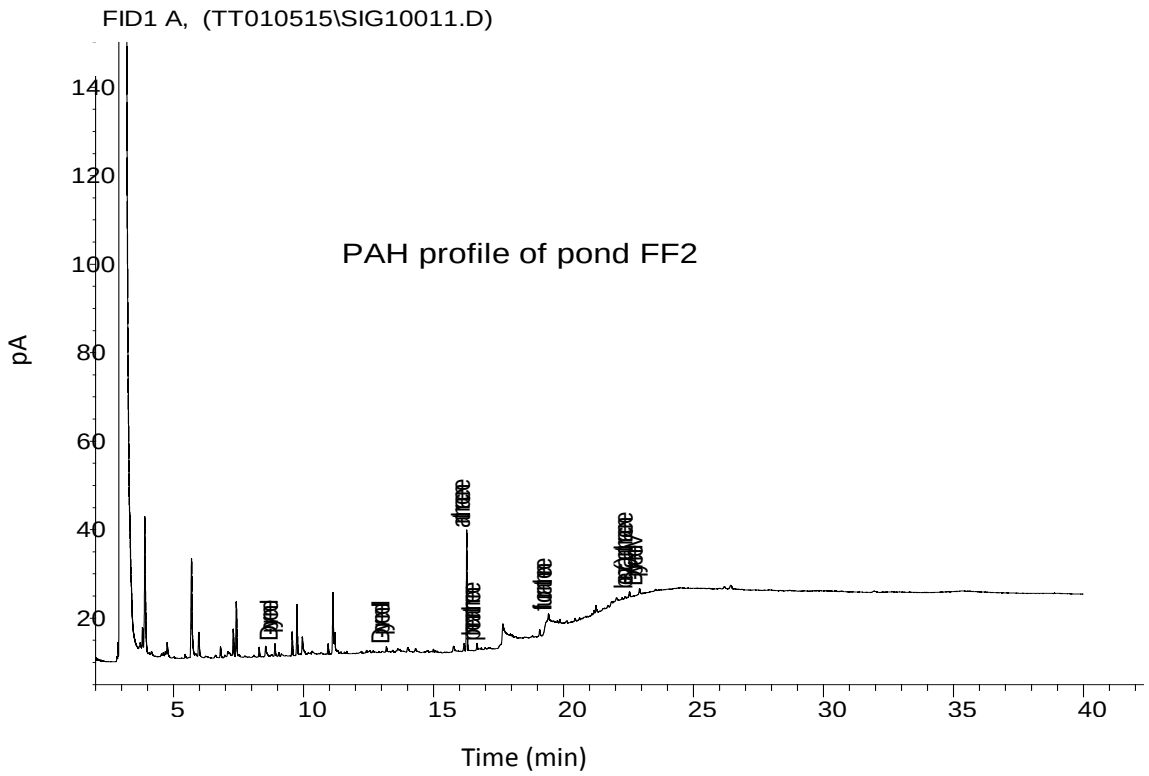

Figure 1. PAHs profile of fresh pond catfish oil sample 2 in the first sampling month.

those from Escravos Estuaries ranged from 0.003 to 0.0029 and 0.00 to 0.029 mg/kg for fresh fish as well as 0.001 to 0.012 and 0.001 to 0.029 mg/kg for dried fish (*M. undulantes*). This value is far more than that reported by Pierre et al. (2012) on smoked fish samples sold in Abobo Abidjan. Benzo[a]pyrene may be bio transformed in humans and animals to 3-OH benzo[a]pyrene (3-OH-B[a]P) (Rey-Salgueiro et al., 2009). 3,4-Benzopyrene, found in smoked products, serves as an indicator of the possible presence of other PAHs and has been repeatedly used as a quantitative index of chemical carcinogens in foods. It has also been reported by the International Agency for Research on Cancer (IARC) that benzo[a]pyrene and benz[a]anthracene and are probably carcinogenic to humans.

Figure 1 shows a gas chromatogram of PAHs' profile of oil extracted from fresh catfish harvested from pond (Pond FF2) with total PAHs concentration of 0.019 mg/kg which is in the range of 0.035 to 0.069 mg/kg as stated earlier in Table 1. Figure 2 shows gas chromatogram of PAHs profile of oil extracted from fresh croaker fish harvested from Escravos Estuaries (ESC FF2) with a total PAHs concentration of 0.009 mg/kg which agrees with the range stated in Table 2. Table 4 gives average PAHs concentration for *M. undulantes* from Escravos using different drying methods and samples dried using the mangrove wood charcoal were found to have the highest average concentration of 0.457 mg/kg; The ones dried using oven and wood shavings were almost having the same values of 0.244 and 0.221 mg/kg, respectively; For individual PAHs 0.136 mg/kg was recorded for pyrene in mangrove wood charcoal dried samples as the highest and is found to be the reason why the mangrove wood charcoal dried samples have the unusually high PAHs concentration when compared with the rest samples.

Figures 3, 4 and 5 give a PAHs profile of oil extract of catfish harvested from the pond but dried using different drying methods namely wood shavings, oven and mangrove wood charcoal, respectively. Figure 6 is a gas chromatogram of oil extracted from croaker fish harvested from the Escravos Estuaries and dried using mangrove wood charcoal.

The results for test of variability using one way analysis of variance show that there was no significant difference in concentration of various PAHs except for *M. undulantes* samples collected from Escravos Estuaries dried using the four different drying methods mentioned in this study [drying with Mangrove wood charcoal, forest wood charcoal (Owewe), oven and wood shaven)]. This result showed that there was significant variation among the values of PAHs in the samples tested as $F_c > F_{t\alpha}$ (3.69>2.45). This may be due to difference in combustion rate of the drying method. According to Rey-Salgueiro et

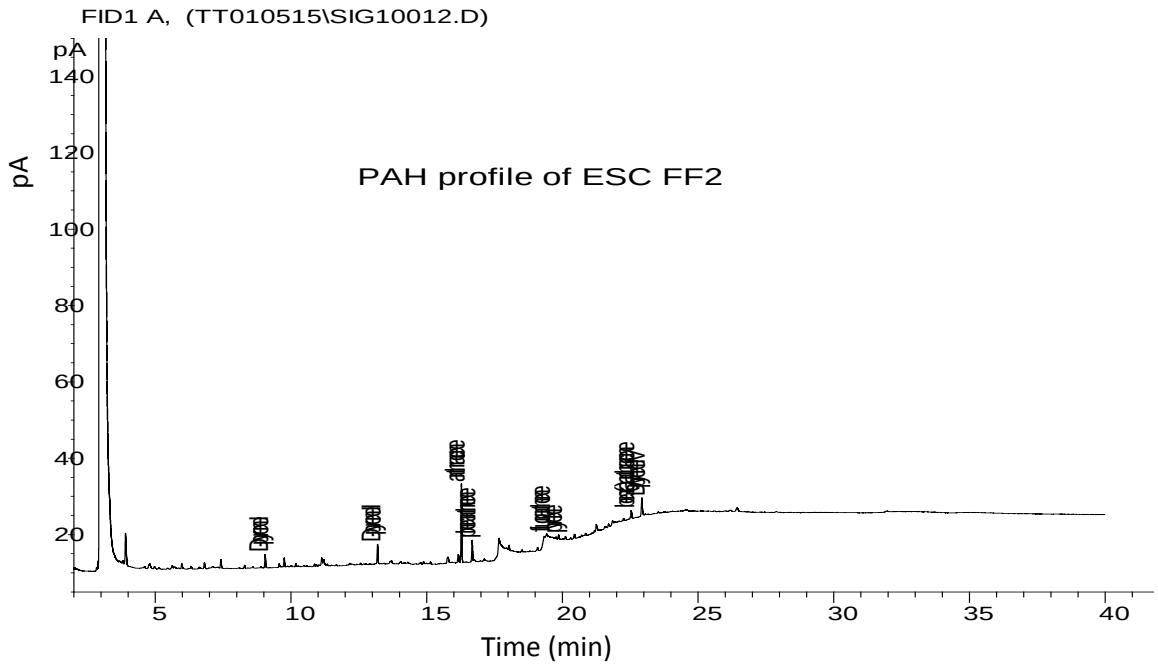

Figure 2. PAHs profile of fresh Escravos fish oil sample in the first sampling month.

Table 2. Average PAHs level of *M. undulantes.*

Parameter	ESC FF	ESC DF	2ESC FF	2ESC DF	3ESC FF	3ESC DF	4ESC FF	4ESC DF	5ESC FF	5ESC DF
Naphthalene	0.000	0.005	0.000	0.003	0.001	0.021	0.016	0.016	0.005	0.039
Acenaphthalene	0.001	0.005	0.013	0.012	0.007	0.023	0.016	0.019	0.008	0.016
Acenaphthene	0.002	0.001	0.006	0.007	0.002	0.005	0.018	0.008	0.022	0.027
Florene	0.001	0.004	0.007	0.006	0.006	0.014	0.010	0.012	0.005	0.014
Phenathrene	0.001	0.002	0.011	0.010	0.009	0.009	0.009	0.010	0.004	0.001
Anthracene	0.000	0.000	0.006	0.025	0.004	0.014	0.001	0.010	0.001	0.004
Fluoranthene	0.000	0.001	0.000	0.007	0.002	0.006	0.000	0.000	0.000	0.006
Pyrene	0.002	0.000	0.000	0.000	0.002	0.027	0.001	0.000	0.000	0.007
Benzo(a)anthracene	0.003	0.000	0.029	0.029	0.006	0.003	0.012	0.028	0.013	0.008
Crysene	0.000	0.000	0.000	0.000	0.000	0.004	0.002	0.000	0.004	0.001
Benzo(b)fluoranthrene	0.000	0.002	0.000	0.000	0.003	0.002	0.000	0.003	0.000	0.000
Benzo(k)fluoranthrene	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Benzo(a)pyrene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000
Indeno(1,2,3) perylene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Dibenzo(a,h)anthracene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Benzo(g,h,i) perylene	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Total PAH (mg/kg)	0.014	0.025	0.073	0.099	0.041	0.129	0.084	0.108	0.063	0.124

ESC FF, Average PAHs concentrations (mg/kg) for samples in the first sampling month for fresh fish samples collected from Escravos. ESC DF, average PAHs concentrations (mg/kg) for samples in the first sampling month for dried fish samples collected from Escravos. 2ESC FF, average PAHs concentrations (mg/kg) for samples in the second sampling month for fresh fish samples collected from Escravos. 2ESC DF, average PAHs concentrations (mg/kg) for samples in the second sampling month for dried fish samples collected from Escravos. 3ESC FF, average PAHs concentrations (mg/kg) for samples in the third sampling month for fresh fish samples collected from Escravos. 3ESC DF, average PAHs concentrations (mg/kg) for samples in the third sampling month for dried fish samples collected from Escravos. 4ESC FF, average PAHs concentrations (mg/kg) for samples in the fourth sampling month for fresh fish samples collected from Escravos. 4ESC DF, average PAHs concentrations (mg/kg) for samples in the fourth sampling month for dried fish samples collected from Escravos. 5ESC FF, average PAHs concentrations (mg/kg) for samples in the fifth sampling month for fresh fish samples collected from Escravos. 5ESC DF, average PAHs concentrations (mg/kg) for samples in the fifth sampling month for dried fish samples collected from Escravos.

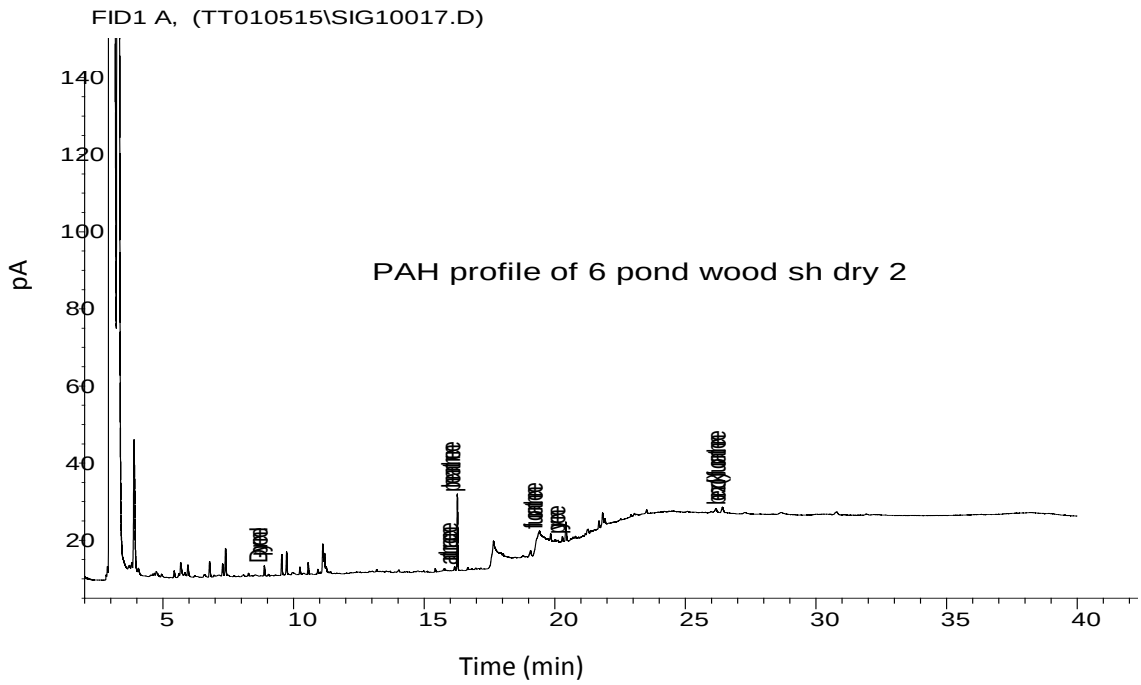

Figure 3. PAHs profile of pond catfish sample dried using wood shavings in the 6th sampling month.

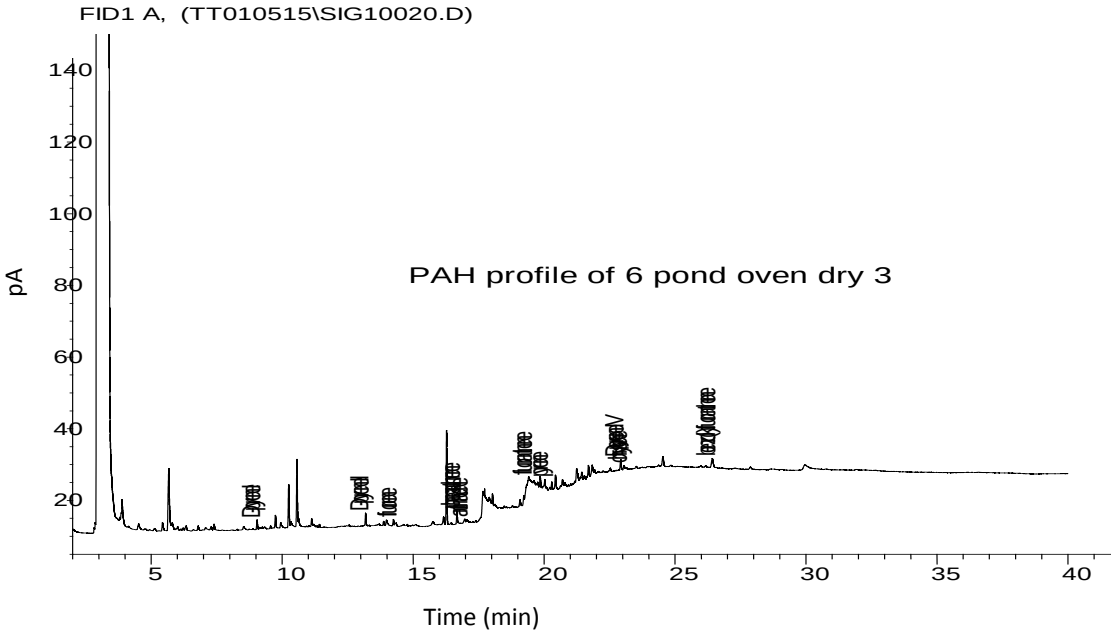

Figure 4. PAHs profile of oven dried pond catfish oil sample in the 6th sampling month.

al., (2004) and Garcia-Falcon and Simal-Gandara (2005), the PAH levels in smoke depend on heat source temperature, flame intensity in flame combustion, and particulate material generated during combustion. The combustion temperature during the generation of smoke seems particularly critical and PAHs are formed during incomplete combustion processes (Muthumbi et al., 2003). According to Zohair (2006), if phenathrene to anthracene ratio is less than 10, combustion is a major source of the PAHs contamination, but if greater than 10

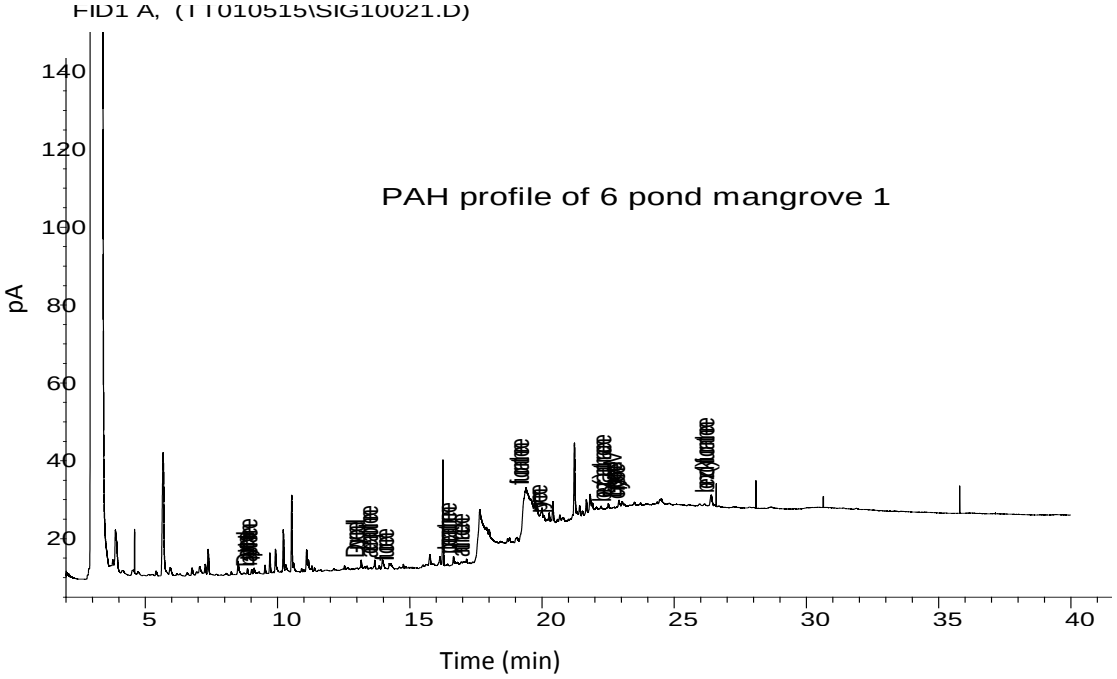

Figure 5. PAHs profile of Mangrove wood charcoal dried pond catfish oil sample in the 6th sampling month.

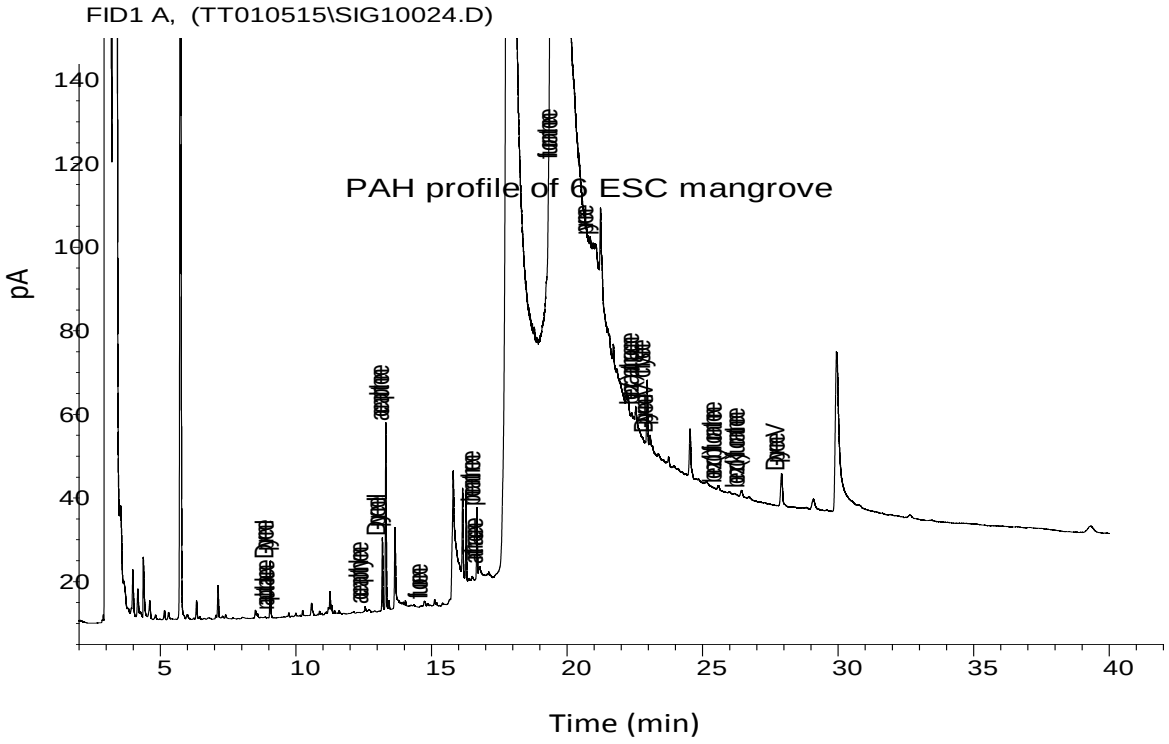

Figure 6. PAHs profile of mangrove wood dried Escravos sample 1 in the 6th sampling month.

it implies that it is petrogenic. From Tables 3 and 4, it was observed that regarding different drying methods for both fish species, the phenathrene to anthracene ratio is less than 10 implying combustion process of the drying source

Table 3. Average PAHs level of *C. gariepinus* using the different drying methods.

Parameter	6pond FF (m/kg)	6pond forest DF (m/kg)	6pond wood shavings DF (m/kg)	6pond mangrove DF(m/kg)	6pond oven DF(m/kg)
Naphthalene	0.007	0.007	0.002	0.013	0.010
Acenaphthalene	0.010	0.000	0.002	0.008	0.007
Acenaphthene	0.018	0.008	0.006	0.012	0.007
Florene	0.010	0.003	0.014	0.006	0.008
Phenathrene	0.009	0.009	0.029	0.009	0.004
Anthracene	0.001	0.001	0.003	0.003	0.007
Fluoranthene	0.000	0.024	0.058	0.086	0.035
Pyrene	0.001	0.001	0.011	0.008	0.014
Benzo(a)anthracene	0.012	0.019	0.003	0.001	0.000
Crysene	0.002	0.000	0.025	0.009	0.005
Benzo(b)fluoranthrene	0.000	0.000	0.006	0.000	0.002
Benzo(k)fluoranthrene	0.000	0.000	0.004	0.004	0.003
Benzo(a)pyrene	0.000	0.000	0.000	0.000	0.000
Indeno(1,2,3) perylene	0.000	0.000	0.000	0.000	0.000
Dibenzo(a,h)anthracene	0.000	0.000	0.000	0.000	0.000
Benzo(g,h,i) perylene	0.000	0.000	0.000	0.000	0.000
Total PAH (mg/kg)	0.069	0.073	0.165	0.159	0.101

6pond FF, Average PAHs concentrations for fresh fish from pond in the sixth sampling month. 6pond forest DF, Average PAHs concentrations for forest wood charcoal dried fish from pond in the sixth sampling month. 6pond wood shavings DF, Average PAHs concentrations for fish dried using wood shavings from pond in the sixth sampling month. 6pond mangrove DF, Average PAHs concentrations for fish dried using mangrove wood from pond in the sixth sampling month. 6pond oven DF, Average PAHs concentrations for oven dried fish from pond in the sixth sampling month.

Table 4. Average PAHs level of *M. undulante* using the different drying methods.

Parameter	6ESC FF (m/kg)	6ESC Oven DF (m/kg)	6ESC forest DF (m/kg)	6ESC mangrove DF (m/kg)	6ESC wood shavings DF (m/kg)
Naphthalene	0.029	0.051	0.005	0.013	0.024
Acenaphthalene	0.003	0.034	0.007	0.011	0.007
Acenaphthene	0.002	0.022	0.006	0.044	0.082
Florene	0.002	0.038	0.026	0.001	0.008
Phenathrene	0.005	0.020	0.012	0.021	0.004
Anthracene	0.003	0.013	0.011	0.064	0.026
Fluoranthene	0.001	0.007	0.000	0.056	0.020
Pyrene	0.003	0.011	0.000	0.136	0.022
Benzo(a)anthracene	0.002	0.031	0.002	0.078	0.009
Crysene	0.008	0.002	0.000	0.028	0.008
Benzo(b)fluoranthrene	0.000	0.000	0.000	0.001	0.011
Benzo(k)fluoranthrene	0.000	0.000	0.000	0.004	0.000
Benzo(a)pyrene	0.000	0.000	0.000	0.000	0.000
Indeno(1,2,3) perylene	0.000	0.015	0.001	0.000	0.000
Dibenzo(a,h)anthracene	0.000	0.000	0.000	0.000	0.000
Benzo(g,h,i) perylene	0.000	0.000	0.000	0.000	0.000
Total PAH (mg/kg)	0.059	0.244	0.069	0.457	0.221

6ESC FF, Average PAHs concentrations for fresh fish from Escravos in the sixth sampling month. 6ESC Oven DF, average PAHs concentrations for oven dried fish from Escravos in the sixth sampling month. 6 ESC Forest DF, average PAHs concentrations for forest wood charcoal dried fish from Escravos in the sixth sampling month. 6ESC Mangrove DF, average PAHs concentrations for Mangrove wood charcoal dried fish from Escravos in the sixth sampling month. 6ESC Wood Shavings DF, average PAHs concentrations for fish dried using wood shavings from Escravos in the sixth sampling month.

as an agent of petroleum hydrocarbon. Effect of combustion from drying method followed the sequence, wood shaving > forest wood charcoal > mangrove wood > oven for *C. gariepinus* and oven > forest wood charcoal > mangrove > wood shaving for *M. undulante*.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Clausenidin upregulated p53 and caused apoptosis in HT-29 tumor cell lines

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Clausenidin is a pyranocoumarin majorly found in medicinal herbs of the Rutaceae family used to treat cancer patients locally in Asia. The compound is presumed to have anti-cancer cell effect but its exact mechanism of action is still unknown. The study aimed to evaluate the effect of pure clausenidin on p53-mediated apoptosis as well as other cell death pathways in colon cancer (HT-29) cell lines. The anti-proliferative effect of clausenidin by cell cycle and annexin V assay using flow cytometry was evaluated. Morphological analysis of the treated cells was performed using scanning and transmission electron microscopy. Furthermore, the effect of p53 mRNA on cell cycle and apoptosis-related genes and proteins in clausenidin-treated HT-29 cells was investigated using qPCR and Western blot assays, respectively. Clausenidin induced a p53 dependent G0/G1 cell cycle arrest in HT-29 cells. It was also observed that the anti-cancer cell effect of clausenidin occurred via p53 mediated activation of p21, bax and transrepression of survivin and bcl 2 that culminated in the apoptosis of colon cancer cells. The transmission electron microscopy (TEM) micrographs confirmed the occurrence of apoptosis in clausenidin-treated HT-29 cells. Clausenidin is a potent anti-HT-29 cell agent that can be used to treat colon tumors.

Key words: Clausenidin, p53, p21, apoptosis, cell cycle.

INTRODUCTION

The protein, P53 (TP53) was first identified in 1979 and named after its molecular weight, which is 53 kDa (Levine et al., 1991). The protein, P53, is one of the best-known tumor suppressor protein that is encoded by the TP53

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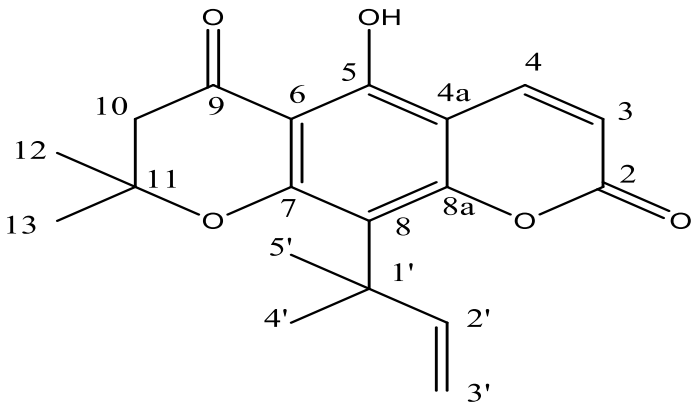

Figure 1. Chemical structure of clausenidin.

gene located on the short-arm of chromosome 17. Besides its involvement in the induction of apoptosis, it is also a key regulator of DNA recombination, gene amplification, segregation of chromosomes, cell cycle and differentiation and cellular senescence (Oren and Rotter, 1999). Deletion of the p53 gene in cells leads to the decrease in rate of apoptosis (Slatter et al., 2011; Vikhanskaya et al., 2007). Because this protein play a profound role in the maintenance of cell integrity, it is often referred to as 'guardian of the genome' (Lane, 1992). In human cancers, the p53 gene is generally impaired (Bai and Zhu, 2006). P53 can detect DNA damage and direct the cells to undergo DNA repair or apoptosis.

Clausena excavata Burm *f*. is a native of the Asian tropical forest commonly found in India, Thailand and Malaysia (Huang et al., 1997; Manosroi et al., 2004). The plant is claimed to have numerous therapeutic benefits that includes anti-bacteria, anti-fungal, anti-inflammatory (Wu et al., 1994) and anticancer effects (Manosroi et al., 2004; Arbab et al., 2013) but its detailed mechanism of action is yet to be understood. Thus far, coumarins and alkaloids are the major bioactive components to have been isolated from *C. excavata*. Previous study reported that clausenidin (Figure 1) isolated from *C. excavata* induces caspase-dependent cell death in colon cancer cell line (Waziri et al., 2016). Since p53 is the most frequent mutated gene in cancerous cells, the current study investigates its role in clausenidin-treated HT-29 cells. Specifically, the study aims to unravel the effect of clausenidin on p53-dependent-cell cycle arrest and -apoptosis in colon cancer cell line.

METHODOLOGY

Cell line and cell culture

HT-29 cell line was purchased from American Type Culture Collection (ATCC) and maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS). The cells were grown in an incubator at 37°C and 5% CO_2.

Extraction of clausenidin

The clausenidin was isolated from the fresh roots of *Clausena excavata* (Voucher number, 2991/16) as described previously (Waziri et al., 2016). The IC_{50} of the pure clausenidin at 72 h was 13.8 ±2.89 µg/mL (Waziri et al., 2016).

Scanning electron microscopy (SEM)

To prepare the cells for SEM, HT-29 cells were seeded at a density of 1×10^6 cells/T-25 mL flask overnight, and treated with the IC_{50} of clausenidin (13.8 µg/mL) for 24, 48 and 72 h while the negative control cells were treated with 0.1% (v/v) DMSO. At the end of the treatment period, cells were harvested with trypsin and washed three times with PBS by centrifugation at 1000 x *g* for 10 min at 4°C before fixing with 4% glutaraldehyde and 1% osmium tetraoxide for 6 and 2 h respectively at 4°C. The cells were washed three times (10 mins each) after each fixing with 0.1 M sodium cacodylate buffer and centrifuged at 1000 x *g* for 5 min to collect the cell pellets followed by successive dehydration with 35, 50, 75 and 95% acetone (10 min each). The cells were further dehydrated three times (15 min each) with 100% acetone before drying for 30 min on a critical drier. Finally, the cells were placed on stubs, coated with gold particles and then viewed under the JSM 6400 scanning electron microscope (Joel, USA).

Transmission electron microscopy (TEM)

For TEM, cells (1×10^6 cells/flask) were seeded overnight in T-25 mL flask and treated with the IC_{50} of clausenidin (13.8 µg/mL) for 24, 48 and 72 h while negative control cells were treated with 0.1% (v/v) DMSO. The cells were processed by fixing with 4% glutaraldehyde and osmium tetraoxide, and dehydrated with acetone as described previously. Thereafter, infiltration was done with acetone:resin mixture successively for 1 and then 3 h in a ratio of 1:1 and 1:3, respectively. Further infiltration was done overnight with 100% resin, and the cells were embedded by inserting into a beam capsule containing the resin. The embedded samples were polymerized in an oven at 60°C for 2 days before cutting into thick sections (1 µm) using an ultramicrotome. The thick sections were stained with toluidine blue and cut further into thinner sections of 60 to 90 nm that were stained further with uranyl acetate and lead for

Table 1. Gene and primer sequences used in the assay.

Gene	Forward sequence	Reverse sequence
P53	AGGTGACACTATAGAATAGATCATTGCTCCTCCTGAGC	GTACGACTCACTATAGGGACGTTGTTTTCAGGAAGTAGT
P21	AGGTGACACTATAGAATAAGCTGAGGTGTGAGCAG	GTACGACTCACTATAGGGACCCAGGCGAAGTCAC
Cyclin A	AGGTGACACTATAGAATACAGCCCGTGGAGTCT	GTACGACTCACTATAGGGACTGATCCAGAATAACACCTG
Cyclin D	AGGTGACACTATAGAATACCTGTGCTGCGAAGT	GTACGACTCACTATAGGGAGAAGCGGTCCAGGTAG
Cyclin E	AGGTGACACTATAGAATACAGGATCCAGATGAAGAA	GTACGACTCACTATAGGGACCTTAAGTATGTCTTTTCCTT
JNK	AGGTGACACTATAGAATACAGAAGCTCCACCACCAAAGAT	GTACGACTCACTATAGGGAGCCATTGATCACTGCTGCAC
β-actin	AGGTGACACTATAGAATAGATCATTGCTCCTCCTGAGC	GTACGACTCACTATAGGGAAAAGCCATGCCAATCTCATC

P53, Tumor suppressor protein 53; P21, Tumor suppressor protein 21; JNK, c-Jun N-terminal kinases.

15 and 10 min, respectively. The fully stained sections were then viewed under the H-7100 transmission electron microscope (Hitachi, Japan).

Cell cycle assay

The cell cycle was monitored by flow cytometry (Becton Dickinson, USA) using the CycleTest^{TM} plus DNA reagent kit according to manufacturer's protocol. The cells were seeded overnight at a density of 1×10^6 cells/flask in a T-25 mL flask and treated with 5, 15, 30 and 40 µg/mL of clausenidin while negative control cells were treated with 0.1% (v/v) DMSO, both for 24 h. The cells were then washed twice with PBS and centrifuged at 1000 × *g* for 5 min at 4°C and the supernatant discarded after each washing. The pellets were suspended in 250 µL of solution A (trypsin buffer), mixed gently and incubated at room temperature for 10 min. Then 200 µL of solution B (trypsin inhibitor and RNase) was added and the cells incubated for another 10 min at room temperature. Finally, 200 µL of ice-cold solution C (125 µg/mL PI final conc.) was added to each reaction tube and the cells incubated on ice for 10 min in the dark and analysed by flow cytometry (BD FACS, Calibur).

Annexin-V assay

Annexin-V assay was performed using the FITC Annexin-V Apoptosis Detection Kit I (BD Pharmingen, US) according to manufacturer's instruction. Briefly, cells were seeded in a 6-well plate at a density of 3×10^5 cells/well and treated separately with 5, 15, 30 and 40 µg/mL of clausenidin while negative control cells were treated 0.1% (v/v) DMSO both for 24 h. After harvesting with trypsin, cells were washed twice with PBS and suspended in 5 µL each of annexin-V and PI, vortexed gently and incubated in the dark for about 15 min. This was followed by the addition of 400 µL of binding buffer to each reaction tube and the cells were analyzed by flow cytometry (BD FACS, Calibur).

Gene expression studies

The extraction of RNA was basically done to determine gene expressions of clausenidin-treated cells.

RNA isolation

The cells were seeded overnight in T-25 mL flask at a density of 1×10^6 cells/well and treated with 13.8 µg/mL clausenidin for 12 or 24 h. Negative control cells were treated with 0.1% (v/v) DMSO for 24 h. The cells were then harvested after detachment with trypsin and washed with PBS. The RNA was extracted using Total RNA extraction kit (GF-1 TRE kit, Vivantis technologies) according to the manufacturer's protocol and its purity determined and amount quantified at 260nm in the nanodrop spectrophotometer (Eppendorf, United Kingdom).

Primer design

The primers for the genes of interest and housekeeping gene used in this study were designed on the NCBI website using PRIMER-BLAST software (Table 1). The primers were purchased from Biosune (Shanghai, China), while the internal control (Kanr) was supplied by Beckman Coulter (USA). The quality of the designed primers was checked using the Oligocal software.

RT-qPCR

The reverse transcriptase quantitative PCR (RT-qPCR) was carried out according to the GenomeLab GeXP Kit (Beckman Coulter, USA) protocol, in an XP Thermal Cycler (Bioer Technology, Germany). Reverse transcription (RT) and PCR were done according to manufacturer's instructions. The conditions were set as follows; RT reaction was at 48°C for 1 min; 37°C for 5 min; 42°C for 60 min; 95°C for 5 min; then held at 4°C, while PCR was as follows: Initial denaturation at 95°C for 10 min, followed by two-step cycles of 94°C for 30 s and 55°C for 30 s, ending in a single extension cycle of 68°C for 1 min. The PCR products were finally analyzed on the GeXP genetic analysis system and the results normalized on express Profiler software. The β-actin gene was used for normalization.

Protein expression studies

To further ascertain the findings of the gene expression analysis, protein profile array and Western blot assays were done to determine expression of apoptotic protein in clausenidin-treated HT-29 cells.

Human apoptosis protein profile array

The protein profile array was performed using the proteome profiler antibody array kit (Raybiotech Inc., USA) according to the manufacturer's protocol. Briefly, the cells were seeded overnight at a density of 1×10^6 cells/well and treated with 15 µg/mL clausenidin while negative control cells were treated with 0.1% (v/v) DMSO,

both for 24 h. Thereafter, protein samples from clausenidin-treated HT-29 cells were incubated overnight at 4°C on primary antibody coated slides (Raybiotech Inc., USA) and washed with wash buffer I before incubating with the biotin-conjugated antibody at 25°C for 2 h. The slides were washed with wash buffer I, incubated with 1,500-fold diluted Hilyte Plus™-conjugated streptavidin in the dark for 2 h and washed again with wash buffer I before disassembling the slides from the incubation frame and chamber. Further washing of slides was done with wash buffer II for 30 min and air-dried for about 1 h and then the result analyzed using the analysis tool software (RayBio Human Apoptosis Array C-series 1).

Protein assay

The cells were seeded in a 6-well plate overnight at a density of 5 × 10^5 cells/well before treating with either 5, 15, 30 or 40 µg/mL clausenidin while negative control cells were treated with 0.1% (v/v) DMSO, both for 24 h. The cells were washed with PBS by centrifugation at 5000 × *g* for 5 min at 4°C. The cell pellets were suspended in 200 µL RIPA buffer (Thermo Fisher scientific, USA) for 30 min, on ice with vortexing at 10-minute intervals for 20 s. The cell suspension was then centrifuged at 14000 × *g* for 25 min at 4°C. The supernatant collected were transferred to fresh tubes. To quantify protein, 250 µL Bradford reagent was added to 5 µL sample in a 96-well plate and the plate incubated for 5 min at room temperature in the dark. A protein standard curve was prepared with bovine serum albumin in PBS. The absorbance was determined at 570 nm in an ELISA plate reader (BioTek, USA) and protein concentration of samples obtained from the standard curve.

SDS-PAGE and Western blot

Protein samples were separated by electrophoresis for 90 min at 120 V on a 10% sodium doedecyl sulphate-polyacrylamide gel. The samples were run using Tris running buffer (25 mM Tris base, 192 mM glycine, 0.1% SDS, pH 8.3) medium. The resolved proteins from SDS-PAGE gel were transferred to the polyvinylidene difluoride (PVDF) membrane (Biorad, USA) using the wet transfer method (Gels, 2001). The transfer of proteins was done with the use of transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) at 100V for 90 min. The membrane was then blocked for 2 h with 5% skim milk in an orbital shaker (Heidolph, Germany) before washing trice for 10 min each time with PBS-Tween 20 on an orbital shaker (Heidolph, Germany). Primary and secondary antibodies (Santa Cruz, USA) were prepared in 5% skim milk at a concentration of 1:2000 and 1:3000 respectively. The membranes were incubated with the primary antibodies at 4°C for 24 h followed by secondary antibody at 25°C for 1 h. The membranes were washed trice for 10 min each time with PBS-Tween 20 after incubation with either primary or secondary antibodies. The membranes were developed using the chemiluminescent substrate (ThermoFisher Scientific, USA) according to manufacturer's protocol. Solutions A and B were mixed in equal proportions and 1 mL of the mixture was added to the membrane while ensuring the surface is completely covered with the substrate. The membranes were incubated for 5 min and then viewed on a ChemiDoc™ imaging system (Biorad, USA) and the protein expressions determined.

Statistical analysis

One way analysis of variance (ANOVA) was used to determine the level significance at 95% confidence interval ($p<0.05$) using the SPSS 22 software (SPSS Inc, Chicago IL, USA).

RESULTS

Effect of clausenidin on cell morphology and nuclear fragmentation

Morphological aberrations of clausenidin-treated cells were examined using scanning electron (SEM) and transmission electron microscopy (TEM). The SEM analysis reveals features of apoptosis that includes membrane blebbing and cytosolic modifications (Figure 2). Further investigation using TEM showed chromatin condensation and margination, convolution of nuclear outline as well as vacuolation (Figure 3). The fragmentation of nucleus was observed at 48 and 72 h of treatment with clausenidin (Figure 3C-D).

Clausenidin inhibited the proliferation of HT-29 cells and caused a G0/G1 cell cycle arrest

The effect of clausenidin on cell cycle progression in HT-29 cells was investigated by flow cytometry. It was observed that clausenidin inhibited the proliferation of HT-29 cells and also caused a G0/G1 arrest (Figure 4F). The cell cycle distribution shows an increase in the percentage of sub G0/G1 cells (apoptotic cells) after treatment with clausenidin in a dose dependent manner. Furthermore, clausenidin caused a significant decrease ($p<0.05$) in the proportion of viable cells.

Clausenidin induced apoptosis in HT-29 cells

Clausenidin treatment caused a significant ($p<0.05$) dose dependent apoptosis in HT-29 cells after 24 h of treatment (Figure 5). In addition, the viable cells decreased significantly ($p<0.05$) in a dose dependent manner after treatment with clausenidin for 24 h.

Gene expression studies

The gene expression studies showed the effect of clausenidin on mRNAs that regulate cell cycle in HT-29 cells. Clausenidin caused over 10 fold increase in the expression of p53 mRNA after 12 hours of treatment. Similarly, the treatment significantly ($p<0.05$) upregulated the expressions of cyclins A, D and E mRNAs at 12 and 24 h of treatment (Figure 6).

Protein expression studies

The analysis of the apoptotic pathway related proteins showed that clausenidin upregulated the expression of

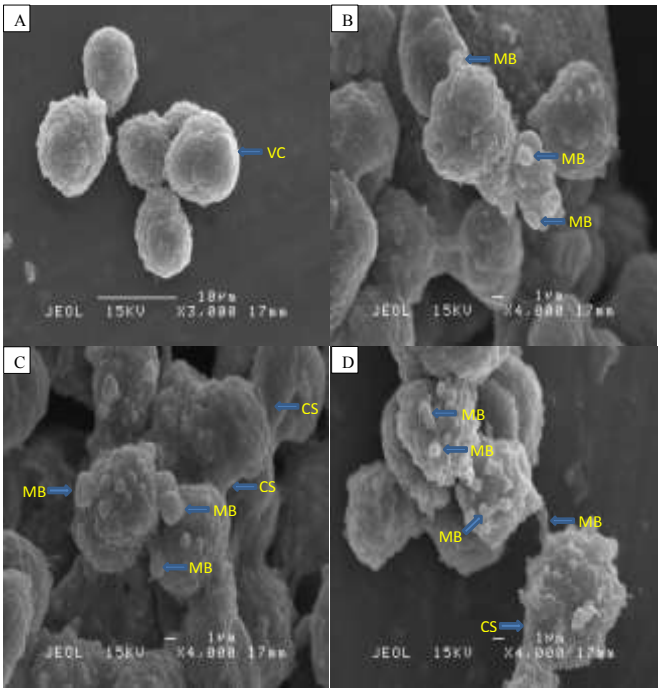

Figure 2. HT-29 cells treated with 13.8 µg/mL clausenidin at (B) 24, (C) 48 and (D) 72 h. (A) is the untreated control. VC=viable cells; CC=chromatin condensation; MB=membrane blebbing; CS=cytosolic shredding.

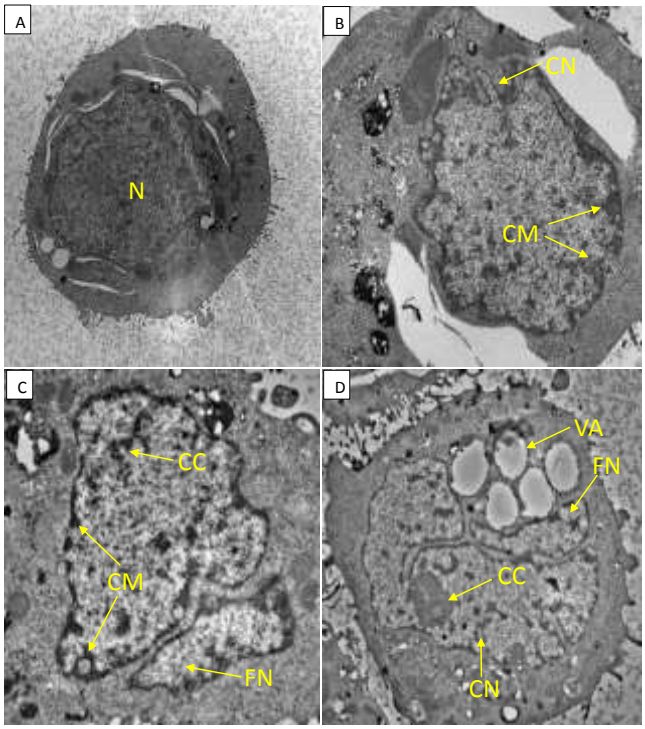

Figure 3. Clausenidin-treated HT-29 cells at (B) 24, (C) 48 and (D) 72 h. A is untreated controls. N=intact nucleus; VA=vacuole; CC=chromatin condensation; CM=nuclear chromatin margination; CN=nuclear convolution; FN=nuclear fragmentation (Magnification × 5000).

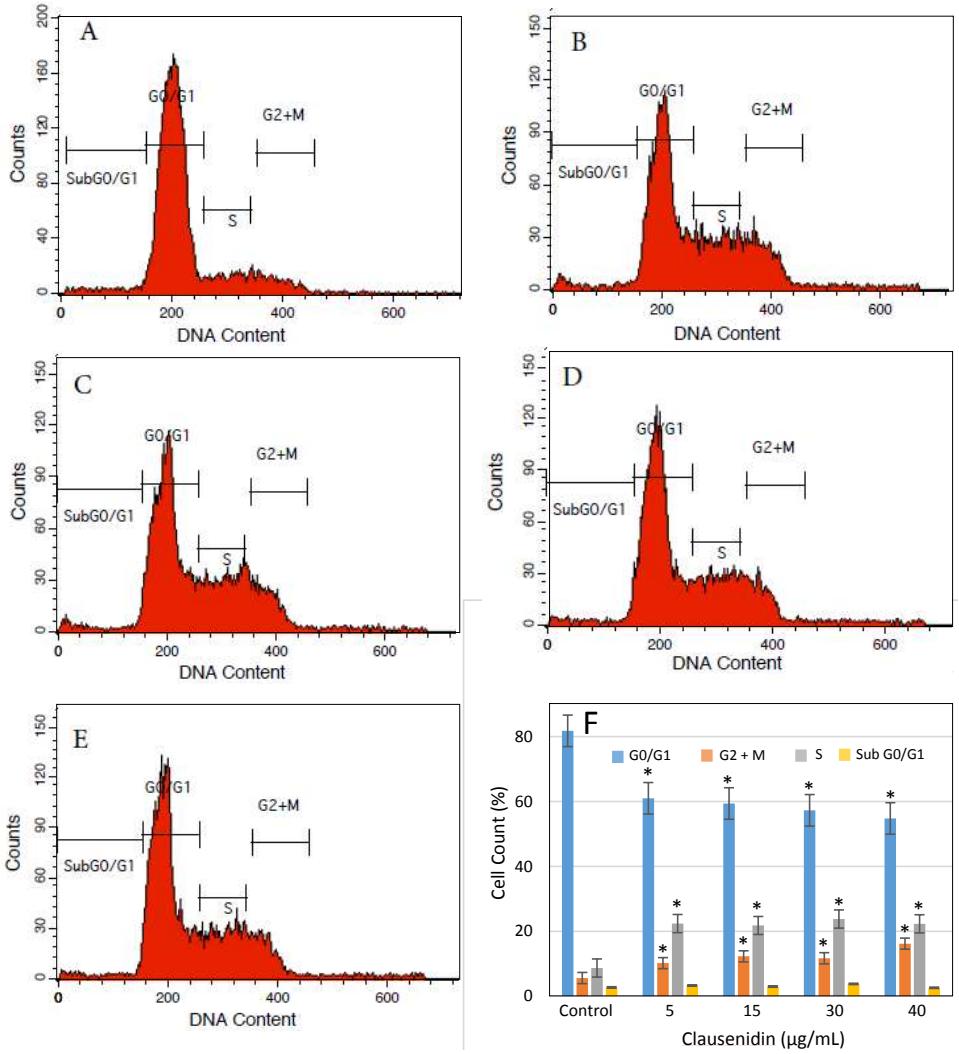

Figure 4. DNA content of HT-29 cells treated with clausenidin at (B) 5, (C) 15, (D) 30 and (E) 40 µg/mL. (A) is untreated control. (F) Distribution of cells in cell cycle phases. *Means significantly ($p<0.05$) different from control.

the pro-apoptotic proteins and down-regulated the expression of the anti-apoptotic proteins (Table 2). The only exceptions are the anti-apoptotic proteins, HSP 60 and 70 that were upregulated following clausenidin treatment. More importantly, the upregulation of p53 and p27 were observed and this confirms the finding of out gene expression analysis. In addition, the Western blot analysis was performed to further validate the findings of the protein profile array. It was observed that clausenidin significantly increased and decreased the expression of the pro-apoptotic bax, and JNK, and anti-apoptotic, bcl 2 proteins respectively (Figure 7).

DISCUSSION

The traditional use of *Clausena excavata* and its compounds for the treatment of cancer is based on Asian folklore that is devoid of scientific evidence. Previous study reported the anti-tumor properties of clausenidin isolated from the root of *C. excavata* (Waziri et al., 2016). However, the detailed mechanism of anti-cancer cell effect of clausenidin is poorly understood. The current study investigates apoptosis mediated by p53 in clausenidin-treated HT-29 cells.

Apoptosis is a coordinated cell death process that is accompanied by changes in the morphology of tumor cells that affects both cytoplasm and nucleus. This process usually takes several hours depending on the cell type and initiating apoptotic stimuli (Häcker, 2000), and begins with chromatin condensation and fragmentation of the nucleus as well as cellular shrinkage (Kroemer et al., 2005). Furthermore, the chromatin moves to the margin or periphery of the nucleus and condenses further until it breaks up inside into smaller materials (Majno and Joris, 1995). Our TEM micrographs

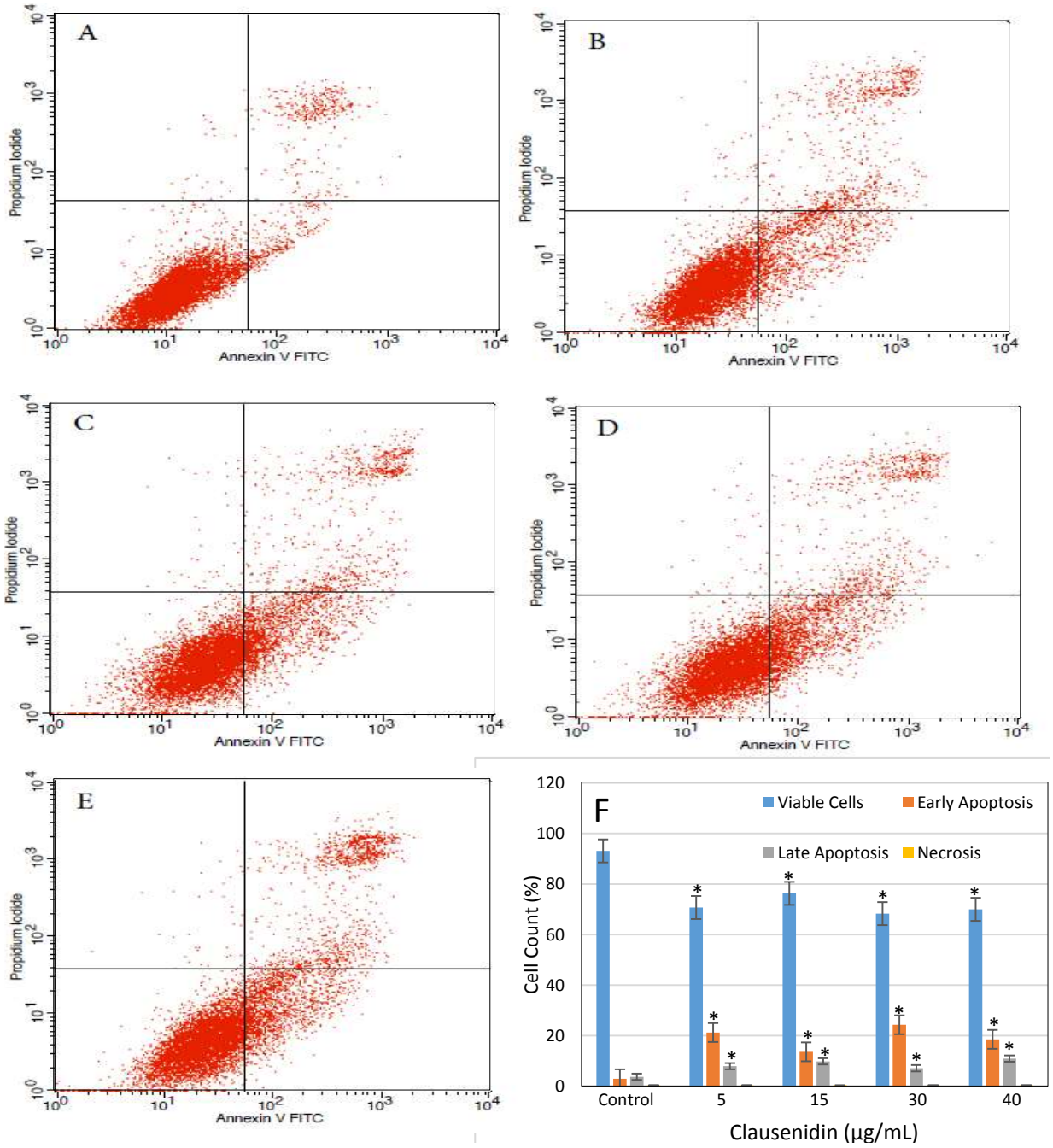

Figure 5. Annexin V assay of HT-29 cells treated with clausenidin at concentrations of (B) 5, (C) 15, (D) 30 and (E) 40 µg/mL. (A) Untreated control. (F) Distribution of cells at different stages of viability. *Means significantly ($p<0.05$) different from control.

material to the margins of the reveals the migration of the condensed chromatin nucleus. Also, the fragmentation of the nucleus progressed with time reaching its peak at 72 h. In addition, the SEM micrograph showed membrane blebs and cytosolic destruction of the clausenidin-treated HT-29 cells. Membrane blebbing, cytosolic damage and loss of membrane integrity are events that characterize the latter stages of apoptosis (Kroemer et al., 2005).

P53 is a tumor suppressor that promotes apoptosis via transcription-dependent or -independent mechanisms in cells (Fridman and Lowe, 2003). The loss of the p53 gene function contributes to carcinogenesis and tumor cell survival (Béroud and Soussi, 2003; Hussain and Harris, 1998). Among the major functions of p53 are the regulation of cell cycle check point and the induction of cell cycle arrest. The lack of cell cycle regulation

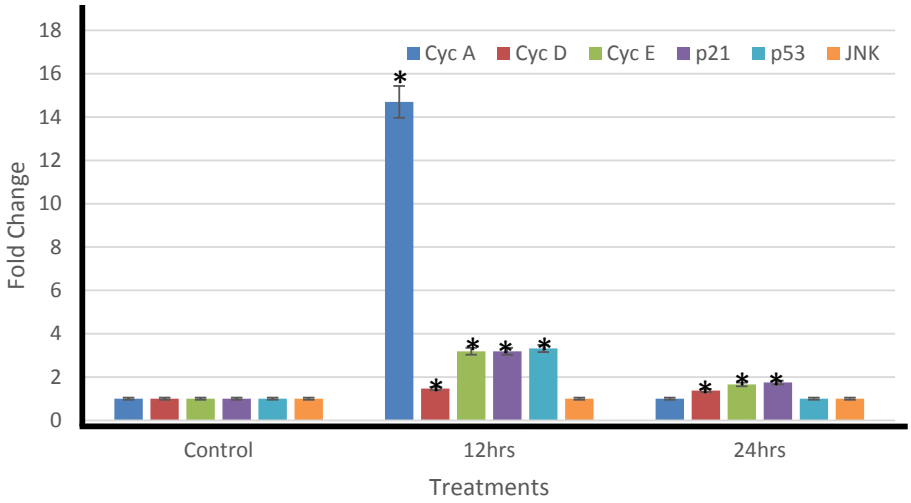

Figure 6. Gene expression in clausenidin-treated HT-29 cells. *Means significantly different from control.

Table 2. Analysis of apoptosis pathway-related proteins in clausenidin-treated HT-29 cells.

Protein	Fold change
Upregulation	
P53	2.19
P27	1.37
Bax	6.20
Bid	65.98
Bim	5.21
Casp 3	21.7
HSP 60	10.06
HSP 70	2215.98
SMAC	192.88
TNF-α	3.43
TNF-β	5.31
Downregulation	
Bcl-2	-91.38
Bcl-w	-57.38
HSP 27	-21.0
Survivin	-2.08

culminates in the development of cancers (Hartwell and Kastan, 1994). In this study, clausenidin affected the progression of cell cycle by causing a G0/G1 cell cycle arrest in the treated HT-29 cells. The induction of cell cycle arrest is an indicator of apoptosis (Kummalue et al., 2007). On the other hand, the activation of p21 gene by p53 comprises one of the main surveillance mechanisms in cell cycle regulation (El-Deiry et al., 1994). For the cell cycle to progress from G1 to S phase, there must be accumulations of cyclins A, D and E that activate the cyclin dependent kinases (CDKs) (Sherr, 1996). The p53 gene transactivates target genes like p21, which is an inhibitor of cell cycle. Increased expressions in the p53 and p21 mRNAs were observed in the clausenidin-treated HT-29 cells. At the same time, there were also increased expressions of the cyclins A, D and E in the treated cells. The upregulation of the cyclins in the clausenidin-treated HT-29 cells was countered by the significant upregulation of p53 and p21 genes that prevented the progression of the cell cycle from G1 to S phase. However, based on the findings, it was postulated that one of the mechanism of action of clausenidin on the HT-29 cells is through the activation of p53 that increased expression of p21 leading to G0/G1 phase cell cycle

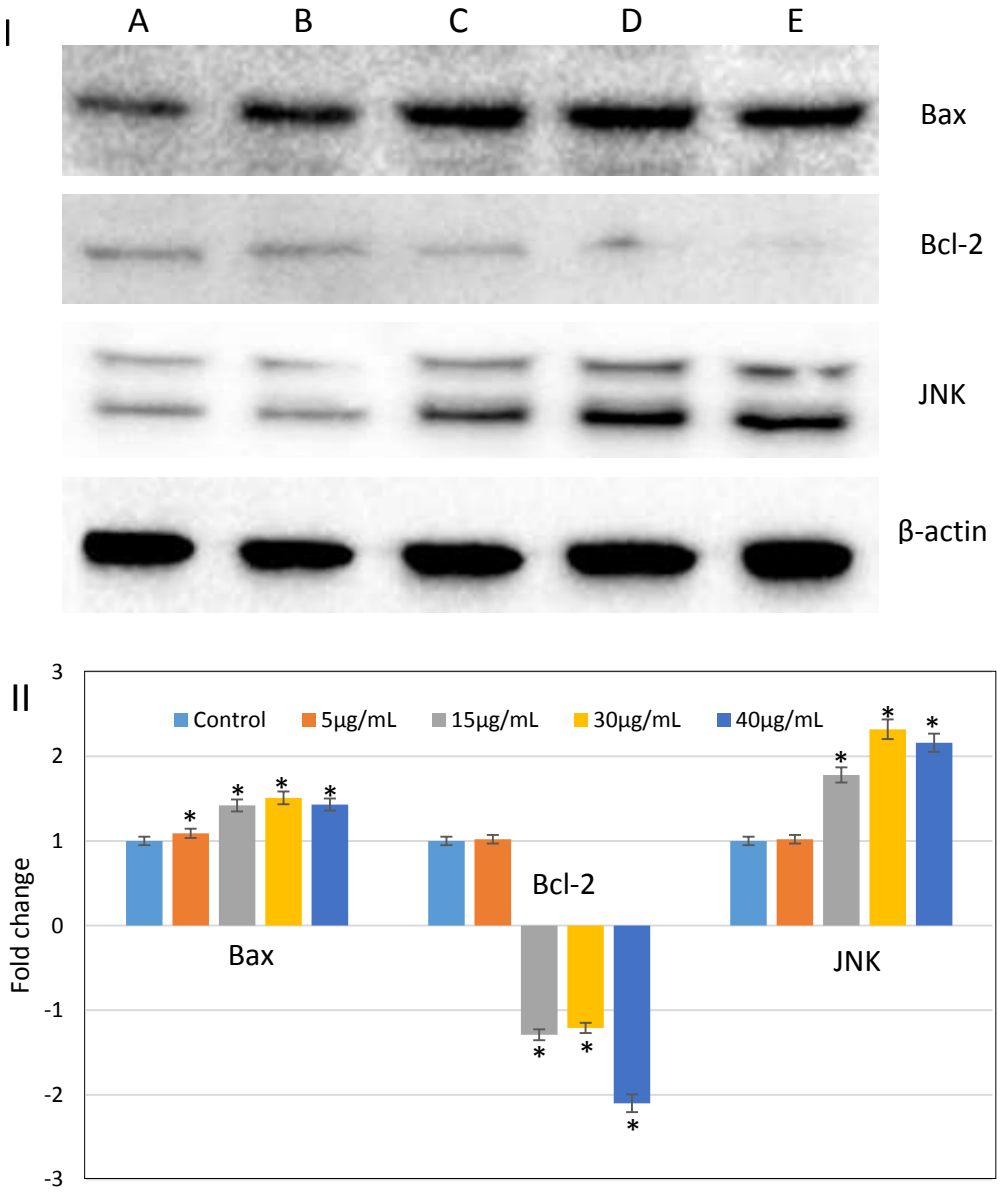

Figure 7. Protein expression in HT-29 cells after treatment with various concentrations of clausenidin. (I) Western blot; (II) Relative protein expression. *Means significantly ($p<0.05$) different from control.

arrest. Similarly, p53 transactivates the pro-apoptotic members of the bcl 2 family, bax (Miyashita et al., 1994) and bid (Sax et al., 2002). In this study, significant expressions of bax and bid in clausenidin-treated HT-29 cells that increased the ratio of pro- to anti-apoptotic bcl 2 proteins and subsequent activation of apoptosis was observed. The ultra-structural micrographs observed in this study confirm the execution of apoptosis in the clausenidin-treated HT-29 cells. More so, p53 is known to prevent the expression of cell survival factors and inhibitors of apoptosis (IAPs). The anti-apoptotic protein, survivin is another target of p53 that encodes an IAP, which promotes cell survival (Ambrosini et al., 1997). Clausenidin treatment caused a decreased expression of survivin in HT-29 cells which we presumed to be mediated by the significant expressions of p53. Furthermore, decreased expressions of the anti-apoptotic proteins, bcl 2, bcl w and HSP 27 were observed which we suspected may have been triggered by p53 in the clausenidin treated HT-29 cells. The non-transcriptional roles of p53 involves mitochondrial activity, eviction of cytochrome c and the activation of caspases among others (Mihara et al., 2003). This was observed in our previous study on clausenidin-treated HT-29 cells.

In conclusion, the current study suggests that bax, survivin and p21 are the main effectors of p53 mediated

apoptosis in clausenidin-treated HT-29 cells. Furthermore, the current study provides more insight on the anti-cancer cell effect of clausenidin isolated from the roots of *C. excavata*.

ABBREVIATIONS

P53, Tumor suppressor protein 53; p21, tumor suppressor protein 21; CDK, Cyclin dependent kinase; **DMSO,** dimethyl sulfoxide; **DMEM,** Dulbecco's Modified Eagle's Medium; **HT-29,** Human colorectal adenocarcinoma cell line; **mRNA,** messenger RNA; **DNA,** deoxyribonucleic acid; **JNK,** c-Jun N-terminal kinases; **SEM,** scanning electron microscopy; **TEM,** transmission electron microscopy.

CONFLICT OF INTEREST

The authors declare no conflict of interests.

ACKNOWLEDGEMENT

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Full Length Research Paper

Quali-quantitative characterization of the honey from *Myracrodruon urundeuva allemão* (Anacardiceae - Aroeira): macroscopic, microscopic, physico-chemical and microbiological parameters

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The objective of the present study was to evaluate the external and organoleptic characters, microscopic, physico-chemical, chromatic and microbiological parameters of the honey from *Myracrodruon urundeuva allemão* (Anacardiceae - Aroeira) of the Jequitinhonha River Valley / Brazil, in order to create its geographical indication. In the first stage a data survey was carried out to characterize the production of honey in the Jequitinhonha Valley. In order to evaluate the use of land as well as the forms of agriculture in relation to beekeeping. For characterization of the aroeira honey of the area, the microscopic, physico-chemical and microbiological quality of the honey was evaluated in samples collected in AAPIVAJE. Samples were collected for convenience. Honey production did not correlate with the area occupied, in hectare, by none of the types of land use in the middle region of the Jequitinhonha River Valley. There was also no significant correlation between honey production and the form of agriculture. In general, all samples of honey analyzed had satisfactory physico-chemical characteristics in terms of parameters of sugars, moisture, ashes, pH, acidity, color, taste and consistency. The analyzed samples were free of commercial sugar and dyes and present diastase activity. In this study we can see that all honey samples had a low microbial count of coliforms at 30°C and coliforms at 45°C (thermotolerant), indicating good hygienic-sanitary quality of the honey extraction and processing procedures. In the same way, it was observed that the counts of fungi and yeasts of all the honey were within the recommended parameters in the legislation of Apis. The presence of *Salmonella* was not detected in any sample of aroeira honey. This study will contribute to the geographical indication for aroeira honey, produced only in the middle region of the Jequitinhonha River Valley.

Key words: *Apis mellífera*, honey quality, sugars, diastase enzyme, moisture, ashes, acidity, microbial.

INTRODUCTION

Beekeeping is a source of work and income, mainly in rural property formed basically by family farming, making it a sustainable strategy for the small farmer. This activity, in addition to being profitable, is capable of promoting regional development, reducing rural exodus, while preserving the environment (Freitas et al., 2004; Kizilaslan and Kizilaslan, 2007).

The main products derived from the apicultural practice are honey, beeswax, propolis, royal jelly, pollen and apitoxin. Honey, possessing unique nutritional qualities and a huge market, is the most important (Ozcan and Olmez, 2014). Honey is a complex natural product produced by bees from flower nectar, tree and plant secretions or exudates from plant-sucking insects (Pires et al., 2009; Feás et al., 2010; Ozcan and Olmez, 2014). This product is increasingly valued by consumers and the food industry because of its strong and characteristic taste (Castro-Vazquez et al., 2006; Jerkovic et al., 2010).

The growing demand for honey in many countries in the world requires its diversification of other types of honey as a response to the consumer request (Simova et al., 2012). Therefore, the determination of botanical essences of apicultural interest as an influencer of honey characteristics becomes essential, since the specific composition may depend on the diversity of flower sources (Anjos et al., 2015; Laallam et al., 2015).

In the Jequitinhonha River Valley, little attention has been focused on beekeeping and the various components of beekeeping efficiency despite the availability of a number of techniques to stimulate efficiency components in production units. In this region, the production of honey has presented very marked fluctuations over the decades. Factors related to climate, diseases of bees, incentives of prices paid in production and development of technologies associated with the production process are some causes that determine the success or failure of the harvest. Besides that, the vegetation this region is threatened by economic activity and therefore beekeeping represents a sustainable alternative income.

For beekeeping to develop in a sustainable way, it is essential, among other needs, to know the flora that provides resources to bees. Aroeira (*Miracrodruon urundeuva allemão*) is a native tree from the Jequitinhonha region and other regions of Brazil, which has excellent characteristics for apiculture pasture, releasing its flowering from May to July. Generally, at this time the aroeira is one of the few sources of nectar (Nunes et al., 2008). The predominance of *M. urundeuva* pollen grains indicates that this specie is the main nectar source for *Apis mellífera* in this Brazilian Dry Forest during the hyper dry period (Bastos et al., 2016).

Despite the empirical knowledge of beekeepers about the potential of Aroeira, there is no study that actually proves their real qualitative and quantitative importance for beekeeping activity. In view of the above, and knowing that the quality control analyses are extremely important in the evaluation of origin, quality, adulteration, storage conditions, and contamination of honey, the objective of the present study was to evaluate the external and organoleptic characters, microscopic, physico-chemical, chromatic and microbiological parameters of the honey from *M. urundeuva allemão* (Anacardiceae - Aroeira) of the Jequitinhonha River Valley/Brazil, in order to create its Geographical Indication.

MATERIALS AND METHODS

Data collection on honey productivity

In this stage a data survey was carried out to characterize the production of honey in the Jequitinhonha River Valley. The Jequitinhonha River Valley region is located northeast of Minas Gerais and is cut by the Jequitinhonha River and its main tributary is the Araçuaí River. The region is divided into Upper, Middle and Lower Jequitinhonha. The relief is formed by large flat plateaus and deep caves. The climate is semi-arid with predominance of the Caatinga, Cerrado and remnants of Atlantic Forest. The average temperature is between 21 and 24°C, with annual rainfall below 1,000 mm (Diniz et al., 2001).

The territory of the middle region of the Jequitinhonha River Valley covers 18 municipalities: Águas Vermelhas, Araçuaí, Berilo, Cachoeira do Pajeú, Chapada do Norte, Comercinho, Coronel Murta, Francisco Badaró, Itaobim, Itinga, Jenipapo de Minas, José Gonçalves de Minas, Medina, Padre Paraiso, Pedra Azul, Ponto dos Volantes and Virgem da Lapa. The population in this part of Jequitinhonha valley is 279,326 inhabitants (IBGE, 2010). The data collected at this stage of the work were related to the municipalities of Francisco Badaró, Jenipapo de Minas, Berilo, Araçuaí and Chapada do Norte. Information about the production of honey specific for aroeira flowers were extracted from the database of – the Association of Beekeepers of the Jequitinhonha Valley (AAPIVAJE), from March to September, 2017.

In order to evaluate the use of land as well as the forms of agriculture (family and non-family) in relation to beekeeping, the Brazilian Statistic and Geographic Institute (IBGE) secondary database was used for the agricultural sense of 2006. The area occupied by the landscape units: pasture, matas and tillage were quantified in hectare and in % and related to the honey production (kg) of each municipality in 2006.

Characterization of the aroeira honey of the Jequitinhonha River Valley

In this stage of the work the microscopic, physical-chemical and microbiological quality of the honey was evaluated in samples collected in AAPIVAJE, from the five municipalities that were part of

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Table 1. Description of the samples analyzed in this study.

Sample	Flowering	Municipality
1	Aroeira	Araçuaí
2	Aroeira	Berilo
3	Aroeira	Berilo
4	Aroeira	Francisco Badaró
5	Aroeira	Francisco Badaró
6	Aroeira	Berilo
7	Aroeira	Francisco Badaró
8	Aroeira	Berilo
9	Aroeira	Jenipapo de Minas
10	Aroeira	Virgem da Lapa

the study. Samples were collected for convenience, depending on the supply availability of the combination. In this way we were able to collect the following samples in Table 1. The analyses performed in the present study are described as follows:

i) External and organoleptic characters and microscopic analyzes

The color classification of the honey was carried out in a spectrophotometer, which will consist of a reading at 560 nm (Abs560) using pure glycerine as white. The reading was later transformed into color expressed in millimeters (mm) by the Pfund scale (BRASIL, 1985). The color of the honey is variable, depending on its composition the darker (coffee color) richer in minerals and stronger is its flavor; the clearer, the poorer in minerals, and may even appear almost colorless, in which case the taste is mild (Crane, 1996). Thus, the taste of the samples was classified as strong or soft. The consistency of the honey was classified as liquid, liquid-crystallized, liquid-granulated, crystallized, granulated and creamy (Couto, 2002). Microscopically, the honey samples were analyzed under optical microscope at the Laboratory of Chemistry of the IFNMG - Campus Araçuaí. One drop of honey was deposited between the slide and the coverslip and observed in a 10x and 40x objective, to investigate dirt and foreign material.

ii) Pollen analysis

Samples of aroeira honey were sent to a reference laboratory for analysis of pollen that could prove the presence of the respective flowers in the product.

iii) Physical-chemical and chromatic analysis

The physico-chemical analyses included among the maturity indicators of honey: moisture; indicators of honey deterioration: pH and acidity; and sensory characteristics: color, taste and consistency. All analyzes were performed in triplicate, following the methods recommended by the Brazilian legislation (BRASIL, 2000). The procedures used were in accordance with the methodology of the Association of Official Analytical Chemists (AOAC, 1998). All the physical-chemical analyzes carried out are as follows:

(a) Moisture: The determination of the moisture of the samples was performed by the refractometric method. An Abbe benchtop refractometer was used. The measurement of the refractive index (IR) of the sample was converted to a percentage of moisture, based on the relationship between the refractive index and the moisture (%) of the honey.

(b) Determination of ashes: The ashes were determined by weighing about 10 g of honey in a tared porcelain capsule. The honey was carefully heated in flame until the swelling ceased, taking care to avoid projecting droplets. The sample was then incinerated at 450°C until white residue was obtained (about three hours) (UFPR - Laboratory of bromatology).

(c) pH and Acidity: A pre-calibrated digital pH (microprocessor / DLA-pH) pH was used for pH measurement. The method of measuring the acidity of honey was based on the determination of free, lactonic and total acidity, with the aid of pH meter. The free acidity was the measurement obtained from titration with sodium hydroxide (0.05 N) to the equivalence point (pH 8.5). The lactonic acidity was obtained by the addition of 10 ml of sodium hydroxide, later titrated with hydrochloric acid. Total acidity was the sum between free acidity and lactonic acidity. 10 ml of honey (beaker) were weighed and 75 ml of CO_2 free water was added, and the pH of this solution was checked. It was titrated with stirring of the solution with a magnetic stirrer and electrode dipped in the solution (honey + water), initially with sodium hydroxide (NaOH) at a rate of 5.0 ml per minute in the solution to pH 8.5 (free acidity). 10 ml of NaOH was quickly added to the solution (honey + water). The last titration was with hydrochloric acid (HCL) until it reached pH 8.3 (lactonic acidity). For the purposes of calculations and corrections it was necessary to prepare the blank, which consisted in measuring the pH of the distilled water and titrating with NaOH up to pH 8.5 (AOAC, 1998).

Calculation of acidity: Free acidity = (corrected NaOH volume corrected - white) × 50 × (correction factor/sample weight)
Lactone acidity = (10 - corrected HCl volume) × 50 ×(correction factor/sample weight)

(d) Sugar detection: Honey samples were sent to a reference laboratory for the analysis of non-reducing sugars (in sucrose) and reducing sugars (in glucose). The methodology used followed what is recommended by Ministry of Agriculture, Livestock and Food Supply (MAPA) - Administrative Rule No. 1 of 07/10/1981 (BRASIL, 1981).

In order to verify a possible addition of commercial sugar to honey, the Jagerschmidt reaction was performed. To begin this analysis, about 10 g of honey and 10 ml of acetone were crushed in porcelain grains. After decanting the solvent, about 2-3 ml was transferred into a test tube containing equal volume of concentrated HCl. And finally, the mixture was cooled in an ice bath or running water. The appearance of strong violet color indicates the presence of commercial sugar. If honey is natural product, a slight amber coloration may appear that turns to violet after some time

(Department of Health/SC, 1985).

(e) Research of diastase enzyme: Another way of verifying adulteration in honey was through the diastase enzyme research. In this analysis, 1 g of honey was dissolved in 20 ml of distilled water previously boiled and cooled to 45°C. In a pre-washed test tube with boiled water, 10 ml of the honey solution (unfiltered) was added and then 1 ml of freshly prepared, clear, 1% solution of starch. The remaining 10 ml were stored in another blank test tube to be done at the end of the experiment.

Thereafter, the tube containing the starch solution was well shaken, leaving in a water bath at exactly 45°C for 1 h. A few drops of lugol solution were then added to the two tubes (blank and assay) and the color the liquid developed was observed. The expected coloration should be around a greenish or yellowish brown, proving the presence of diastase enzymes, natural in honey. If it causes a violet color or lack of expected color, this will indicate poor extraction conditions or fraud, respectively (Department of Health/SC, 1985).

(f) Search for dyes: To verify the presence of dyes in honey, 1 g of honey was weighed, dissolving it in 10 ml of distilled water. Then, about 2 ml of 5% sulfuric acid solution was added. The honey should remain unchanged. If there are coloring substances added to honey, the color gradually changes from violet to pink (Department of Health/SC, 1985).

iv) Microbiological analyses

Microbiological analysis of the research focused on the occurrence of *Salmonella* spp., the most probable number of coliforms at 35 and 45°C, which were based on the methodologies described in Normative Instruction No. 62 (BRASIL, 2003). For standard counts on filamentous fungi and yeast plaques, and identification of fungal species, the serial decimal dilution methodology described by Pitt and Hocking (2009) was followed. From each sample 25 g of honey were collected and weighed aseptically and added to 225.0 mL of 0.1% peptone saline, thereby obtaining an initial dilution of 10^{-1} and from that dilution dilutions were prepared to 10^{-3}.

(a) *Samonella* spp search: For the research of *Salmonella* spp. the pre-enrichment was done by transferring 25 ml of honey to 225 ml in lactose broth incubating at 35°C for 24 h. For the selective enrichment, the Rappaport-Vassiliadis broth and the cystine broth selenite were used, transferring 0.1 and 1.0 ml, respectively, being incubated at 45 and 35°C respectively. In isolation, Hektoen Enteric agar (HB), bismuth sulphite agar (BS) and deoxycholate-lysine-xylose agar (XLD) were used and the inocula were incubated at 37°C for 24 h. Characteristic colonies were transferred to the medium sugar-iron triple agar and lysine-iron agar for preliminary biochemical characterization.

(b) Most probable number of coliforms at 35 and 45°C: The determination of total coliforms will be performed by the multi-tube fermentation method; using series of three tubes in the presumptive procedures by inoculating 1.0 ml of each dilution in the Lauryl Sulfate Triptose broth (LST) and the brilliant green bile lactose broth (BGBL) a for the confirmatory tests, with incubation at 36.0 ± 1°C for 24 to 48 h. Confirmation of the presence of coliforms at 45°C will be performed by inoculating the suspect colonies in EC broth and subsequent incubation at a selective temperature of 45 ± 0.2°C in a water bath with constant shaking for 24 h.

(c) Filamentous fungi and yeast counts: The count of filamentous fungi and yeasts was performed according to serial decimal dilution methodology described by Pitt and Hocking (2009). 25 g of the sample was homogenized in 225 ml of 0.1% peptone water. From this initial dilution (10^{-1}) serial decimal dilutions were prepared up to 10^{-3}. The inoculums were 0.1 ml aliquots per petri dish on the surface of the culture medium (in duplicate) potato dextrose agar (PDA), of each dilution, used for general counting (King et al., 1979). BDA plates were incubated at 25°C for seven days in the absence of light. All plaques were observed, being selected those that presented UFC.g-1 around 10 to 100 (Dalcero et al., 1997, 1998).

Statistical analyses

A preliminary exploratory and descriptive research was carried out to collect data on honey production in the middle region of the Jequitinhonha River Valley, as well as the microscopic, physico-chemical and microbiological characterization of honey produced in the region. The statistical analysis used to evaluate the relationship between honey production and land use and form of agriculture was the Pearson correlation, due to the data presented in the Normal distribution, in which each type of land use or form of agriculture was correlated with the production of honey. The significance level (alpha) considered was 5%. For these analyses, we used the program SPSS®, version 13.0, for Windows.

RESULTS

Honey productivity and relation to land use and forms of agriculture with beekeeping activity

It is verified that the pasture occupation was bigger in all municipalities (Figure 1A), pointing out the more recent tendencies of development and replacement of traditional activities by cattle raising with the use of native and artificial pastures. The occupation by forests and / or forests was lower in the municipality of Jenipapo de Minas, although this represents 21.10% of the total landscape, very similar to the municipality of Virgem da Lapa, which presented a greater occupation by forests and / or forests. Araçuaí presented the largest area occupied by forests and / or forests, represented by 32.99% of the total landscape. The occupation by crops and agroforestry varied among the municipalities, being the municipality Francisco Badaró with less occupation by agroforest, in absolute numbers and percentages, and the Araçuaí municipality with greater area occupied by agriculture, representing 10.33% of the total landscape (Figure 1A).

Honey production did not correlate with the area occupied, in hectare, by none of the types of land use in the middle region of the Jequitinhonha River Valley. The p values were greater than 0.05 under all conditions.

In the same way that the correlation of honey production with the occupation of the area in hectare occurred, also occurred with the occupation of the area in percentage. No significant correlation was observed ($p>0.05$).

Regarding the form of agriculture in the five municipalities contemplated in this study, family agriculture is predominant in all municipalities, with the largest area occupied by family agriculture located in the Araçuaí municipality and the smallest in the municipality

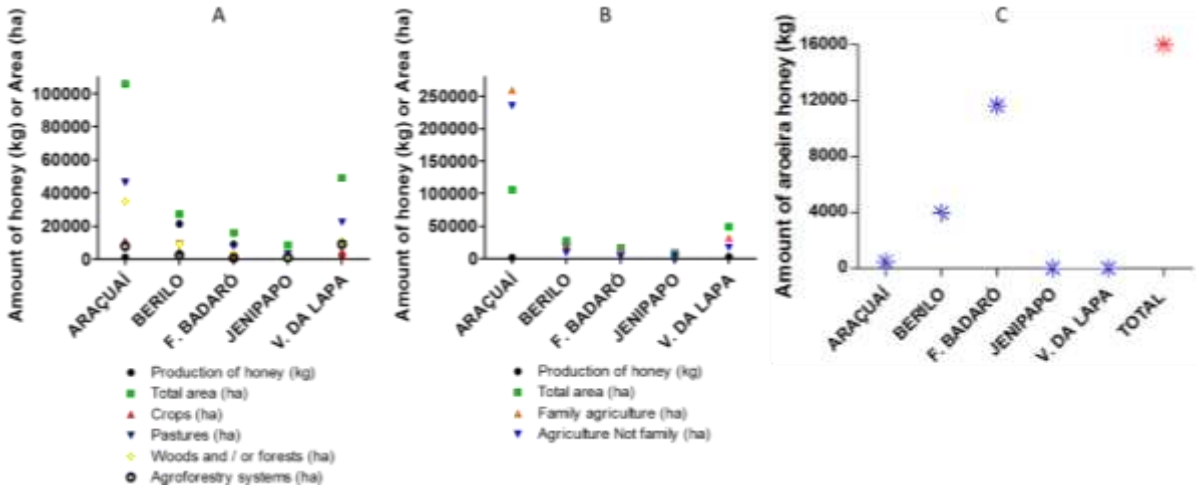

Figure 1. Forms of land use (ha) and honey production in 2006 in the five sampled municipalities of Jequitinhonha River Valley (A). Form of agriculture (ha) and honey production in 2006 in the five sampled municipalities of Jequitinhonha River Valley (B). Production of honey delivered to the association of beekeepers in the five sampled municipalities of the Jequitinhonha River Valley from March to September 2017 (C).

of Jenipapo de Minas. These being the municipalities that present the largest and smallest territorial extension, respectively. There was also no significant correlation between honey production and the form of agriculture ($p>0.05$) (Figure 1B). The production of honey delivered to the association of beekeepers was expressive in the five sampled municipalities of the middle region of the Jequitinhonha River Valley, from March to September 2017, with the production of honey of aroeira reaching 16 tons (Figure 1C).

Microscopic characterization and qualitative analysis of pollen

All samples were free from foreign matter as recommended by current legislation. The standards of honey identity and quality (BRASIL, 2000) require, for macroscopic and microscopic aspects, that the product be free of foreign substances of any nature, such as: insects, larvae, grains of sand and others. Samples of aroeira honey registered in the laboratory under the number of Reg. 17.5806 showed a positive pollen result for the aroeira flowering analyzed (Test code = 093).

External and organoleptic characterization of honey samples

As to the color of the honey samples, it was observed that the majority presented a dark amber coloration, with absorbance values at 560 nm ranging from 0.3876 to 1.8252, corresponding to light amber and dark amber, respectively. The flavors and consistencies of the honey samples were in accordance with the characteristics of honey from aroeira (Table 2).

Physico-chemical and chromatic characterization of aroeira honey

The results of the physical and chemical analyzes were expressed by means of the triplicates and compared to the values suggested by Normative Instruction No. 11 of the Ministry of Agriculture and Supply (BRASIL, 2000), when possible.

In general, all samples of honey analyzed had satisfactory physico-chemical and chromatic characteristics (Figures 2 to 4). As observed in Figure 2A, all samples of aroeira honey presented values of moisture within normality, that is, not exceed the limits recommended by the current legislation. The maximum moisture content for honey samples, considering the parameters established by the Brazilian Ministry of Agriculture and Supply is 20% (m/m). Implicating results within the law for samples of honey of aroeira, whose maximum value of moisture reached 16% (Figure 2A).

Soluble solids correspond to all substances that are dissolved in a given solvent. They are mainly composed of sugars, variable with the plant species and climate. They are designated as Brix and tend to increase with maturation. In honey, the soluble solids content is very close to the total sugars content, which makes this simple and economical technique very useful (Gois et al., 2013).

In this study the soluble solids content was between 80 and 90% (Figure 2B), values considered normal in accordance with regulation N° 11 of 2000 (Ministry of Agriculture, Livestock and Food Supply - Brazil), which

Table 2. Classification of color, taste and consistency of aroeira honey.

Sample	Absorbance at 560 nm	Color	Taste	Consistency
Aroeira 1	0.6669	Amber	Strong	Liquid
Aroeira 2	1.8025	Dark amber	Strong	Liquid
Aroeira 3	1.733	Dark amber	Strong	Liquid
Aroeira 4	1.4045	Dark amber	Strong	Liquid
Aroeira 5	1.6872	Dark amber	Strong	Liquid
Aroeira 6	1.6777	Dark amber	Strong	Liquid
Aroeira 7	1.8252	Dark amber	Strong	Liquid
Aroeira 8	1.7986	Dark amber	Strong	Liquid
Aroeira 9	1.7615	Dark amber	Strong	Liquid
Aroeira 10	0.3876	Light amber	Soft	Liquid

establishes a minimum of 65% for reducing sugars and 6% for sucrose (BRASIL, 2000).

The ash content indicates the amount of minerals found in the honey, determining its color. In this study the ash content varied from 0.21 to 0.37% (Figure 2C), which are values below the maximum limit (0.6%) determined by the Ministry of Agriculture, Livestock and Supply. The color of the honey was not altered when performing dye research, as shown in Figure 2D. This indicates that the honey samples were free of the addition of these compounds.

Although it is not mandatory, by official legislation, the pH analysis as indicative of the physico-chemical quality of the honey, it was performed as a complementary parameter for the evaluation of the total acidity. pH values ranged from 3.86 to 4.55 (Figure 2C). The value for free acidity varied from 17.32 to 30.64 Meq.kg $^{-1}$. Therefore, no sample was found to be in disagreement with the current legislation (Figure 2D). The Brazilian legislation accepts maximum acidity of 50 Meq.kg $^{-1}$ of honey (BRASIL, 2000). Codex and the European Union also determine a maximum acidity of 50 Meq.kg $^{-1}$, but specify that the parameter to be measured is free acidity (CAC, 2001; UNIÃO-EUROPÉIA, 2001). For MERCOSUL the maximum acidity limit is 40 Meq.kg $^{-1}$ (MERCOSUL, 1999). The content of individual sugars such as glucose, fructose and sucrose are important when evaluating the degree of sweetness of products, since the sweetness of these products is varied and increases in the glucose sequence: sucrose: fructose (Chitarra and Chitarra, 2005). In the present study, the mean values obtained for reducing sugars and non - reducing sugars were 66.44% (65.40 - 67.90%) and 2.99% (2.00 - 5.30%) respectively (Figure 4A – B).

In the Jagerschmidt reaction there was no evidence of strong violet color in any of the samples, which indicates the presence of commercial sugar (Figure 4C). Diastase enzyme research revealed violet staining for one sample, and in the other samples yellow staining. The result of the violet sample indicates a decrease in the activity of the diastase enzymes, probably due to the heating of the honey. The yellow staining of the other samples indicates the presence of the diastase enzymes (Figure 4D).

In the correlation analysis performed between the physical-chemical parameters, a negative correlation was observed between moisture and free acidity (Figure 3 and Supplementary Table 1). The acidity of the honey is due to the amount of minerals and the variations of the organic acids. With the increase of the water content in the honey, the minerals and organic acids become more diluted, and therefore diminished. The correlation between ash content and honey color was not significant ($p = 0.123$), based on absorbance values at 560 nm. Most of the samples showed dark amber coloration and for this color the ash contents ranged from 0.22 to 0.37%. It is important to note that the samples that presented lighter coloring also had lower gray levels.

Microbiological characterization of honey

Microbiological quality may be associated with the hygienic conditions of food production, processing and handling. In this work we can see that all honey samples had a low microbial count of coliforms at 30°C and coliforms at 45°C (thermotolerant), indicating good hygienic-sanitary quality conditions (Table 3). In the same way, it was observed that the counts of fungi and yeasts of all the honey presented within the recommended in the legislation of Apis (BRASIL, 1985, 1997, 2003). The presence of Salmonella was not detected in any sample of aroeira honey (Table 3).

DISCUSSION

Beekeeping is considered an important activity for the agricultural sector at the national level, and in the Jequitinhonha River Valley it has been highlighted in recent years, due to the income opportunity given to small producers. Thus, in the Jequitinhonha River Valley, beekeeping is among the most promising economic

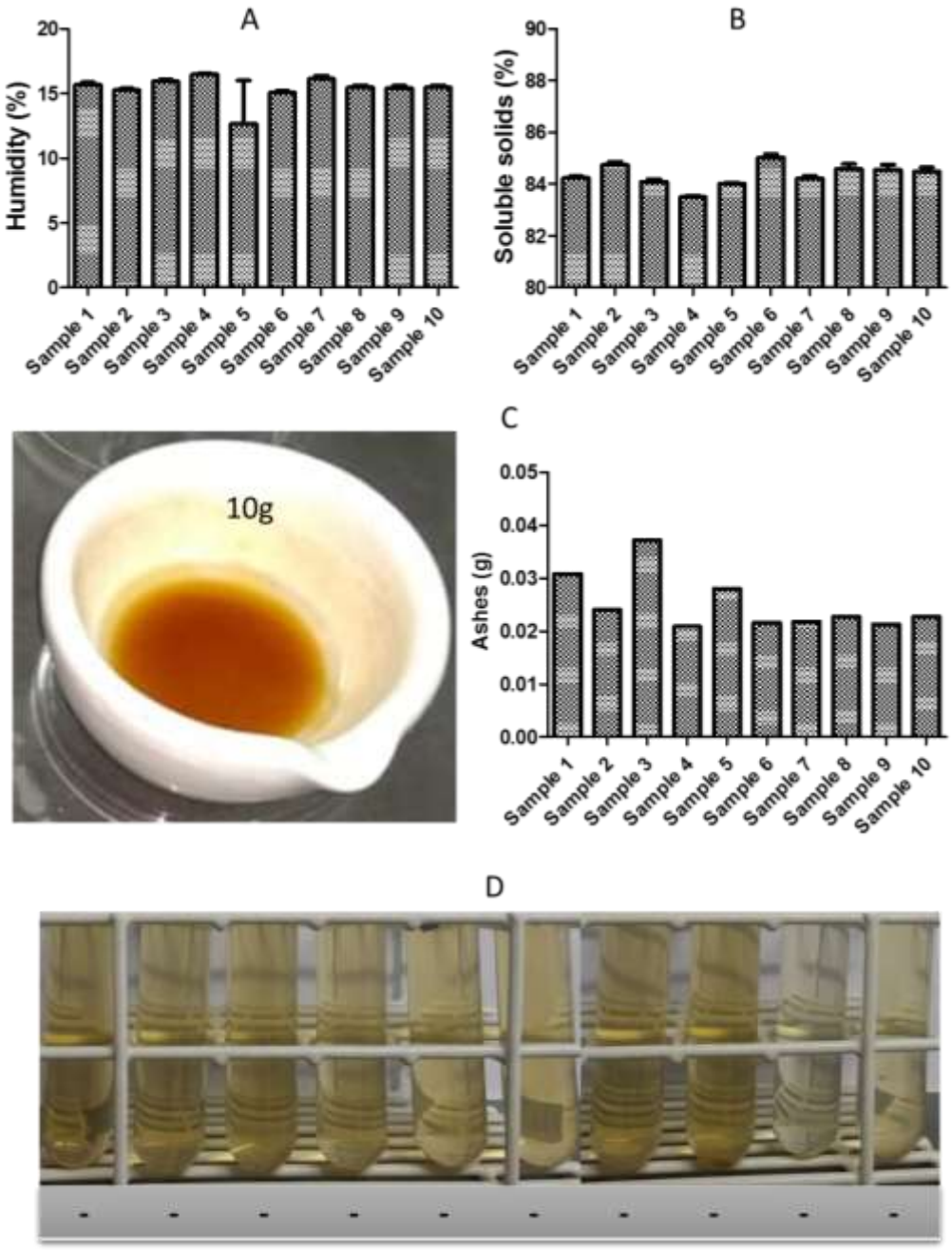

Figure 2. Physico-chemical parameters evaluated: Moisture (A), soluble solids (B) content of aroeira honey using the Chataway table. The values presented are triplicate averages. Ashes (C) and dye research (D) in aroeira honey.

activities in the region, especially due to favorable environmental conditions. However, the region has untapped beekeeping potential. In addition to the economic aspects, brazilian beekeeping, and thus the Jequitinhonha River Valley, meets some requirements that also credence as an activity with a high potential for social inclusion, considering the economic, social and environmental characteristics, which contributes to higher levels of profitability (Freitas et al., 2004).

In the present study we tried to investigate in which landscapes or in what form of agriculture the production of honey predominates. No significant correlation was found in the analyses. The absence of correlation between the production of honey and the landscape units may be due to the bees finding nectar resource in all landscape. In the same way, whether family farming or

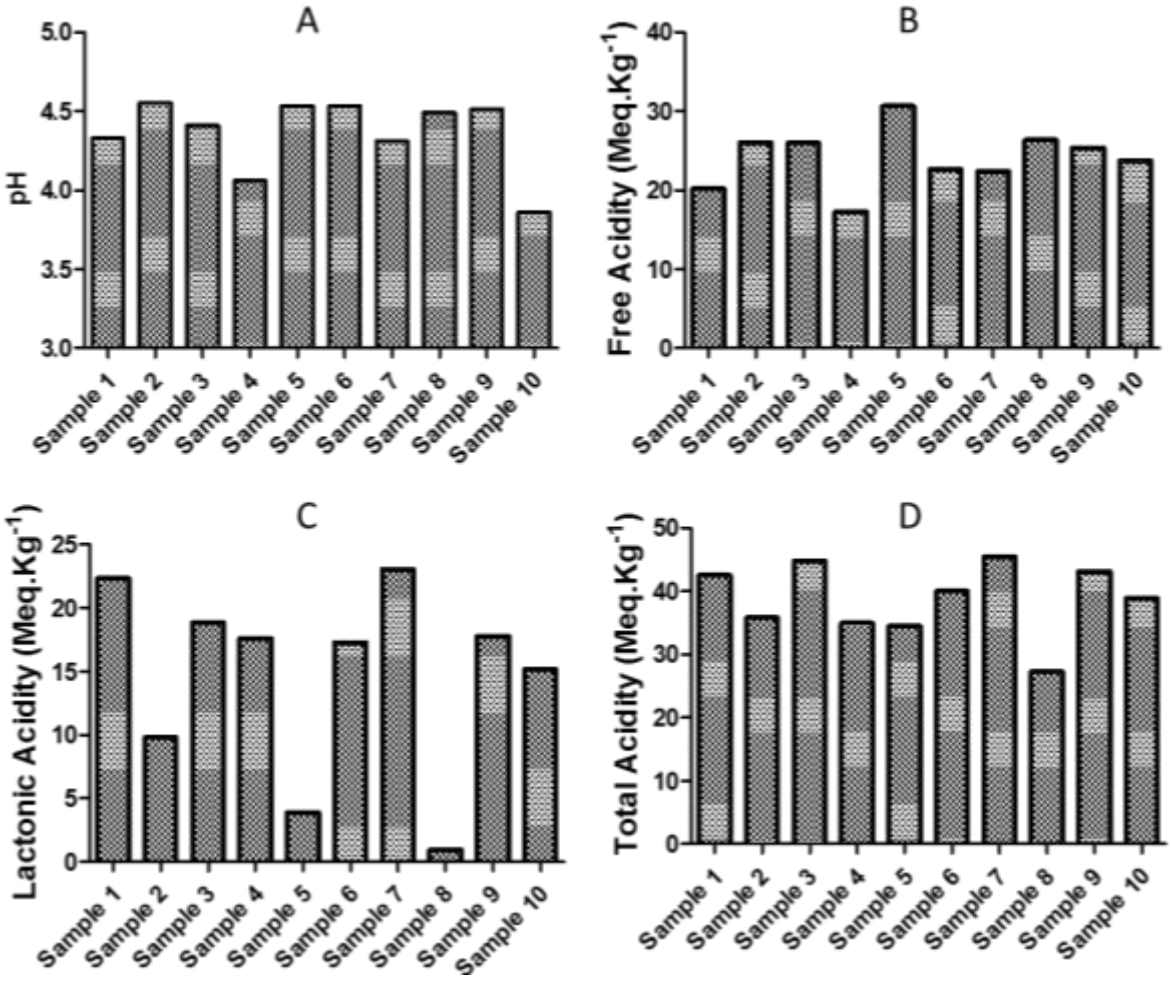

Figure 3. Physico-chemical parameters evaluated: pH (A), Free acidity (B), Lactonic acidity (C) and Total acidity (D) values of aroeira honey.

not, both are apt to the development of beekeeping fully. It is also worth mentioning that the honey of aroeira has found a prominent place in the production of honey in the region, due to the presence of the extensive forests of aroeira that beautify the region. In this sense, the quantity of producers, and consequently the production of aroeira honey has expanded in the region.

The honey produced in Brazilian Dry Forest, also present in the Jequitinhonha River Valley, is characterized as a *M. urundeuva* (aoriera) honey and in this biome, the scarcity of floral resources, associated with high temperatures and low humidity, induce bees to seek this massive food sources to ensure their food supply (Bastos et al., 2016). As an animal product, all honey marketed is subject to the standards foreseen by the legislation. Therefore, make sure that aroeira honey meets the requirements of the current standards, can be an attraction to the marketing of honey. In addition, demonstrating the benefits of aroeira honey can add value to the product, enhancing production in the region and promoting expansion. So, providing support for the creation of a standard identity and specific quality for aroeira honey is crucially increases knowledge of the product, and thus allows supervision by food control authorities which, in turn, guarantees the quality of the honey for consumers.

The flavor and aroma are directly linked to the honey color. The darker the honey, the richer in minerals, consequently stronger flavor and aroma, which was the case of the majority of honey samples evaluated in this study. The aroma and flavor of the honey is that of the original flower, it goes from sweet to sweet and strong and may have acid or bitter taste (Ajlouni and Sujirapinyokul, 2010). The consistency of the honey can be liquid, liquid-crystallized, liquid-granulated, crystallized, granulated and creamy. In the Brazilian market there is a greater trend of consumption of liquid honey, while in Europe, the most sought-after honey is the creamy honey, there are equipment to beat the honey to make it creamy and consequently light in color (Couto, 2002).

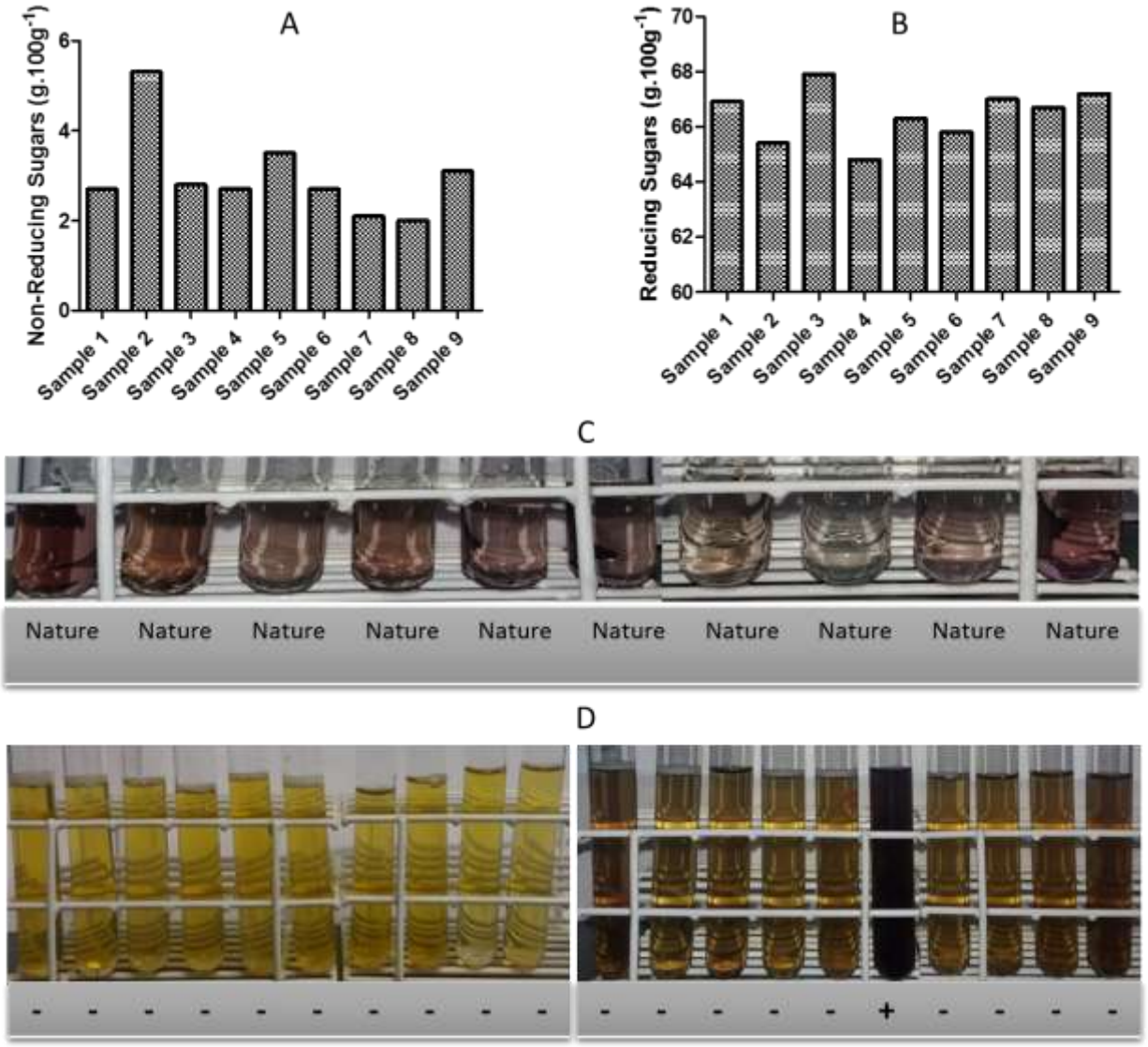

Figure 4. Physico-chemical and chromatic parameters evaluated: Detection of non-reducing sugars (in sucrose) (A), reducing sugars (in glucose) (B) in samples honey. Jagerschmidt reaction (C) and Diastase enzyme research (D) in aroeira honey.

Honey from aroeira usually presents in liquid form and is hardly presented in crystallized or granulated form.

In the physico-chemical analyzes of honey, it was verified that the moisture values of the samples, ranging from 15.07 to 16.47%, did not exceed the limits recommended by the current legislation (BRASIL, 2000). The water content in honey is one of the most important characteristics and constitutes the second component in quantity, varying according to the climate, floral origin and harvesting season. The moisture content is the main determinant of parameters such as viscosity, specific gravity, crystallization and flavor, besides being an important indicator of the fermentation tendency, mainly influencing the conservation of the product (Abramovic et al., 2008; Al-Ghamdi et al., 2017). In this way, it is evident that the conditions of processing and storage of the analyzed samples are adequate to guarantee this parameter.

Ash content provides important information about the quality of honey, as floral honey has lower ash content than honeydew honey (Almeida-Muradian et al., 2013). In the present study the ash content did not exceed 0.37%, indicating a good quality for the Jequitinhonha River Valley honey and also its floral origin. According to Brazilian legislation, the ash content in blossom honey should be at maximum 0.6%, and at maximum 1.2% for honeydew honey (BRASIL, 2000). The absence of significant correlation between the ash content and the

Table 3. Investigation of Total Coliforms, Thermotolerant Coliforms, *Salmonella*, Fungi and Yeasts in aroeira honey. The values presented are triplicate averages.

Sample	Total Coliformes MPN/ml	Thermotolerant Coliforms MPN/ml	*Salmonella* / 25 g	Fungi and Yeasts UFC/ml
1	< 3.0	< 1.0	Absence	40.0
2	< 3.0	< 1.0	Absence	1.5
3	< 3.0	< 1.0	Absence	< 1.0
4	< 3.0	< 1.0	Absence	< 1.0
5	< 3.0	< 1.0	Absence	2.6×10^{2}
6	< 3.0	< 1.0	Absence	< 1.0
7	< 3.0	< 1.0	Absence	< 1.0
8	< 3.0	< 1.0	Absence	< 1.0
9	< 3.0	< 1.0	Absence	< 1.0
10	< 3.0	< 1.0	Absence	< 1.0

The values presented are triplicate averages, MPN = Most Probable Number.

color of the honey can be attributed to the small number of samples evaluated in the present study, since it is known that the mineral content is also associated with sensorial properties as color and flavor, which are important for honey commercialization (Escuredo et al., 2013).

Regarding the pH parameter, there is no indication of pH analysis as mandatory for evaluation of honey quality; however, it was performed as a complementary parameter for the evaluation of total acidity. The pH ranged from 3.86 to 4.55. During the hyper dry season, the blooming period of *M. urundeuva*, pH was significantly higher (4.35) than in the dry season (3.90), showing a less acid honey during this period (Bastos et al., 2016).

The pH varies very little between the samples, because of the presence of organic acids honey is naturally acidic (Welke et al., 2008). However, it has been identified that honey with a quantity of acidity above the permitted level have been identified (Araújo et al., 2006). Other studies indicated values within the parameters established by the legislation (Welke et al., 2008). Free acidity is characterized by the presence of organic acids in equilibrium with their respective lactones, esters, and inorganic ions (Moreira et al., 2007; Gomes et al., 2010). The aroeira honey of the Jequitinhonha River Valley presented values from 17.32 to 30.64 $Meq.kg^{-1}$, indicating a good state of conservation of the product, since this parameter used to evaluate honey deterioration, as fermentation of sugar into organic acids increases its value (Almeida-Muradian et al., 2013). Studies have shown an average value for total acidity of 30.21 $Meq.kg^{-1}$ in honey from the cashew tree bloom and attributed the physical and chemical characteristics found in the samples to the specificity of the flowering (Bendini and Souza, 2008). In the present study, the honey from aroeira presented average values of 27.27 and 45.45 $Meq.kg^{-1}$, indicating that the honey of the Jequitinhonha River Valley present important characteristics that help to preserve the product for long periods.

The negative correlation between moisture and free acidity suggests that increasing the water content dilutes the amount of acids present in honey. At least 18 organic acids have been reported, some of which are volatile and others are inorganic. Gluconic acid is the main one, which is formed by the action of the glucose oxidase enzyme produced by the hypopharyngeal glands of bees and by the action of bacteria during the process of honey maturation (De-Melo et al., 2017). The samples of honey analyzed are within the standards required by the legislation, which classifies honey from colorless to dark amber. The color of honey is associated with its floral origin, but the substances responsible for color are still unknown (Moraes et al., 2014). The predominance of dark colors in aroeira honey may result in a product of high acceptance in the national market, if it is reported that the dark color may be associated with the high concentration of minerals and other nutrients.

Honey is composed of different sugars, predominating the monosaccharides glucose and fructose. These attributes depend on climate, floral source and individual beekeeping practices (Racowski et al., 2007). Of the total samples analyzed in this study, 100% presented values below that allowed by the legislation for reducing sugars (minimum of 65%) (BRASIL, 2000). All samples were within the standard for non-reducing sugars according to the legislation.

Glucose is a sugar with little solubility, determines the tendency to crystallize and fructose by having high hygroscopicity, determines its sweetness. The mean proportion of fructose in honey is 38.5%, while glucose is 31.0%, and a honey with high fructose rates may continue to be liquid for a long period or never crystallize (Tette et al., 2016). However, the sucrose content is important to know if there was adulteration of the honey by the direct addition of sucrose or if the bees were fed at

the beginning of the flowering with sugar (Puscas et al., 2013). The aroeira honey from the Jequitinhonha River Valley, presenting sugar levels below the limits established by current legislation and a negative Jagerschmidt reaction, shows the absence of adulteration of the product.

The measure of diastase activity is an indicative of honey's freshness and is useful to detect improper storage conditions (Almeida-Muradian et al., 2013) and it is may be also an indicative of honeybees fed artificially with glucose (Guler et al., 2014). Therefore, the presence of diastase activity in the honey samples evaluated in the present study points to an absence of adulteration of honey by addition of sugar and adequate conditions of manipulation and storage.

According to the literature, honey has low susceptibility to the proliferation of microorganisms due to its physicochemical characteristics, such as antimicrobial substances, low moisture content, low pH, and oxidation reduction potential, among others (Ananias et al., 2013). Therefore, the microbiological quality of honey is often related to the hygienic conditions of food production and handling. In this way, the results obtained in the present study, once again evidence the care given by beekeepers in the production and management of aroeira honey. In Brazil, the Ministry of Agriculture, Livestock, and Supply (MAPA) published the Technical Regulation of Identity and Quality of Honey (BRASIL, 2000). Regarding microbiological criteria, the MERCOSUL/GMC/RES document (n° 15/94) has the following technical specifications for honey: total coliforms/g: absence; *Salmonella* spp. and *Shigella* spp./25 g: absence; enumeration of molds and yeasts: maximum of 100 CFU/g (MERCOSUL, 1994).

The total coliform group includes four genera: *Escherichia, Klebsiella, Citrobacter*, and *Enterobacter*. Literature data confirm that the presence of these bacteria in food indicates that there was fecal contamination (Marquele-Oliveira et al., 2017). The analyzes of the present study showed that there was absence of coliforms in honey samples, that is, the harvesting, the management, and the processing of the samples were made as recommended in order to obtain a good quality of honey.

The genus *Salmonella* includes several pathogenic serotypes, which can cause from gastroenteritis to serious systemic infections, like *Salmonella typhi* that causes typhoid fever. However, gastroenteritis is the most common form of salmonellosis and the major mode of transmission is by means of contaminated food (Marquele-Oliveira et al., 2017). Some studies showed that there was an absence of Salmonella species in Brazilian honey samples from different regions of Brazil (Schlabitz et al., 2010; Tavares et al., 2015). Likewise, in the present study, all samples of aroeira honey from the Jequitinhonha River Valley were free these bacteria.

Presence of fungi and yeasts in the samples analyzed in this study were inexpressive and this is another positive point that makes the Jequitinhonha River Valley honey an attractive product. Brazilian honey samples produced in several cities of the state of Minas Gerais were according to Brazilian law, as regards the presence of fungi and yeasts (Tavares et al., 2015). Microbial measurements allow the hygienic evaluation of a product with regard to the application of hygiene practices throughout its production chain and exposure to consumption; however, the presence / absence or low numbers of these microorganisms is not sufficient and is not directly related to conclusions about consumer risk.

In view of the above, it was understood that the Jequitinhonha River Valley honey mostly met the basic requirements of quality control, that is, the set of inspection actions on the properties of a food, aiming to maintain these properties according to norms and standards. Aroeira honey of the Jequitinhonha River Valley was shown to be free from evidence of fermentation and was handled properly while maintaining the conditions for storage and provides high protection against contamination. Due to the concern of the consumers to acquire quality products, it is necessary that the honey meets the requirements demanded by the market and for this one must obtain a broad knowledge of its physicochemical and microbial characteristics.

Conclusion

By means of this study it was possible to characterize the honey of the region of the Jequitinhonha River Valley, proving through macroscopic, microscopic, physico-chemical and microbiological analyses the good practices of production and management adopted in the region. The honey of aroeira was characterized by the compliance with the current norms, presenting good quality and own characteristics, such as, color dark amber, moisture low, and less acid pH and acidity. All samples followed current Brazilian legislation, showing the good management practices adopted by the beekeepers of the Jequitinhonha River Valley. The honey produced in the Jequitinhonha River Valley is informally recognized for its quality, so the analysis of the macroscopic, microscopic, physico-chemical and microbiological identity was important, since it was possible to prove the quality of the honey, market and open up new markets. It is always important to note that to maintain the characteristics and quality, the local flora should be maintained. This study will contribute to the geographical indication for aroeira honey, produced only in Jequitinhonha River Valley. Once this geographical indication is established beekeepers will be able to export it and this product will have its market value increased.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Supplementary Table 1. Correlation datas between the physical-chemical parameters evaluated in the aroeira honey samples.

		Moisture	Soluble_sólids	pH	Free_acidity	Lactonic_acidity	Total_acidity	Non_Reducing_Sugars	Reducing_Sugars
Moisture	Pearson Correlation	1	-0.135	-0.425	-0.761*	0.606	0.301	-0.328	0.037
	Sig. (2-tailed)		0.711	0.221	0.011	0.063	0.397	0.389	0.925
	N	10	10	10	10	10	10	9	9
Soluble_sólids	Pearson Correlation	-0.135	1	0.422	0.311	-0.165	-0.014	0.206	0.101
	Sig. (2-tailed)	0.711		0.224	0.381	0.649	0.969	0.594	0.796
	N	10	10	10	10	10	10	9	9
pH	Pearson Correlation	-0.425	0.422	1	0.551	-0.336	-0.083	0.396	0.274
	Sig. (2-tailed)	0.221	0.224		0.099	0.343	0.819	0.291	0.476
	N	10	10	10	10	10	10	9	9
Free_acidity	Pearson Correlation	-0.761*	0.311	0.551	1	-0.673*	-0.234	0.339	0.350
	Sig. (2-tailed)	0.011	0.381	0.099		0.033	0.515	0.372	0.355
	N	10	10	10	10	10	10	9	9
Lactonic_acidity	Pearson Correlation	0.606	-0.165	-0.336	-0.673*	1	0.877**	-0.241	0.215
	Sig. (2-tailed)	0.063	0.649	0.343	0.033		0.001	0.532	0.578
	N	10	10	10	10	10	10	9	9
Total_acidity	Pearson Correlation	0.301	-0.014	-0.083	-0.234	0.877**	1	-0.096	0.511
	Sig. (2-tailed)	0.397	0.969	0.819	0.515	0.001		0.806	0.160
	N	10	10	10	10	10	10	9	9
Non_Reducing_Sugars	Pearson Correlation	-0.328	0.206	0.396	0.339	-0.241	-0.096	1	-0.382
	Sig. (2-tailed)	0.389	0.594	0.291	0.372	0.532	0.806		0.311
	N	9	9	9	9	9	9	9	9
Reducing_Sugars	Pearson Correlation	0.037	0.101	0.274	0.350	0.215	0.511	-0.382	1
	Sig. (2-tailed)	0.925	0.796	0.476	0.355	0.578	0.160	0.311	
	N	9	9	9	9	9	9	9	9

*Correlation is significant at the 0.05 level (2-tailed).

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Full Length Research Paper

Determination of trace elements and heavy metals content of green and black tea varieties consumed in Iran

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The aim of the present study was to determine the level of selected heavy metals in green and black tea varieties consumed in Iran. Samples were prepared with the acid digestion method and measured by polarography. The mean concentration of Zn, Cd, Pb and Cu was 0.505±1.632, 0.417±0.268, 0.325±0.249 and 4.151±3.739 mg/100 g for black tea, and 2.442±4.003, 0.284±0.198, 0.395±0.279 and 5.723±4.286 mg/100 g for green tea, respectively. Results showed no statistically significant difference among the samples with various flavors. Green tea samples were accompanied with high concentration of Zn, Pb and Cu and lower concentration of Cd in comparison with black tea samples. The mean concentration of Cu and Cd had no significant difference in the level of the reference article. Although the level of heavy metals in samples varied from non-detectable doses to extremely high concentration, the mean concentration of each element was in an acceptable range.

Key words: Cadmium, *Camellia sinensis*, copper, lead, zinc.

INTRODUCTION

Known to be one of the most popular and globally consumed beverages, tea is produced from the leaves of a shrub called *Camellia sinensis* (Chen and Lin, 2015). Some specific characteristics such as pleasant flavor, cool effect on the mind, and refreshing mood, affordable price and being easy to brew, have made tea to be a first choice beverage in the daily diet in many parts of the world (Chen and Lin, 2015). Iranian people show huge interest in drinking tea. With an old history of presence in social and private life, tea has been a fixed part of the daily diet for majority of the people. With an extensive area appropriated for tea cultivation in the northern provinces together with the same imported volume, a large retail market of tea exists in Iran (Ghoochani et al., 2015; Rezaee et al., 2014; Salahinejad and Aflaki, 2010). Today, with the improvement of processing and

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producing techniques, an extensive spectrum of choices with various smells and tastes, is prepared for consumers. There are various types of tea (green, black, red, white, etc) obtained from *C. sinensis* plant species and are especially produced from the leaves, since the leaves undergo fermentation procedures and excess oxidation to make the ordinary black tea (Chen and Lin, 2015).

In the last decades, an increasing number of studies were done on different properties of tea and the impact on human health. It has been well known that by having unique bioelements and organic ingredients like flavonoids and catechins, which express antioxidant and anticarcinogenic roles, regular drinking of tea would have beneficial effects on human health. There have been documented events on a possible role of tea in preventing cardiovascular diseases, neurologic diseases, metabolic diseases, and inhibiting lung, gastrointestinal tract, breast and prostate cancers (Chen and Lin, 2015).

Zinc deficiency through growth periods causes growth failure. Epidermal, gastrointestinal, central nervous, immune, skeletal and reproductive systems are the organs most affected clinically by zinc insufficiency. Clinical diagnosis of marginal Zn deficiency in humans remains controversial. So far, blood plasma/serum zinc concentration, dietary intake and stunting prevalence are the best-known indicators of zinc deficiency (Roohani et al., 2013). Copper (Cu), a redox active metal, is a crucial nutrient for all species studied till now. During the last decade, there was an increasing interest in the concept that marginal deficits of this element can contribute to the development and progression of a number of disease states including cardiovascular disease and diabetes. Deficiency of this nutrient during pregnancy can result in gross structural deformities in the embryo, and perpetual neurological and immunological malformations in the children. Excessive amounts of Cu in the body can also be harmful. Acute Cu toxicity can result in a number of diseases, and in severe cases, death (Uriu-Adams and Keen, 2005).

Due to the growing tendency of industrialization and use of chemical agents (Roohani et al., 2013; Seenivasan et al., 2016; Brzezicha-Cirocka et al., 2016), heavy metals contamination has become a concern in food and beverage business. Recent studies in Iran have reported a probable risk of exposure to heavy metals, to consumers (Ghoochani et al., 2015; Rezaee et al., 2014; Falahi and Hedaiati, 2013). The aim of this study was to determine heavy metals and trace elements level in green and black tea consumed in Iran.

MATERIALS AND METHODS

Polarography

Acetic acid glacial, copper (II) nitrate, lead (ii) nitrate, zinc (II) nitrate and cadmium (II) nitrate, sodium acetate and tartaric acid were bought from Merck (Germany), and nitric acid (65%) was obtained from Applichem (Germany).

Preparation of standard solutions

For preparation of standard solution, 17 g of sodium acetate trihydrate was liquefied in 30 mL of distilled water, and 0.75 g of tartaric acid was added to produce sodium acetate buffer. To produce a limpid and monotonous solution via ultrasound waves, a sonicator device was utilized. Glacial acetic acid for setting the pH of the solution in the range of 4.6 to 4.8 was utilized and then distilled water was added to increase the volume up to 100 ml. Next, 0.080 g lead (ii) nitrate, 0.191 g copper (ii) nitrate, 0.137 g cadmium (ii) nitrate and 0.214 g zinc (ii) nitrate, respectively, were dissolved in 100 mL double distilled deionized water to prepare the standard solutions of Pb, Cu, Cd and Zn. The calibration curves of the Zn, Cd, Pb and Cu by the standard addition technique are available in Figure 1.

Sampling and preparation

This study was conducted to obtain comprehensive information on heavy metals and trace elements level in most current green and black varieties of tea in the retail markets of Iran.

The level of four heavy metals and trace elements including Cd, Pb, Zn and Cu was monitored in 25 different commercial kinds of green and black tea. Samples were purchased from the local markets in Tehran, Iran. They covered almost all types of green and black tea found in the retail market. The collected tea brands included Golestan, Mahmud, Ahmad, Dr Bin, Lipton, Golkuh, Miaad, Doghazal, Mehregiah, Zarnush, Twinings, Shahrzad, Shahsevand, Dr. Soleymani, Fumanat, Dineh, Mohsen, Mardin, Bamdad, Gozal, Tashrifat and Newsha**.**

The collected green and black tea brands were in 7 different flavors, including Ordinary (non-flavored), lemon, mint, cinnamon, jasmin, honey and Tab (tablet). Tea flavor, commercial brands and types (green or black) were considered as variables to study their influence on heavy metals and trace elements concentration**.**

To measure heavy metals and trace elements concentration, a group of 60 samples consisting of 33 black and 27 green tea species with different flavors was set to be surveyed for each element. The sample group was the same for each element. The total number of samples was 180 (n=180). For each sample, about 1 g of commercial tea was accurately weighed into a glass beaker. All glassware were cleaned by soaking in dilute sulfuric acid and rinsed with distilled Milli-Q water. Each tea sample of 1 g was put into a clean 100 ml beaker. 20 ml of concentrated nitric acid was added, the beaker was covered with a watch glass and material was boiled gently on a hot heater equipped with a tunable thermostat until digestion was complete. The complete digestion took about 1 h. A 2.0 ml portion of 70% perchloric acid was then added and gentle heating was performed for 1 h. Small fractions of double purified water were added to prevent dryness caused by evaporation. After cooling down gently to room temperature, the mixture was filtered and transferred to a 100 ml volumetric flask that was rinsed with distilled and deionized water. The concentration of heavy metals was then measured with a polarography machine.

Measurement

Voltammetry comprises a group of electroanalytical methods widely employed in analytical chemistry and other industrial processes. In voltammetry, the information on the analyte was obtained from the measurement of current as a function of applied potential, under the conditions of complete concentration polarization.

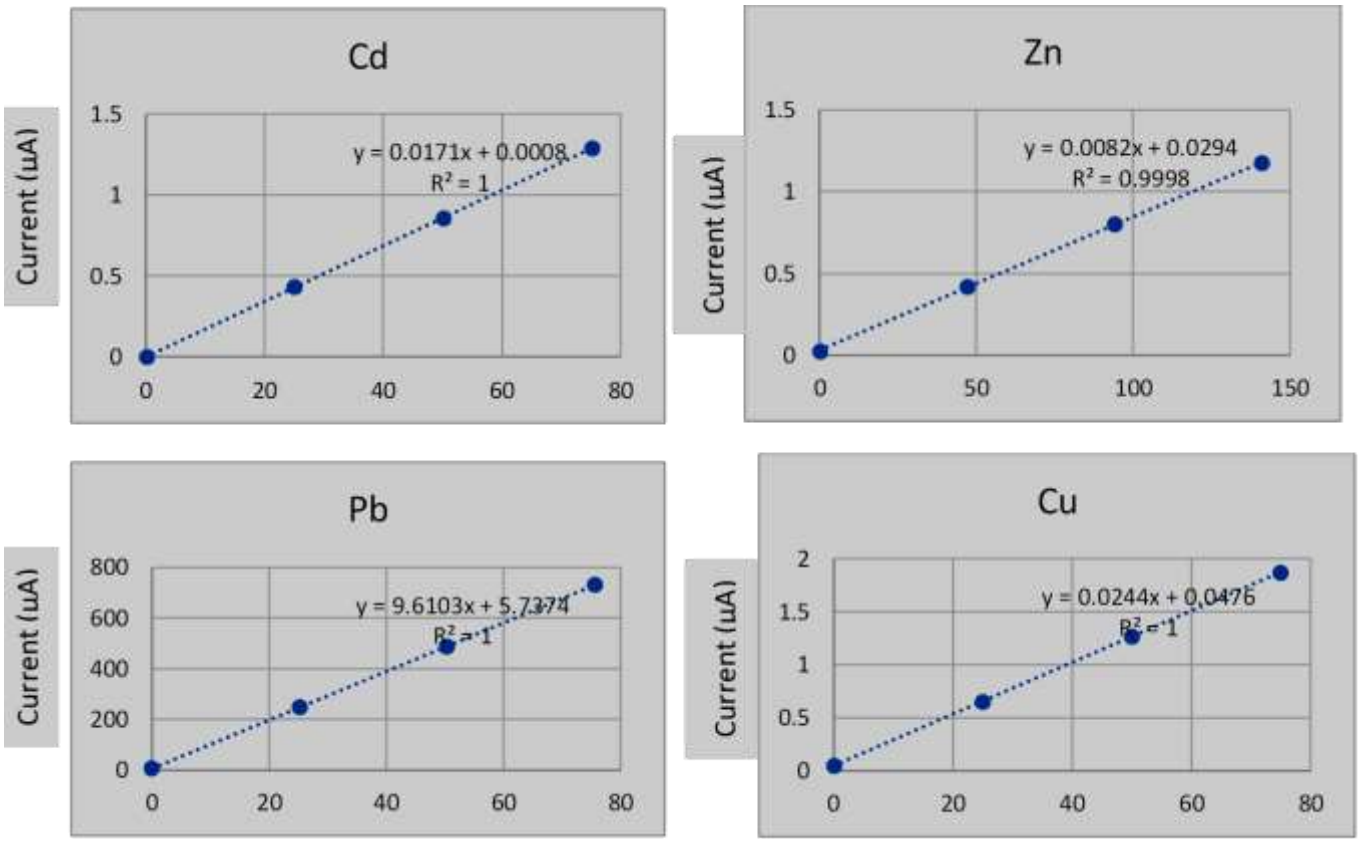

Figure 1. Calibration curves of Zn, Cd, Pb and Cu using standard addition technique for tea.

In this study, the polarograph cell was filled with 10 mL of sodium acetate buffer and the solution was stirred at 2000 rpm for 1 min. The polarograph was measured by drawing a baseline utilizing a Hanging Drop Mercury Electrode (HDME). The polarograph was conducted with the initial electrolysis of 800 mV potential applied for 90 s. The stirring was stopped for 10 s through the resting step so that the solution can be ready for measurements. After the polarograph baseline was drawn, 500 µL of the prepared solution was added to the cell and stirred for 100 s at 2000 rpm. By repeating these processes in the next steps, the voltamogram was obtained. Then, 100 µL of the quadruple standard solution was added to the cell in three different stages. The polarograph was used to compute the mean of the three measurements for the evaluation. The device drew the polarogram curves of Zn, Cd, Pb and Cu for black and green tea samples as illustrated in Figure 2.

RESULTS AND DISCUSSION

The level of the selected heavy metals in 25 commercial green and black teas is presented in Tables 1 to 5 and Figures 3 to 6. Concentrations of categories of tea and the samples' flavor are also shown. Analysis of variances (ANOVA) showed no significant statistical difference between green and black tea in terms of heavy metals concentration. Sample 1 groups were ignored with regards to effect of tea flavor in the distribution of heavy metals in the samples. Flavor was not a significant factor in determining the level of heavy metals in tea samples. T-test was conducted to compare the mean concentration of each element with acceptable or allowable daily intake (ADI) as reported by WHO. The mean concentration of Zn and Pb in this study was less than the ADI of reference article and the mean concentration of Cu and Cd had no significant statistical difference with ADI of reference article (by 95% of confidence interval). Cu had the highest mean concentration, while Pb showed the minimum level among the studied elements. The mean concentration of Zn was 0.505±1.632 mg per 100 g for black tea and 2.442±4.003 mg per 100 g for green tea, which varied through a range of 0.00 (non-detectable) to 12.52 mg/100 g with a standard deviation of 12.52. The mean concentration of Cd was 0.417±0.268 mg for black tea and 0.284±0.198 for green tea. The minimum and maximum level for Cd was 0.00 (non-detectable) and 0.996 mg/100 g, respectively. The mean concentration of Pb was 0.325±0.249 mg/100 g for black tea and 0.395±0.279 mg/100 g for green tea. The level of Pb varied between 0.00 (non-detectable) and 0.933 mg/100 g. The average concentration of Cu was 4.151±3.739 mg/100 g for black tea and 5.723±4.286 mg/100 g for green tea. The minimum and maximum concentration of Cu was 0.00 (non-detectable) and 13.82. There is a significant difference between black and green tea content

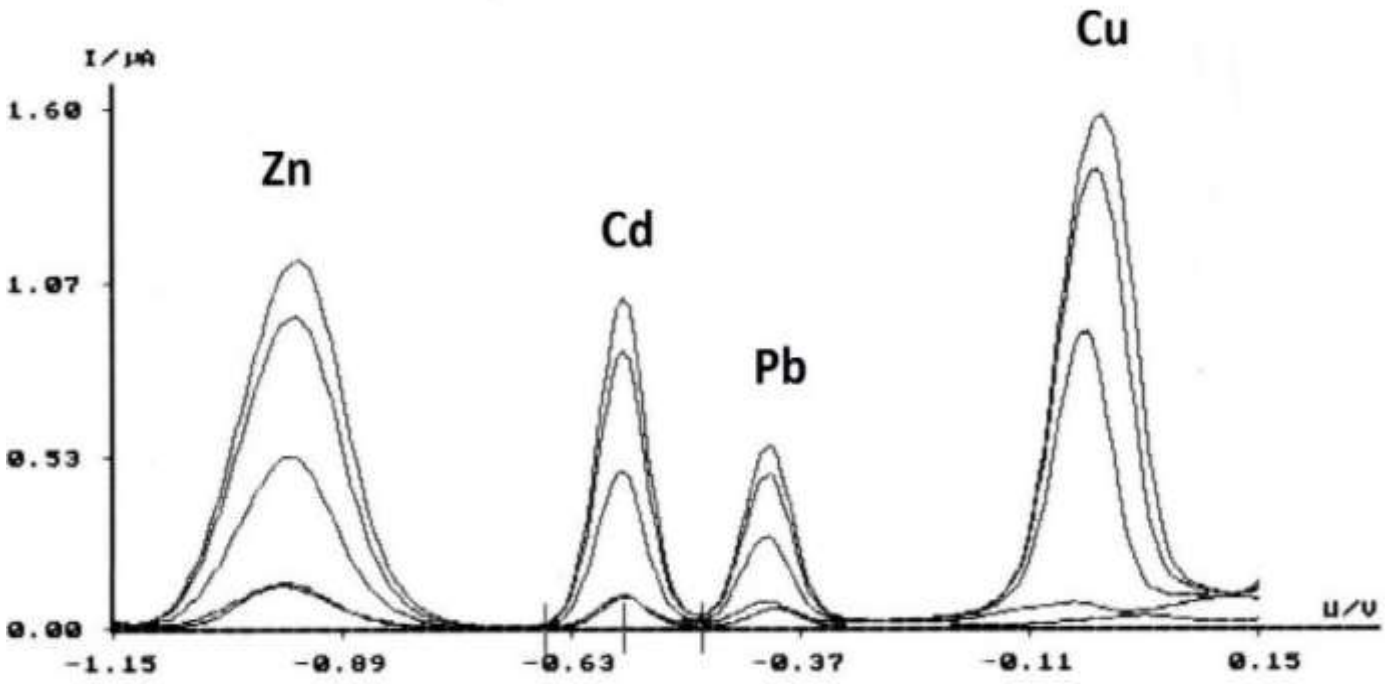

Figure 2. Polarogram curves of the four elements in tea samples.

Table 1. Zinc levels in selected group of Black tea samples (mg/100 g).

Brand	Mean	Standard deviation
Miaad	0.000	0.000
Doghazal	0.000	-
Lipton	1.049	1.483
Zarnush	0.000	-
Golestan	2.104	2.976
Twinings	0.000	0.000
Ahmad	1.650	3.691
Shahrzad	1.829	-
Shahsevand	0.000	-
Fumanat	0.000	0.000
Fenjun	0.000	0.000
Mohsen	0.196	0.392
Bamdad	0.000	-
Gozal	0.000	-
Tashrifat	0.000	-
Famila	0.000	0.000
Newsha	0.000	-
Total	0.401	0.723

of zinc and cadmium in all samples ($P < 0.05$) and with regards to lead and copper, there is no significant differrence between black and green tea samples ($P > 0.05$).

In a survey, Nkansah et al. (2016) in Ghana, after monitoring 15 green and black commercial teas, reported the average concentration of Zn, Cd and Pb to be 0.02, 0.036 and 0.016 mg per 100 g, respectively. All the reported sums are less than the ones obtained in the

Table 2. Zinc levels in selected group of green tea samples (mg/100 g).

Brand	Mean	Standard deviation
Miaad	3.265	-
Doghazal	0.000	-
Mehre Giah	0.000	-
Dr Bin	0.000	-
Lipton	5.579	-
Golkuh	0.000	-
Zarnush	0.000	-
Mahmud	10.281	-
Golestan	6.071	4.773
Twinings	3.781	6.549
Ahmad	0.000	0.000
Shahrzad	0.000	0.000
Shahsevand	0.000	-
Mardin	0.000	-
Dr Soleymani	5.111	-
Dineh	0.000	-
Fumanat	0.000	-
Fenjun	0.000	0.000
Total	1.893	3.069

Table 3. Mean concentration of Zn in Black and Green tea samples (mg/100 g).

Tea	Number	Mean	Standard deviation
Black	33	0.520	1.632
Green	27	2.442	4.003
Total	60	1.385	3.072

current study, taking into account the difference between the experimental techniques performed. In both studies, Pb had the least concentration of the selected elements.

Falahi and Hedaiati (2013), through a related previous study in Iran, reported a mean concentration of Zn, Cd, Pb and Cu to be 2.88, 0.0134, 0.021 and 1.59 mg/100 g, respectively. The concentrations in the current study, on the other hand, were 1.38, 0.357, 0.357 and 4.85 mg/100 g. The selected elements in this study show a noticeable rise in concentration except for Cu in comparison with the previous study. In a study, Raj et al. (2011) measured the concentrations of some metals in green and black teas in India. The results showed that the concentration was 0.231 for lead, 0.089 for cadmium, 2.539 for zinc and 1.434 for copper per 100 g of black tea, whereas the amounts in green tea were less than LOQ (0.5 mg/kg) for lead and 0.159, 2.639 and 1.128 per 100 g for Cd, Zn and Cu, respectively.

In another study, Hosseni et al. (2013) compared 10 brands of Iranian black tea and 10 brands of imported black tea in terms of contamination with lead and cadmium. The results of their study revealed that the amount of lead and cadmium in Iranian tea is higher than that of imported tea. The average value measured for lead in Iranian and imported black tea was 0.04975 and 0.02933 mg/100 g, respectively. As for cadmium, the average amount of 0.0045 mg/100 g for Iranian and 0.000914 mg/100 g for imported black tea was reported. Values for lead and cadmium in these two categories of tea were less than the maximum allowable concentration and did not pose any problem to human health.

In a research, Sadeghi et al. (2016) measured concentration of zinc, copper, lead and cadmium in four brands of baby food (rice and wheat based) and powder milk with DPASV and polarograph set. Total mean ± SE of zinc, copper, lead and cadmium in baby foods (n = 240) were 11.86 ± 1.474 mg/100 g, 508.197 ± 83.154 µg/100 g, 0.445 ± 0.006, 0.050 ± 0.005 mg/kg, respectively. Also, these amount in powder milk (n = 240) were 3.621 ± 0.529 mg/100 g, 403.822 ± 133.953 µg/100 g, 0.007 ± 0.003, 0.060 ± 0.040 mg/kg, respectively. Zinc level in baby food type I was higher than labeled value (P = 0.030), but in other brands, there was no difference. Concentration of copper in all the samples was in labeled range (P > 0.05). In each of the four products, level of lead and cadmium was lower than the standard limit (P <0.05). Amount of zinc and lead in baby food type I, was different compared to other products. Concentration of zinc and cadmium in baby food type I, was higher than in type II (P = 0.043, 0.001 respectively). Concentration of lead and cadmium in baby food type II, was higher than

Table 4. Copper levels in selected group of black tea samples (mg/100 g).

Brand	Mean	Standard deviation
Miaad	11.552	3.217
Doghazal	1.281	-
Lipton	6.125	6.618
Zarnush	2.194	-
Golestan	5.506	0.017
Twinings	7.080	6.607
Ahmad	3.118	2.282
Shahrzad	8.022	-
Shahsevand	1.753	-
Fumanat	1.220	0.184
Fenjun	1.587	7.617
Mohsen	1.575	0.133
Bamdad	2.066	-
Gozal	1.641	-
Tashrifat	4.916	-
Famila	6.971	2.849
Newsha	1.0711	-
Total	4.548	3.393

Table 5. Comparison of black and green tea metals with standard limits (mg/100 g).

Green tea					Black tea			
Element	N	Mean	Std. deviation	Std limit	Element	N	Mean	Std. deviation
Zn	27	2.442	4.003	5	Zn	33	0.505	1.632
Cd	27	0.284	0.198	0.01	Cd	33	0.417	0.268
Pb	27	0.395	0.279	0.1	Pb	33	0.325	0.249
Cu	27	5.723	4.286	5	Cu	33	4.151	3.739
Total	108	2.211	2.542		Total	132	1.349	1.869

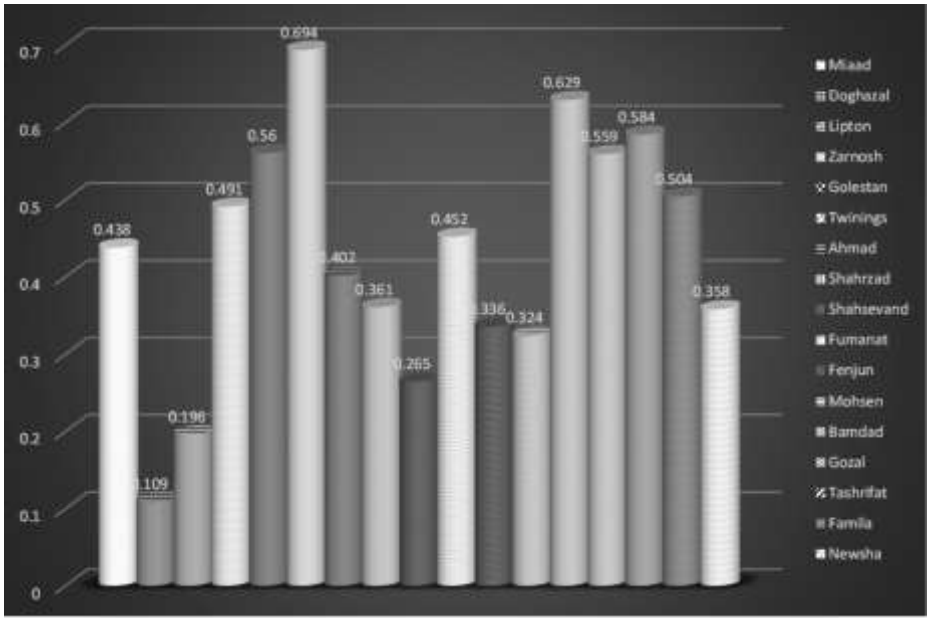

Figure 3. Cadmium concentrations of commercial black tea samples (mg/100 g).

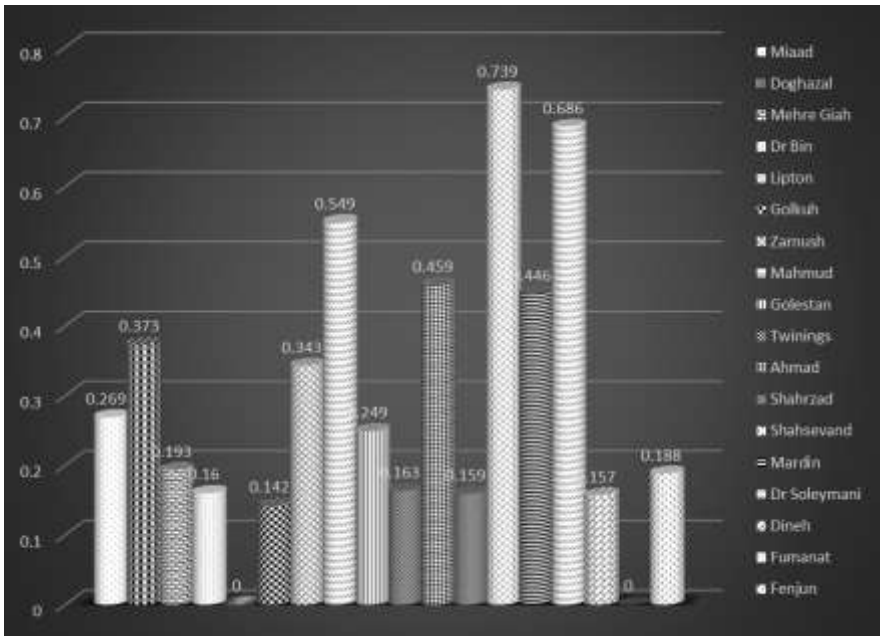

Figure 4. Cadmium concentrations of commercial green tea samples (mg/100 g).

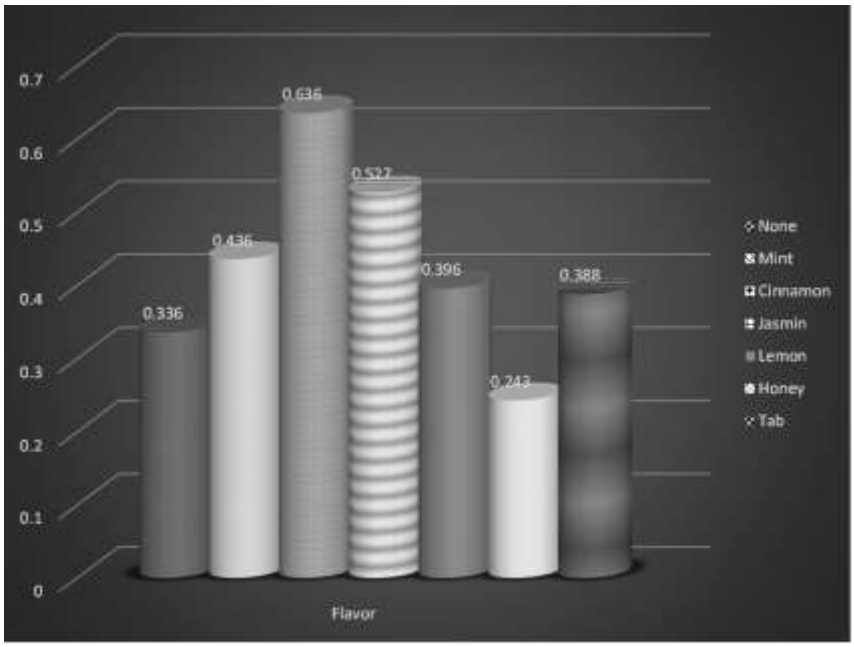

Figure 5. Lead levels in different flavors of green tea samples (mg/100 g).

infant formulas, but are in standard limit (Sadeghi et al., 2014). Sadeghi et al. (2014) aimed to measure the levels of trace elements: zinc, copper and heavy metals: lead and cadmium by differential pulse anodic stripping voltammetry in 19 barley grain cultivars and their malts in Iran. Ferric reducing antioxidant power (FRAP) assay was also used for determination of antioxidant activity of the samples. The mean levels of Zn, Cd, Pb and Cu were measured to be 18.813 ± 8.575, 0.212 ± 0.116, 0.278 ± 0.163, 3.746 ± 1.118 mg/100 g in the barley samples and 14.364 ± 6.391, 0.153 ± 0.098, 0.179 ± 0.082 and 3.033 ± 1.392 mg/100 g in the malt samples, respectively. The highest concentration of Zn was measured in the Bahman cultivar of barley and Mb-82-4 sample of malt, while the Sahra cultivar of barley and Valfajr sample of malt had the lowest concentration of Pb and the Nimrooz

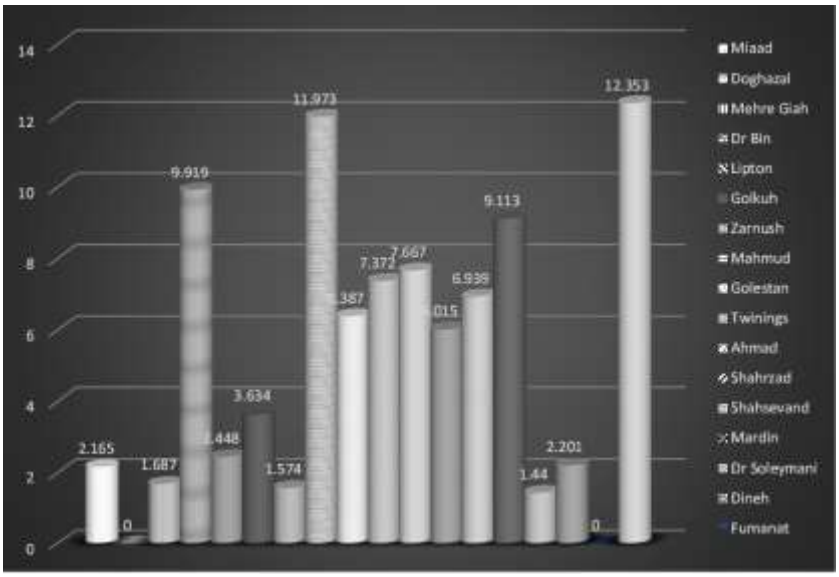

Figure 6. Copper concentrations of commercial green tea samples (mg/100 g).

cultivar of barley and Rihane-03 sample of malt had the lowest concentration Cu. The mean levels of zinc and lead in the evaluated samples of barley and the mean levels of zinc, lead and cadmium in the samples of malt were significantly lower than the standard limits (Sadeghi et al., 2016). Maleki et al. (2010) in a research investigated the cadmium and lead content of several commercially available brands of tea (*Camellia sinensis*) in Iran and compared the release of cadmium and lead from them by infusion. Ten of the most consumed brands of Iranian and imported black tea were bought from local markets. The results showed that the min and max lead contents in the studied tea were 0.66 ± 0.14 and 15.48 ±0.58 µg/g for Sharzad and Sharyat tea, respectively. In addition, the minimum and maximum cadmium content was 0.09 ± 0.013 and 1.92 ± 0.38 µg/g for Ahmad and Sharyat tea, respectively (Maleki et al., 2010).

Conclusion

This study evaluated the health risk of Cd and Pb as well as the health benefits of Zn and Cu in tea of 25 different brands in Iran. The results of the analysis of black and green tea samples are presented. There were wide variations in the heavy metal contents of black and green tea. Consumer brand teas are the blend of various individual teas from different regions across the country. The results of the mean concentration of the selected heavy metals in the current study are in agreement with the results of other studies. The higher levels of some elements in this study as compared to the ones in other studies may be explained by the sensitivity of the laboratory technique applied in this study. The level of the selected heavy metals in green and black tea in this study is in the acceptable range, although, based on some high levels seen in few samples, the risk of exposure to heavy metal intoxication for Iranian tea consumers could not be ignored. There was noticeable amount of zinc and copper in black and green teas. Zinc is an important mineral that is found in every single cell of the body. The red and white blood cells, bones, retina, kidneys, pancreas and liver, all store zinc. The tenth most common element in the human body, zinc, is vital for the functioning of more than 300 hormones and countless enzyme systems in the body. Zinc is also vital for cells to divide and replicate during the production of new tissues. Copper is a key mineral in different body systems. It is central to building strong tissue, maintaining blood volume, and producing energy in cells.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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