Research Article

Title: ***Evaluation of ELISA, Four Plate and HPLC Tests in the detection of Tylosin residue in raw and heat treated chicken***

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Abstract

Tylosin residues (TR) contaminated chicken constitute health risk to humans. TR concentration in chicken from Ikpa slaughterhouse, Nsukka and effect of heat treatment were evaluated using enzyme linked immunosorbent assay (ELISA), four plate test (FPT), and high performance liquid chromatography (HPLC). The muscles, liver, and kidney tissues of 60 randomly processed chickens were harvested and tested for TR at zero-twenty minutes of cooking and microwaving. Of the 180 tissues, 93 (51.7%) were positive; 24 (40%), 33 (55%), and 36 (60%) in muscles, liver and kidney samples respectively with ELISA test. Six out of the 33 (18%) positive liver samples were >100 µg/kg Maximum Residue Level (MPL). The mean inhibition zones were 100% reduced from (9.7- 0) and (11.25 - 0) mm in the muscle and liver tissues after 0-20 minutes of cooking respectively and 100% from (9.5 - 0) and 87 % from (11.5 - 1.5) mm in muscle and liver tissues after 0-20 minutes of microwaving respectively using FPT. Furthermore, 98.7% and 99% reduction from (36.1-0.4) and (47.6 - 0.6) µg/kg in muscle and liver tissues respectively were observed after 0-20 minutes of cooking in the same order using HPLC. Likewise, 100 % and 97.1% from (36.6 - 0) and (47.6 - 1.4) µg/kg in muscles and liver after 0-20 minutes of microwaving in the same order. Chicken at Ikpa slaughterhouse, Nsukka are contaminated with TR even in concentrations above the MRL but were significantly eliminated ($P < 0.05$) after 20 minutes of cooking or microwaving.

Abbreviations

TR =Tylosin residues

ELISA= Enzyme linked immunosorbent assay

FPT= Four plate test

HPLC= High performance liquid chromatography

MPL=Maximum Residue Level

CRD= Chronic respiratory disease

Introduction

Chicken is the second most widely produced, exported and consumed meat worldwide [1, 2]. It has contributed significantly to the supply of about 40% of protein need as demanded by the increasing human population globally [3]. However, the modern-day integrated and intensive production system has been associated with unwarranted use and misuse of antimicrobials in preventing disease occurrences and as growth promoters for poultry in compounded animal feeds [4]. Tylosin is a 16-membered macrolide approved for therapy for a variety of infectious disease agents including *Mycoplasma gallisepticum* and *M. synoviae* that causes chronic respiratory disease (CRD) in poultry. They are metabolized in the liver where the highest tissue concentrations are found especially in chickens and turkeys [5]. TR should not be detected in the edible tissue of treated bird and other products of animal origin in concentrations exceeding the recommended MPL of 100µg/kg [6]. Hence, the recommendation that chickens should not be slaughtered for human consumption 6 days after the last oral tylosin administration [7]. The excess of the residues in meat above MPL could pose high toxicologically, microbiologically or immuno-pathological damage to the consumers of contaminated meat hence the needs to effectively prevent and reduce the TR occurrence in meat for human consumption.

Chicken is usually cooked or roasted ("suya") with or without food additive to increase taste, shell life, digestibility and other sensory properties thereby making them appetizing to the consumers [8]. Nevertheless, the knowledge on the reductive impact of cooking or any other thermal processing methods on TR in heat-and-serve meat are still very limited [9]. There has been a report of an overall reduction rate of TR in meat by 35.3% and 79.9% after 2 and 30 minutes

of microwaving and boiling respectively [10]. Meanwhile, other factors including the concentration of TR in raw meat before processing have been reported to influence the rate of TR reduction in meat [11].

The use of ELISA kit has been reported to have good performance in the analysis of antimicrobial residues like Tylosin in meat as it has the advantages of specificity and sensitivity [12]. It allows the analysis of a large number of samples per kit in few hrs without the requirement of sophisticated instrumentation unlike the Four Plate Test (FPT) [13]. HPLC on the other hand, quantifies the concentration of the residue in meat unlike FPT [14]. It has more prospect of repeatability, selectivity, resolution, high recovery, and ease of application compared to others [15, 16]. However, Enzyme-linked immunosorbent assay (ELISA), high-performance liquid chromatography (HPLC) and other chromatography-mass spectrometry types are generally used in diverse analytical techniques for the detection of antimicrobial residues in foods of animal origin [12, 17, 18]. Tylosin is indiscriminately used by farmers in the study area while data on the contamination rates in the processed chicken are unavailable in the literature to best of our knowledge. Hence it has become necessary to constantly monitor and periodically assess the risks of exposure to TR associated with misuse or abuse in poultry production and take apt action in ensuring meat safety [19]. Moreover, the detection rates with different test and effect of thermal meat processing could be validated to guarantee consumers confidence and meat safety. The study therefore evaluates the concentration of TR in raw, cooked, and micro waved chicken sold to consumers in Nsukka, Nigeria using ELISA kit, Four Plate and HPLC tests.

Results

Detection of TR in muscles, kidney and liver tissues of slaughtered chicken using ELISA kit

Out of 180 tissues of sampled birds, a total of 93 (51.7%) was positive for TR with 24 (40%), 33 (55%) and 36 (60%) of the detected proportion in muscles, liver and kidney respectively while 6 out of 33 (18%) positive liver samples were above the MRL of 100 µg/kg. However, there was no association between the type of tissue and the occurrence of TR ($x^2 = 5.206$; P = 0.0741) (Table 1).

Table 1.

Detection of Tylosin residue in raw chicken tissues from Ikpa slaughter, Nsukka, using ELISA test

Tissue (60 each)	**Frequency (concentration (µg/kg)**		**Proportion detected (%)**	**Above MRL 100 µg/kg (%)**
	Undetected (≤ 2.0)	Detected (> 2.0)		
Muscle	36	24	24 (40)	0 (0)
Liver	27	33	33 (55)	6 (18.0)
Kidney	24	36	36 (60)	0 (0)
Total	87	93	93 (51.7)	6 (6.5)

($x^2 = 5.206$; P = 0.0741). MRL (Maximum Residue Level) WHO (2004)

Effect of cooking and microwaving on TR in muscles and liver tissues of chicken using FPT

The effect of cooking at different time (10, 15 and 20) minutes revealed reduction rates of TR concentration at 28.2%, 64.1% and 100% with the decreasing mean inhibition zone from 9.75mm to (7, 3.5 and 0) mm respectively in muscle tissues, and 22.2%, 53.3% and 100% with decreasing mean inhibition zone from 11.25mm to (8.75, 5.25 and 0) mm in the same order of cooking in the liver tissue (Table 2).

Table 2.

Effect of cooking versus microwaving on TR concentrations in chicken tissues using FPT and HPLC

Detection Method	Tissues	Mean inhibition zone raw chicken (mm)	Mean inhibition zone (mm) and reduction rate (%) after 10-20 (mins) cooking			Mean inhibition zone (mm) and reduction rate (%) after 10- 20 (mins) of microwaving		
			10	15	20	10	15	20
FPT	Muscle	9.75	7.0 (28.2)	3.5 (64.1)	0 (100)	6.7 (29.5)	5.1 (46.3)	0 (100)
FPT	Liver	11.25	8.75 (22.2)	5.25 (53.3)	0 (100)	7.75 (32.6)	5.1 (55. 7)	1.5 (87)
HPLC	Muscle	31.6	16.4 (48.1)	9.6 (69.6)	0.4 (98.7)	22.2 (29.7)	5 (84.2)	0 (100)
HPLC	Liver	47.6	31.8 (33.2)	15.8 (66.8)	0.6 (99)	36.6 (23.1)	16.8 (64.7)	1.4 (97.1)

On the other hand, the impact of microwaving revealed 29.5%, 46.3% and100% reduction rate after 10, 15, and 20 mins with the decreasing mean inhibition zones from 9.5mm to (7.75, 5.1 and 1.5) mm respectively in the muscle tissue and 32.62%, 55.7% and 87% with decreasing mean inhibition zone of (6.7, 5.1 and 0) mm in the same order in the liver tissue (Table 3).

Effect of cooking and microwaving on TR in muscles and liver tissues of chicken using HPLC

The use of HPLC revealed reduction rate of 48.1%, 69.6% and 98.7% in the TR concentration from 31.6 µg/kg to (16.4, 9.6, and 0) µg/kg respectively after (10, 15 and 20) minutes of cooking in the muscle tissue in same order, while 33.2%, 66.8% and 99% reduction in concentration from the initial 47.6 µg/kg to (31.8, 15.8, and 0.6) µg/kg in the same order of cooking

were revealed for the liver tissue. Significant differences exist between contamination rates in raw and cooking of both muscle and liver tissue at 20 mins ($p < 0.05$) (Table 4), (Figs. 2 and 3).

Moreover, the effect of microwaving on TR concentration reduction using HPLC also revealed 29.7%, 84.2% and 100% reduction rate with corresponding values from 31.6 µg/kg to (22.2, 5 and 0) µg/kg after (10, 15, and 20) mins of microwaving respectively in the muscles tissue and 23.1%, 64.7% and 97.1% with corresponding values from 47.6 µg/kg to (36.6, 16.8 and 1.4) µg/kg in the liver tissues in the same order (Table 5). Statistical significant differences exist between the raw and after 20 mins microwaving of both muscle and liver tissues ($P<0.05$) (Figs. 4 and 5).

Discussions

The contamination rate of TR in slaughtered chicken at 51.7% is of public health concern especially with the values above the MPL in the liver tissues. Humans especially the consumers of such muscle parts and liver of contaminated chicken are at the risk of the many chronic health challenges which have been associated with the TR accumulation in the body system [23]. The residue is known to interrupt the colonization barrier of the gastrointestinal tract in humans because of their antibiotic activities against bacterial strains in the human colonic flora [23, 24]. This could lead to antibiotics resistance development and the resistant gene transfer especially when such contaminated chicken are not properly processed via cooking or other thermal heat methods before consumption [25]. The effect is equally of economic consequences with regards to resistant or difficult to treat infections, high cost and longer duration of medication or stay at the hospitals [26]. The health implications of the findings in this study could involve wider range of consumers population as the meat from Ikpa slaughterhouse, Nsukka, Enugu State, Nigeria are usually transported to the neighboring states of the country including Kogi and Benue where they are

processed and consumed in the form of pepper soup by the street meat vendors or as road side ready-to-eat meat 'suya' joints [27]. The accumulation of TR in chicken as revealed in the study could be as a result of constant abuse of the drug in poultry production in Nsukka ranging from wrong dosage, wrong route of administration, non adherence to the withdrawal minimum period of 3 days before slaughtering of treated birds among other factors by farmers and poultry processors [11, 28]. In fact, there are enough evidence that majority of the farmers are ignorant of the consequences of antibiotics misuse or abuse in poultry in Nsukka area in particular and Nigeria at large [29, 30].

The detected concentration (51.7%) of the TR using ELIZA test, serves as true reflection of the contamination rate in the study area since it screened tylosin specifically not just the group of macrolid. The observed rate is higher than 6.3% in meat samples in Croatia using the same ELISA test [31]. Furthermore, the detection rate of TR at 40% in the muscle in this study was slightly lower than 50.6 % in chicken breast meats as reported in Oman using ELISA method.[12] The differences may be a reflection of differences in the level of exposure of poultry to tylosin or abuse by farmers in the developing countries where farmers easily assess drugs over the counter without prescription compared to the developed countries where restrictions on drug use are fully implemented. The higher distribution of the residue in the liver tissues with values above the MPL was in agreement with the report of Pavlov *et al.* (2008) that recorded higher residues in kidney and liver tissues and this could be attributed to the biotransformation and detoxification actions of the liver with slower depletion rate when compared with the muscle tissues [32]. This further agrees with JECFA (2006) report of positive meat samples with higher TR in liver and kidney than muscle tissues [33]. However, the estimated daily intake of muscle tissues is more than that of liver and kidney thereby making muscle tissues more important risk assessment point for TR effect

in humans. The 18% rate of TR exceeding MRL as found in the study was lower than 47.83% in chicken meat with the use of high-performance liquid chromatography method [34]. Meanwhile, the range of tylosin concentration in the study (31.6-47.7) µg/kg was within the average amount of tylosin (38.8) µg/kg reported in china even though it was in swine and bovine tissue samples (muscle, liver, kidney) using LC-MS/MS [35]. On the other hand, very high values (105.4-109.2) µg/kg in 2 (0.6%) of 300 chicken meats has been reported using the HPLC method [36].

The effect of the heat treatments on the TR as detected using FPT has revealed total elimination in both the muscle and liver tissues after boiling for 20 minutes compared to 87% and 100% in muscle and liver tissues after 20 mins of microwaving. This was in agreement with the effect of cooking process that significantly reduced the TR in both thermal processing procedures with negative correlations between the length of cooking time and the reduction percentage of tylosin using HPLC [10]. However, it disagrees with the overall effect of cooking time on TR reduction in meat which was reported to significantly decrease after cooking but not microwaving [37]. This may be because of the longer microwaving time of 20 minutes in this study against the two minutes in the reported study. Lower reduction rate of 35.3% has also been reported for TR after two minutes of meat microwaving [11].

The reductive effects of cooking and microwaving was further confirmed with the detection of the TR concentration in the tissues using HPLC that also recorded similar reduction rates with significant impact after microwaving at 20 minutes [38]. The use of both test have shown that raw muscles and liver with high concentration of TR have time dependent reduction rate when cooked or micro waved. The 100% significant reductions in the concentration of TR for both muscle and liver tissues over time between the raw, cooked and microwaved chicken were in agreement with work done by Salaramoli *et al.* (2015) who also recorded significant reduction in TR in chicken

meatballs subjected to microwaving and boiling treatments [10]. Furthermore, other similar studies have reported 90-100% reduction of antibiotics residues including that of ciprofloxacin, oxytetracycline, sulfamethazine in meat tissues using HPLC [11, 39]. It has been reported that the residue levels in meat tissues following heat treatment of different cooking methods and time reduces in the tissues but increases in the juiced water, however, the level in the juiced water was not checked in this study and can be a limitation.

As a result of abuse or mis-use of tylosin, and not complying with the withdrawal period in treated poultry, TR were found in slaughtered chicken tissues from Ikpa slaughterhouse even in concentrations above the MPL hence, constitutes health risk to the consumers. Awareness campaign on health implication of TR in ready to-eat-meat, judicious use of the antibiotics, adherence to withdrawal time before slaughter, the use of probiotics as alternative in poultry production has become inevitable. Adequate heat application on meat either by cooking or microwaving should be encouraged especially for the ready-to-eat meat since 20 minutes of cooking or microwaving have shown to significantly eliminated TR in meat. Further studies to check the concentration of the TR in the juice (broth) of the cooked chicken should be encouraged since consumers also drink the juice of cooked meat in different forms.

Materials and Methods

Ethical approval

The protocol for the research project was approved by the Institutional animal care and use committee of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka with reference no; FVM-UNN-IACUC-2024-03/147.

Study area

The study area is Nsukka while the sample collection site was Ikpa slaughter (Fig. 1) [20]. The Ikpa slaughterhouse was visited twice a week and on each visit, a 1 in 4 systematic random sampling technique was used to select three out of 12 to 15 birds from poultry retailers that bring birds for slaughter from different towns within Nsukka environs including the university community. Two birds were selected using simple random sampling technique from each selected retailer. In each visit, six birds were purchased, i.e.12 birds per week for 5 weeks. A total of 60 birds was purchased and slaughtered; the breast muscles, kidney and liver tissues were harvested. The total of 180 tissue specimens were collected using sterile universal bottles and transported in cold condition which was maintained with ice blocks to the Department of Veterinary Public Health and Preventive Medicine, University of Nigeria, Nsukka laboratory for analysis.

Sample preparation for TR detection

A 2g each of the harvested tissues was weighed, macerated and emulsified with equal volume of distilled water in a 1:1 ratio and introduced into centrifuge tubes. The tubes were centrifuged at 5000 rpm for 10 mins and the supernatant decanted while required quantity of the solution was used for Tylosin detection.

Detection of TR with ELISA test

The microtitre plates of ELISA test kit and the reagents were sourced from Shenzhen Lvshiyuan Biotechnology Company Limited, Shenzhen, China. The microtitre plates of ELISA KIT and the reagents were adjusted to room temperature before use. The lyophilized conjugate was reconstituted first with 1ml of conjugate diluents, vortexed and diluted with the same conjugate diluents at 1:10 ratio. The standards and the control were reconstituted with 1ml of deionized water. The wash buffer (5X) concentrate was diluted at a ratio of 1:5. For each plate, a

working scheme was prepared, the standards and samples were run in duplicates as previously reported [21], and briefly described; Twelve strips, each containing 8 wells, were fixed on the plate. Each of the six provided 50 µl standard solutions (0, 2, 6, 18, 54, and 162 ng/kg) was added in duplicate wells according to the working scheme. 50 µl of each tissue extract sample was added in duplicate wells following the standards according to the working scheme. The antibody conjugate (50 µl) was added to each of the wells. The plate (wells) was covered with a paraffin tape and the content mixed by circular motion on the bench for several seconds and then incubated at 30ºC for 30 seconds. It was tapped from time to time to remove bubbles. The microtitre wells were further washed with a wash solution five times and tapped to remove bubbles completely. Solution A (50 µl) color was added followed by solution B color immediately and mixed thoroughly by shaking. The microtitre plate was incubated at 37ºC for 10 minutes. Stop solution (50 µl) was then added. The absorbance was read at 450 nm wavelength, within 5 min of adding the Stop solution.

Thermal treatments of ELISA positive samples

Each positive sample with high concentration of tylosin after ELISA test was divided into two parts by weight, and then subjected to different processing methods (boiling and microwaving). Twenty gram sample each was placed into a strainer, immersed in 10 ml water bath preheated to 100ºC and cooked for 10, 15, and 20 min, and allowed to cool before extraction while same quantity of sample was placed in a glass tray and microwaved at 450 W for 10, 15, and 20 min and allowed to cool before extraction.

Analysis of raw and heat treated meat samples using FPT and HPLC

All the raw and heat treated samples were subjected to FPT and HPLC analysis to determine the residue level using modified methods as previously reported [21, 22].

Four Plate Test: Briefly, two grams of each organ were macerated with equal volume of sterile water in porcelain mortar and pestle, centrifuged at 3000 revolutions per minute (rpm) in a test tube for 10 minutes after which the supernatant was decanted and stored for analysis. Three batches of Muller Hinton agar were prepared according to manufacturer's recommendations and autoclaved. After cooling to 45 – 50oC, they were adjusted to pH 6, 7.2 and 8 using NaOH (base) and HCL (acid). Ten millilitre of the media was poured on sterile Petri dishes and allowed to solidify. Each plate with pH 6, 7.2 and 8 was seeded with a ready to use suspension of *Bacillus subtilis* (Merck Darmstadt, Germany) and another media at pH 8 was seeded with 24-hour culture of *Micrococuss luteus* bacterial suspension (ATCCR 10240). Four wells were bored, and 80 µl of each tissue extract was inoculated into each of the wells and the fourth well was inoculated with tissue extract from oxytetracycline treated bird as positive control. After, the plates were incubated for 24 hours at 37°C. They were observed for clear zone of inhibition with annular diameter ≥ 2 mm, which indicated positive test for antimicrobial residues.

HPLC-based test: Briefly, tylosin stock solution was obtained from Sigma (St Louis, MO, USA). One mg/ml tylosin was prepared by dissolving 10 mg tylosin tartrate in 10 ml methanol and stored at -18oC. Working standard solutions for calibration curve was prepared by appropriate diluting of the stock solution, using dilution factor. The kidney, liver and muscle samples that were tested and confirmed to be free of macrolide antibiotic residues (control) were used as blanks for the preparation of matrix matched calibration curves. For fortification, standard solutions were prepared by dissolving standard substance in methanol at concentrations 40, 20, 10, 5, and 2.5 mg/ml. Two grams each of the kidney, liver and muscle samples of birds were weighed and macerated with mortar and pestle. 2 ml of distilled water was added, followed by 10 ml HPLC grade acetonitrile. It was then mixed with vortex mixer to homogenize for 1minute. Then, the

sample was centrifuged for 15 minutes at 3,000 rpm. The clear extracted solvent layer was then collected using disposable pasture pipettes and diluted to 50 ml with distilled water. The SPE Cartridges Bond Elute C18 500 mg/3ml were activated with 2 ml of methanol and 5 ml of distilled water. The cartridge was washed with 20 ml of distilled water and allowed to dry. The extracted sample solution was loaded and allowed to elute from the cartridge with 3 ml of HPLC grade methanol. The solution was then filtered using 0.45 micromilllipore syringe filter. The samples were manually injected into the HPLC. Chromatographic analysis was performed with isocratic elution on Zorbax Eclipse XDB - C18 (150 x 4.6mm, 5 µm) analytical column at 30oC. The mobile phase composed of HPLC grade acetonitrile and water (90:10), at the flow rate 1.00 ml/min, 20µl was injected. The chromatogram was monitored at wavelength 292 nm.

Statistical Analysis

Statistical analysis was performed with Graphpad prism statistical software version 5.02. Chi-square (χ^2) test was used to evaluate association between TR concentrations with the tissue types. Kruskal–Wallis test was used to compare differences between the mean concentration of the raw values and each of the different cooking and microwaving time. p-value < 0.05 was considered statistically significant.

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Figure legends

Fig 1. Map of Nsukka urban with blue rectangle showing the study site (Ikpa slaughter)
Source: Nwankwo *et al.* (2021).

Fig. 2. Mean concentration of Tylosin residue in raw muscle tissue and after cooking for 10, 15 and 20 minutes

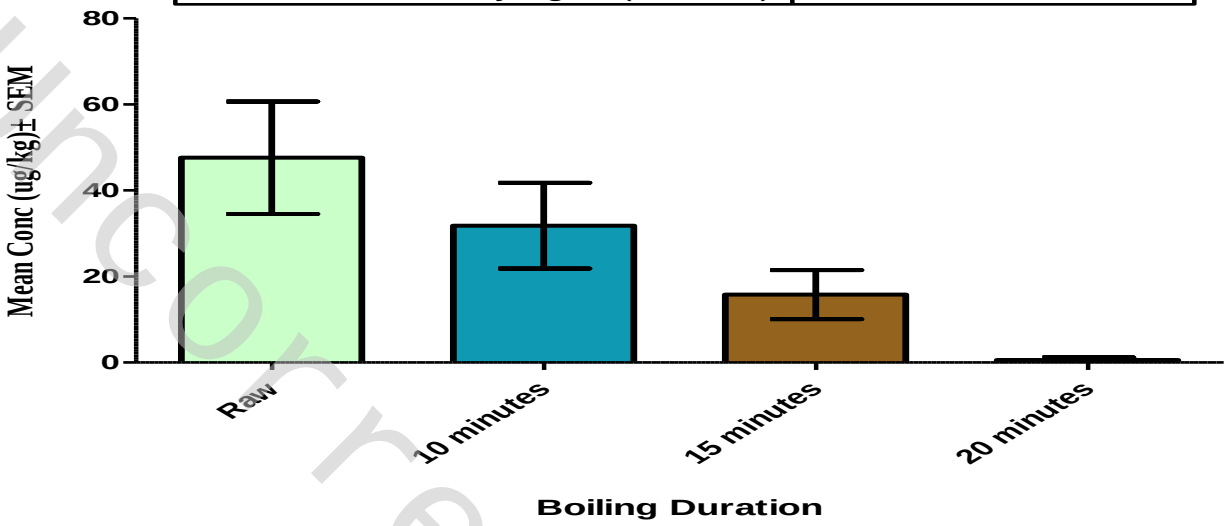
Fig. 3. Mean concentration of Tylosin residue in raw liver tissue and after cooking for 10, 15 and 20 minutes

Fig. 4. Mean concentration of Tylosin in raw liver tissue and after microwaving for 10, 15 and 20 mins

Fig. 5. Mean concentration of Tylosin in raw muscle tissue and after microwaving for 10, 15 and 20 minutes

Figure 3.

Kruskal-Wallis test	
P value	0.0094
Exact or approximate P value?	Gaussian Approximation
P value summary	**
Do the medians vary signif. (P < 0.05)	Yes



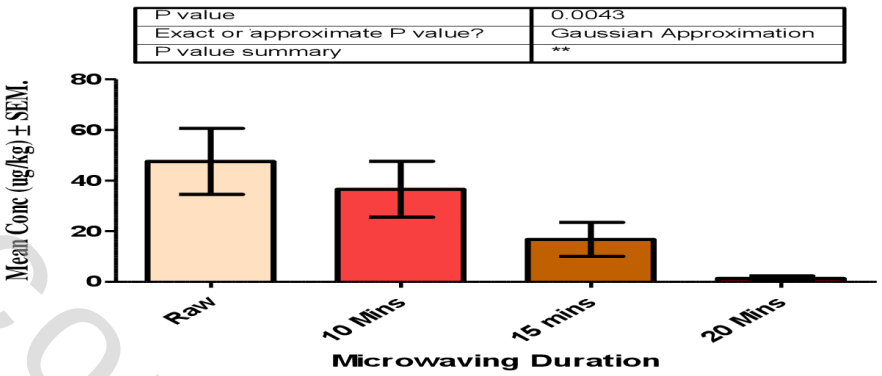


Fig. 4

P value	0.0028
Exact or approximate P value?	Gaussian Approximation
P value summary	**

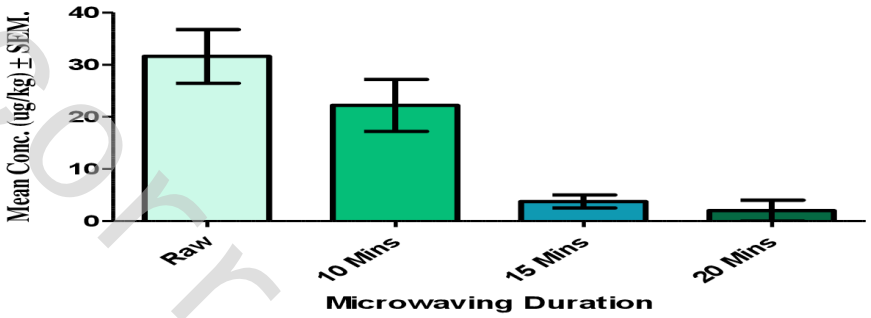


Fig. 5

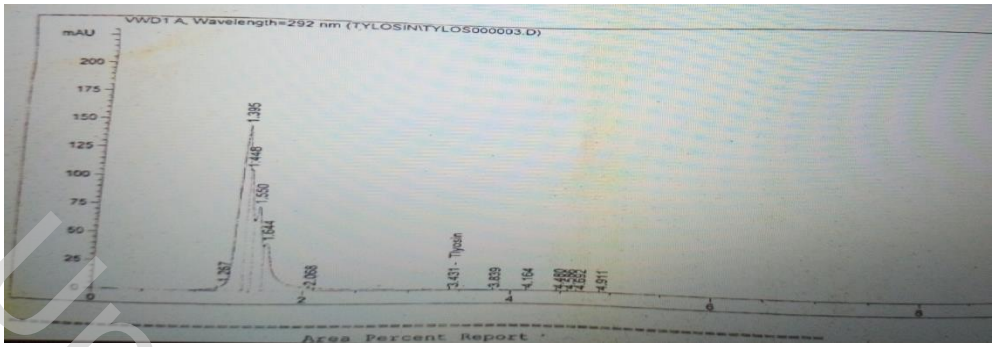


Fig 6.