

Determination of Heavy and Trace Metals in Honey Using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) in South Eastern Zone of Tigray Region, Northern Ethiopia

Kusse Gudishe Goroya,^{1*} Gebrewahd Abadi,¹ and Yoseph Alresawum Asresahegn²

¹Department of Physics, College of Natural and Computational Sciences, Wolaita Sodo University, P. O. Box 138, Wolaita, Ethiopia.

²Department of Physics, College of Natural and Computational Sciences, Kotebe Metropolitan University, Addis Ababa, Ethiopia.

*Corresponding email: anaxorma@gmail.com

Received 20 May 2020; Accepted 20 July 2021

ABSTRACT

The present study aimed to determine the concentration of heavy and trace metals (Fe, Cu, Mn, Zn, Co, Cd, Pb, As, and Hg) in honey by inductively coupled plasma optical emission spectrometry (ICP-OES). Samples passed through wet digestion methods following the optimum digestion condition by applying the reagents (30mL HNO₃:30mL H₂O₂) at a temperature of 270°C for 3 hours. The average concentrations of the metals are found in the range of 5.32-28.6 mg/kg for Fe, 0.24-0.749mg/kg for Cu, 0.627-4.401mg/kg Zn, 0.41-3.15mg/kg for Mn, 0.08-0.112 mg/kg for Co, 0.25-0.325mg/kg for Pb, 0.24-0.46mg/kg for As, ND-0.031mg/kg for Hg and 0.02-0.03mg/kg for Cd. This study shows that the honey in the studied area is a good source of essential metals (Fe, Cu, Mn, Co and Zn) as they are found to be within the permissible limit. Moreover, the maximum concentration of the toxic metals determined in this work are below the level of toxicity as per the standard set by WHO/FAO.

Keywords: Concentration, essential metals, honey, ICP-OES, non-essential metals

INTRODUCTION

Honey is the natural sweet substance produced by honey bees from the nectar of plants which bees collect and transform by combining with specific secretions of their own and deposit, dehydrate, store and leave in the honey comb to ripen and mature [1, 2]. Metal concentrations in different honey types depend largely on the elemental composition of flowers, with regard to their botanical and geographical origin. These metals can damage human life when they accumulate to a toxic concentration level. Heavy metals in foods and beverages are classified in to two: essential trace (Fe, Zn, Mn, Cr, Cu and Co) element, which are indispensable to human body; and toxic (Pb, Cd, As, and Hg) elements, which are toxic even in minute quantity [2-5].

In multiple regions around the world, soil and water reservoirs are contaminated with heavy metals, especially with in and surrounding urbanized and industrialized areas [5]. Many of these heavy metals are taken up by the plants growing in contaminated soil and accumulate to high levels in plant tissues. In addition to affecting plant productivity and survival, this contamination exposes the herbivores and pollinators that depend on these plants to potentially

The journal homepage www.jpacr.ub.ac.id

p-ISSN : 2302 – 4690 | e-ISSN : 2541 – 0733

toxic levels of the metals [6]. Though bees are able to detect some toxics by their sensory structures, there are some toxins that honey bees do not appear to be able to detect through these sensory structures. Investigating the likelihood that honey bees will readily feed on metal contaminated resources helps to determine level of threat a toxin poses to the bee population and indirectly to humans [7, 8].

Mekelle is a capital city of Tigray region in Ethiopia and is among the highly developing and urbanizing cities in Ethiopia. It is also among the few industry zones in Ethiopia. Research conducted by Samuel [9] on the effect of dust from Mesebo cement factory around Mekelle shows that the soil is polluted by heavy metals. According to Fitsum's [5] research on Elala River which crosses Mekele city, the water is polluted by heavy metals. The effluents, sewages, smokes that come out from industries and factories may cause pollution of air, water and soil by heavy and toxic metals. The heavy and trace elements may accumulate more than the allowed content and cause health problems. This work aimed to determine the concentration of these heavy and trace elements in honey in Tigray region, north Ethiopia.

EXPERIMENT

Data collection method

The study area, South Eastern Zone of Tigray, consists of four Weredas/districts (HW= Hintalo Wajrat, DT= Degua Tenben, SS = Saharti Samre and ET= Enderta) and data was taken from four of them. Six samples from one Wereda were collected and then mixed in one holder by taking 500 g from each and take as a representative sample of the Wereda. Total of four honey samples were collected, each with 2000 g weight. The honey samples were collected from areas of intensive beekeeping. Samples were collected directly in June 2019 from model farmers. All the samples were collected in clean and closed glass bottles in order to protect them from contaminations. The samples were given their own label, sealed and put in a refrigerator at 4°C until digestion.

Apparatus and instruments

Digital analytical balance (D-72336 Bvange-Germany) was used for weighing the honey samples. Pipettes and micropipettes syringes were used to measure different amount (volumes) of acids and standard solutions. Test tubes were used to hold digested samples on the auto sampler. Borosilicate volumetric flasks were used to dilute sample solutions and prepare standard solutions. Filter funnel and filter papers were used to filter the digested solution of the samples. A refrigerator was used to keep the sample and digested samples till analysis. Fume hood was used to heat samples. Absorption spectra of heavy and trace metals was recorded using an inductively coupled plasma-optical emission spectrophotometer (ARCOSFSH12 ICP-OES).

Reagents and chemicals

Concentrated nitric acid (con.HNO₃) and hydrogen per oxide are used for digestion of honey samples to ensure the removal of organic impurities. Standards of stock solutions containing concentration of 1000 mg/L heavy and trace metals (Fe, Cu, Co, Cd, Mn, As, Hg, Zn and Pb) were used for the preparation of calibration curves, standardization process and preparation of spike samples. Deionized water was used throughout the experiment for rinsing of the glassware, dilution of the digested samples and intermediate metal standard solutions. All chemicals and reagents used in this laboratory are of the analytical grade and are obtained from in organic vouchers.

Method of sample preparation

Samples from all the four Woredas were heated to 65°C on a fume hood until get liquefied for easier handling and to decrease viscosity for more uniform distribution. Then the samples were then cooled and weighed for subsequent analysis as per previous literatures [10-12].

Optimization of the working procedure

To prepare clear and colorless sample solution that is suitable for the analysis, different working procedures for the digestion of honey samples were assessed using mixture of HNO₃ and H₂O₂ by varying parameters such as volume of reagents, digestion time and digestion temperature. By examining the nature of the final digested honey samples, the optimized procedure was selected based on clearness of the digested sample, less digestion time, minimum temperature applied less reagent consumption and simplicity. A series of procedures involving some changes in reagent volume, digestion time and temperature were tested. Total of nine trials were tested for digestion of the honey samples.

Digestion method

Sample pretreatment is usually required to destroy the organic matrix and to extract the metal ions bound inorganic complexes [13]. In this work, wet (acid) digestion method was used [14]. First, to minimize contamination glass wares were washed with 10% HNO₃ and then all materials were rinsed with distilled water and dried in an oven before use. Accurately weighed 5 grams representative of each honey sample has been taken in to beakers. Then 30 mL grade 70 % HNO₃ and 30 mL 30% H₂O₂ is added to each sample. The solution is then stirred carefully and put on the fume hood for heating by adjusting the temperature at 270 °C for 180 minutes. Organic parts of the sample are evaporated in the form of red gases. During this time the color of the gas gradually changed from red to yellow and then to white. After the color of the samples has changed near to dry and white, samples are then cooled to room temperature. The solution form of the samples was filtered with filter paper and transferred to a volumetric flask and diluted to 50 ml with distilled water and 0.5% HNO₃. The digestion gave a clear colorless solution. In addition to sample digestion, digestion of blank was also prepared for correcting the effect of blank, keeping all digestion parameter the same.

Method validations

Method validation is used to confirm the analytical procedure employed for specific test. Results from method validation can be used to judge the quality, reliability and consistency of the result. In order to validate the analytical method, the following method validation parameters such as recovery test, instrument detection limit, method detection limit, limit of quantification, precision and accuracy and linearity studies were carried out [15].

Recovery test: The recovery test of this work was obtained from spiking experiments as

$$\%Recovery = \frac{Conc. \text{ after spike} - Conc. \text{ before spike}}{Conc. \text{ of added sample}} \times 100\% \quad (1)$$

Recovery values of the method within 80-120% range are acceptable [14].

Instrumental detection limit: The instrumental detection limits (IDL) were obtained from ICP-OES (ARCOSFSH12) manual which are encoded in the equipment itself.

Method detection limit: Method detection limit (MDL) is defined as the minimum concentration of analyte that can be measured and reported with 99% confidence that the analyte concentration is greater than zero, but it may not necessarily be quantified as an exact value. The method detection limit was calculated by multiplying the standard deviation of the blank concentration (SD) by 3. The MDL of each element was determined as per previous literatures [14, 16].

$$MDL = 3 \times SD \quad (2)$$

Limit of quantification: Limit of quantification (LOQ) is the level above which quantitative results may be obtained with specific degree of confidence. The LOQ is equal to 10 times the standard deviation of the results for a series of replicates. It is used to determine a justifiable limit of detection.

$$LOQ = 10 \times SD \quad (3)$$

Precision and accuracy: Precision was evaluated regarding repeatability by calculating the relative standard deviation (RSD) of the recovery percentage for each spiked level. The relative standard deviation for replicate analyses of the same sample was obtained as [2]

$$\%RSD = \frac{SD}{Mean\ value} \times 100\% \quad (4)$$

Linearity: Calibration curves were prepared to determine the concentration of the heavy and trace metals in honey sample solutions from the known standard solutions. Intermediate standard solutions containing 10mg/L were prepared in 100 ml volumetric flasks from the standard stock solutions that contained 1000 mg/L solution for each metal. Nine working standards were freshly prepared for each metal from the intermediate standard by diluting with deionized water for calibration curve purpose. The linearity of the experiment was confirmed from linear equation of the calibration curves.

Statistical analysis

All the determination of concentrations of the heavy and trace metals were conducted in triplicate and results were expressed as mean \pm standard deviation (SD). The results of the minimum, maximum, standard deviation, coefficient of variation and least significant difference values of the measured concentrations of the heavy and trace metals were tabulated. The determined data were analyzed by analysis of variance (ANOVA) using SAS software version 9.1.3. This was used to determine whether there were significant concentration differences of the metals with in honey samples or not. SPSS software version 20 was used to determine Pearson's correlation among the concentrations of the metals in the honey samples determined in this work.

RESULT AND DISCUSSION

Optimization of digestion

Among the nine-serial optimization, the optimal digestion procedure chosen was the method that required 3 hours for complete digestion of 5 g of honey sample with 30ml 70% HNO_3 :30ml of 30% H_2O_2 reagent ration (Table 1).

Table 1. Optimization of the digestion of honey samples in this work.

Trial No.	Reagent used	Volume ratio(ml)	Temp($^{\circ}\text{C}$)	Digestion time(min)	Observation
1	HNO_3 : H_2O_2	30:5	270	180	Light yellow solution
2	HNO_3 : H_2O_2	30:10	270	180	Yellow solution
3	HNO_3:H_2O_2	30:30	270	180	Clear colorless solution
4	HNO_3 : H_2O_2	30:30	270	120	Light yellow solution
5	HNO_3 : H_2O_2	30:30	270	150	Yellow solution
6	HNO_3:H_2O_2	30:30	270	180	Clear colorless solution
7	HNO_3 : H_2O_2	30:30	210	180	Yellow solution
8	HNO_3 : H_2O_2	30:30	240	180	Light yellow solution
9	HNO_3:H_2O_2	30:30	270	180	Clear colorless solution

Validation methods

Calibration curves for all the nine metals are constructed from their respective standard solutions. It was noted that all the calibration curves confirmed the linearity nature of the experiment. Figure 1 only displays the calibration curve for copper standard solution. Table 2 displays method validation results of all the nine metals studied in this work.

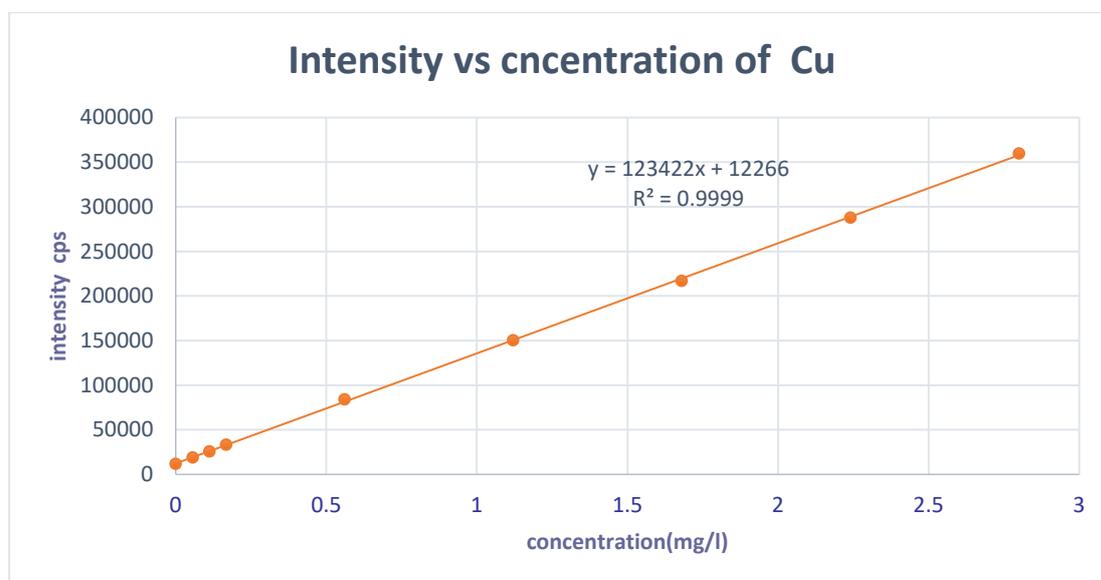


Figure 1. Calibration curve for Fe standard solution.

Table 2. Instrument detection limit, method detection limit, and quantification limit for metals determined in honey samples in this work.

Metals	IDL($\mu\text{g}/\text{kg}$)	MDL ($\mu\text{g}/\text{kg}$)	LOQ (mg/kg)	Recovery test (%)	Precision (%)
Fe	1.80	6.00	0.06	117.97	1.65
Mn	0.20	1.00	0.01	93.24	0.51
Cu	2.50	8.00	0.08	105.71	1.84
Zn	0.90	3.00	0.03	119.63	1.03
Co	0.32	1.00	0.01	106.40	1.65
As	1.20	3.00	0.03	117.03	3.21
Pb	0.70	2.00	0.02	86.83	2.06
Cd	0.10	0.30	0.003	91.41	0.66
Hg	2.80	7.00	0.07	114.07	2.34

IDL=Instrument Detection Limit, MDT= Method Detection Limit, LOQ=limit of quantification

As can be seen from Table 2, the mean presentage recoveries for the studied heavy and trace metals in the spiked sample ranged between 86.83 and 119.63%. All the recovery values are within the acceptable range of 80-120% for metal analysis [14]. Consequently, the proposed method for digestion of honey samples was valid and reliable. The %RSD values obtained for honey spiked samples ranged from 0.656 to 3.21% (Table 3), which is under the required control limits $\leq 15\%$. The results indicate that the proposed method was precise and accurate. The precision determined at each concentration level should not exceed 15% of the coefficient variation [14].

Concentration of essential and nonessential metals

Results of this experiment showed that the concentration of the essential and nonessential heavy metals (Fe, Mn, Co, Cu, Zn, Pb, Cd, Hg, and As) in the digested honey samples were determined. The results indicate that, the honey samples collected from four districts had different concentration of metals. The average concentration of the analyzed heavy and trace metals in all honey samples with their corresponding standard deviation is summarized in Table 4 and 5. Among the metals studied in this work, iron has higher concentration (Table 4). On the other hands, mercury is obtained below instrumental detection limit in samples collected from Saharti Samre and Hintalo Wajrat (Table 5).

Table 4. Mean concentration of the essential trace metals in honey (mean \pm SD, n=3) samples in this work (mg/kg).

Districts	Metal concentrations				
	Fe	Cu	Mn	Co	Zn
ET	5.65 ^c \pm 0.11	0.24 ^d \pm 0.01	1.90 ^b \pm 0.06	0.08 ^c \pm 0.01	0.62 ^d \pm 0.05
DT	5.32 ^c \pm 0.08	0.38 ^c \pm 0.02	0.41 ^d \pm 0.01	0.10 ^b \pm 0.00	1.29 ^c \pm 0.05
SS	10.79 ^b \pm 0.83	0.63 ^b \pm 0.03	1.06 ^c \pm 0.06	0.10 ^{ba} \pm 0.01	2.24 ^b \pm 0.09
HW	28.60 ^a \pm 0.40	0.75 ^a \pm 0.01	3.15 ^a \pm 0.01	0.11 ^a \pm 0.00	4.40 ^a \pm 0.08
LSD	0.87	0.04	0.08	0.01	0.13
CV	3.68	3.70	2.47	6.88	3.22

Means with the same letter in the same column are not significantly different ($p < 0.05$), HW= sample from Hintalo Wajrat district, DT= Sample from Degua Tenben district, SS= Sample from Saharti Samre district and ET=Sample from Enderta district, LSD=list significant difference, CV=coefficient of variation.

Table 5. Mean concentration of the nonessential trace metal in honey samples (mean \pm SD, n = 3) samples in this work (mg/kg).

District	Metal concentration			
	Pb	Cd	As	Hg
ET	0.25 ^b \pm 0.03	0.03 ^a \pm 0.01	10.46 ^{ab} \pm 0.14	0.03 ^a \pm 0.01
DT	0.33 ^a \pm 0.04	0.02 ^a \pm 0.01	0.38 ^a \pm 0.04	0.03 ^a \pm 0.01
SS	0.30 ^{ba} \pm 0.03	0.02 ^a \pm 0.00	0.34 ^{ab} \pm 0.02	ND
HW	0.25 ^b \pm 0.03	0.02 ^a \pm 0.01	0.24 ^b \pm 0.00	ND
LSD	0.07	0.01	0.14	0.01
CV	12.34	28.20	20.78	67.90

Means with the same letter in the same column are not significantly different, ND= not detected

The concentrations of all the essential trace metals in this study (Fe, Cu, Co, Mn, and Zn) are within the detection limit of the ICPOES (Table 2). One-way analysis of variance (ANOVA) was made at 95% confidence level. The results showed that there are significant differences ($p \leq 0.05$) for the metals Fe, Cu, Zn, Co, Mn, Hg, and As among the four districts, except for arsenic.

Pearson's correlation

The correlation with magnitude of $r \geq 0.5$ is said to be significant while with magnitude less than 0.05 is said to be less significant. Accordingly, there is a strong significant negative correlation between lead (As) and (Zn, Cu, Fe), Hg and (Cu, Co). The high negative correlation of indicate the large absorption of one element may affect the absorption of the other element. There is moderate negative correlation between Pb and Mn, Hg and Zn, As and Co. On the other hands, there is positive moderate relationship is observed in Cd and As, Mn and Co. Moreover, zinc has strong positive correlation with Co, Cu, Mn and Fe. Copper has also strong positive correlation with Co and Fe. The high positive association between elements, evidenced by high positive correlation coefficient, can arise from common anthropogenic or natural sources as well as from similarity in chemical properties [23, 24]. As can be seen from Table 6, other elements have weak negative or positive correlation indicating that the presence or absence of one element affect in lesser extent to the other. This poor relationship might be due to soil type, environmental conditions [1, 3, 23].

Table 6. Pearson’s correlation matrices for honey samples.

	Pb	Zn	Cd	Hg	Cu	Co	Mn	Fe	As
Pb	1								
Zn	-0.17	1							
Cd	0.33	-0.35	1						
Hg	-0.49	-0.53	0.05	1					
Cu	0.02	0.93	-0.20	-0.69	1				
Co	0.30	0.83	-0.07	-0.81	0.90	1			
Mn	-0.64	0.68	-0.22	0.08	0.46	0.27	1		
Fe	-0.32	0.97	-0.33	-0.40	0.84	0.72	0.83	1	
As	0.21	-0.78	0.57	0.35	-0.76	-0.60	-0.38	-0.71	1

Comparison of current results with literature values and standards

The concentrations of the heavy and trace metals determined in this work are compared with results of different scholars’ result and standards as shown in Tables 7 and 8.

Table 7. Comparison of the concentration (mg/kg) of essential trace metals in honey samples of present study with literature reviews and standards.

Country/Organization	Metal concentrations				
	Fe	Cu	Zn	Co	Mn
WHO/FAO	30	2	10	NDR	NDR
Turkey	3.5 – 1278	0.22 - 198	1.73 - 245	NDR	NDR
Iran	NDR	5.60	10.9	NDR	NDR
Kenya	0.07 – 1.30	0.03 – 0.12	0.01-0.36	NDR	NDR
Addis Ababa (Ethiopia)	5.37 – 12.43	0.09 – 0.46	1.10 – 4.22	0.60 – 1.17	0.16 – 0.88
Sidama zone(Ethiopia)	NDR	0.03 - 0.07	0.06 - 0.34	ND	0.07 - 0.82
Malaysia	55.83 - 23	0 - 2.93	4.7 - 173.77	0 - 0.12	NDR
Himalya (India)	0.91 - 2.90	0.01 - 0.10	0.13 - 1.50	NDR	0.02 - 0.20
Present study	5.32 - 28.6	0.24 - 0.75	0.63 - 4.40	0.08 - 0.11	0.41 - 3.15

ND=Not detected, NDR=Not determined

Table 8. Comparison of the concentration (mg/kg) of nonessential trace metals in honey samples of present study with literature reviews and standards.

Country/Organization	Metal concentration				Reference
	Pb	Cd	As	Hg	
WHO/FAO	1	0.25	0.5	0.5	[8, 17]
Turkey	3.04	0.30	NDR	NDR	[18]
Iran	0.45	0.01	4.68	NDR	[19]
Kenya	0.06 – 0.49	0.04 – 0.22	NDR	NDR	[20]
Ethiopia	NDR	0.67	NDR	NDR	[21]
Malaysia	ND – 1.02	0 – 1.03	0.03 – 0.13	NDR	[19]
Himalya (India)	0.01 – 0.10	0 - 0.13	NDR	NDR	[22]
Present study	0.25 – 0.33	0.02 - 0.03	0.24 – 0.46	ND – 0.03	

ND=not detected, NDR= not determined

As can be seen from Table 8, all the toxic metals determined in this work are below limit of toxic levels, as compared with standards set by WHO/FAO. Moreover, these limits

are below standard limits set by different countries presented in Table 8. However, according to Samuel's finding, the soil on the farm land investigated in the studied area is heavily contaminated with Cr, Co, Mo and Ni [9]. On the other hands, findings of Ftsum indicated the contamination of Elala River and contains Physico-chemical constituents above limits set by WHO [5].

CONCLUSION

This study in depth focused on determination of concentrations of heavy and trace metals (Fe, Cu, Co, Mn, Zn, As, Pb, Hg, Cd) in honey samples in four districts of Tigray, Ethiopia, using inductively coupled plasma optical emission spectroscopy. The optimized digestion procedure for analysis of the heavy and trace metals in honey samples was found to be effective for all the metals. Moreover, the optimum condition was checked for its validity through spiking from which good percentage recovery 86.83-119.63% was determined for the identified metals. The concentration of all the metals considered in this work are determined. It can be concluded that the honey samples in this study are not free of heavy metals but the levels of the concentrations obtained are found to be below the permitted and toxic levels. And honey in this region could be said out of risk for toxic metals, but still demands close attention.

REFERENCES

- [1] Abebe, Y., Bogale, A., Hambidge, K.M., Stoecker, B.J., Bailey, K and Gibson, R.S, *J. Food Comp. Anal*, **2007**, 20 (3-4), 161-168.
- [2] Hailemariam, A and Bibiso, M, *Int. J. Curr. Res.*, **2019**, 11 (3), 1839-1844.
- [3] Ahmed, K.S and Mohammed, A.R, *Emir. J. Food Agr.*, **2005**, 17 (1), 34-42.
- [4] F.A.O. /W.H.O., **1993**, 54-67.
- [5] Gebreyohannes, F., Gebrekidan, A., Hedera, A and Estifanos, S, *Momona Eth. J. Sci.*, **2015**, 7 (2), 240-257.
- [6] Mineral nutrition, <http://www.esf.edu/efb/course/EFB530/lectures/nutritio.htm>, Accessed, 2019.
- [7] Saxena, S., Gautam, S and Sharma, A *Food chem.*, **2010**, 118 (2), 391-397.
- [8] Escuredo, O., Seijo, M.C and Fernández-González, M, *Int. J. Food Sci. Tech.*, **2011**, 46 (11), 2329-2336.
- [9] Estifanos, S, *J. Environ. Prot.*, **2014**, 5 (2), 144-155.
- [10] Ashenafi, E, *J. Food Chem.*, **2018**, 1, 10-16.
- [11] Afzal, S, Farzanaskandar and Imdadullah, *J. Food Nutr. Res.*, **2014**, 2, 1-7.
- [12] Kambai, C., Popoola, V., Ugbe, C.J., Janfa, N and Ukanyirioha, C, *IOSR J. Environ. Sci., Toxicol. Food Technol.*, **2015**, 9, 94-98.
- [13] Soheil, S and Mona, K, *J. Avicenna Environ. Health. Eng.*, **2016**, 3, 1-6.
- [14] Bogale, Y., Lelago, A and Bibiso, M, *J. Sci. Inclusive Dev.*, **2019**, 1, 16-33.
- [15] Ravisankar, P., Navya, C.N., Pravallika, D and Sri, D.N, *IOSR J. Pharm.*, **2015**, 5 (10), 7-19.
- [16] Farooq Khan, Z and Maqbool, T, *Cien. Inv. Agr.*, **2008**, 35 (2) 199-204.
- [17] Deressa K, *Food Sci. Nutr.*, **2019**, 5, 1-5.
- [18] Sema, S. A., Duygu, T., Filiz, A., Umit, B. B and Mehmet, E. O, *J. Food Sci. Technol.*, **2017**.
- [19] Mohammed, M., Muhammed, A. Z. C., Mohammad, A. R., Siti, A. S and Siew, H. G, *Biomed. Res. Int.*, **2014**, 1, 1-10.

- [20] Maiyo, W.K., Kituyi, J.L., Mitei, Y.J and Kagwanja, S.M, *Int. J. Emer. Sci. Eng.*, **2014**, 2, 1-9.
- [21] Provenzano, M.R., El Bilali, H., Simeone, V., Baser, N., Mondelli, D and Cesari, *Food Chem*, **2010**, 122 (4), 1338-1343.
- [22] Prabhawati, T., Preeti N and Jay, K. T, *World J. Pharm. Pharm. Sci.*, **2016**, 5, 812- 819.
- [23] Zelalem K.A and Chandravanshi, B.S, *Afr. J. Food Agric. Nutr. Dev.*, **2014**, 14(5), 2015-2035.
- [24] Wagesho, Y and Chandravanshi, B.S, *Springer Plus*, **2015**, 4, 107.