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**Mohammed Radi Obaid**

High School, Ministry of  
Education Babel Arrangement  
Directorate, West Bengal, India

## Evaluation of CYP3A4 activity and tetracycline residues in meat poultry after exposure to Hydroxytyrosol, Anethole and Eugenol

**Mohammed Radi Obaid**

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### Abstract

**Background:** The residues of antibiotics in meat especially in chicken meat are a risk source on public health in animal and human. This study aimed to determine tetracycline residues in the chicken samples (thigh, chest and liver) via analytical method HPLC with evaluation of CYP3A4 in liver tissue homogenate using Eliza Kit after administration of Hydroxytyrosol, Anethole and Eugenol. Methods : In this study, sixty chickens chosen according to weight and healthy condition divided into four equal groups, they dosed the antibiotic tetracycline (250 mg / liter) for 3 days. First group as positive control second received orally eugenol (150 mg/kg), third group received orally anethol (100 mg/kg), and fourth group received orally hydroxytyrosol (50 mg/kg). These compounds were administrated only on the 4<sup>th</sup> and 5<sup>th</sup> day of the experiment, on the 6<sup>th</sup> day all poultry were slaughtered and samples were taken from the thigh, liver and chest to evaluate the tetracycline residues. Results: The present study noted that hydroxytyrosol has moderate activity to clear the tetracycline residual from chicken thigh, chest and liver without effect on CYP3A4 activity, a significant induction of CYP3A4 activity was recorded with using of eugenol and anethole that might be was responsible of metabolism and excretion of tetracycline residues.

**Keywords:** CYP3A, hydroxytyrosol, eugenol, anethole, poultry

### Introduction

Poultry is commercially grown for meat consumption as they rapid grow and the fiscal employment is uncostly (Salman *et al.*, 2022) <sup>[26]</sup>. In fact, human consumption for poultry meat is increased day by day, different medication today utilized to stimulate growth and manage their infection. Antibiotics are synthetic compounds, or produced in nature by soil bacteria and fungi that destroy or inhibit the growth of bacteria (Hutchings *et al.*, 2019) <sup>[11]</sup>. The antibiotics widely utilized in veterinary field as a prophylactic, growth-promoter and control of bacterial infection in livestock production and poultry field. In chicken treatment antibiotics has broad methods of administration as mixed with drinking water, feed, or by injection (Om and McLaws, 2016) <sup>[21]</sup>. After antibiotic treatment in poultry, antibiotics accumulate in meat and eggs, after some weeks, the residue drop slowly because of metabolic regression (Jalloob *et al.*, 2022) <sup>[12]</sup>. The antibiotic withdrawal period is mean that last doses of an antibiotic consumed by chicken before slaughter and which accumulate in meat in acceptable limit according to FDA. Before of the withdrawal periods, the meat chicken or eggs are not favorable for humanitarian consumption (Mdegela *et al.*, 2021) <sup>[16]</sup>. The residual of tetracycline or their metabolites may cause adverse effects after consumed poultry meat by humans. Mutagenic, carcinogenic, bone marrow damage, resistance due to Chloramphenicol and Penicillin respectively are produced due to consumption of these residual in food chain (Bilal *et al.*, 2020) <sup>[6]</sup>. The resistance of bacteria to drugs is another important critical issue may be the perturbation of intestinal micro flora (Mukherjee *et al.*, 2018) <sup>[20]</sup> Tetracycline's are a family of antibiotics that block protein synthesis via block bind of aminoacyl tRNA at ribosomal receptor (A) site (Patrabansh *et al.*, 2020) <sup>[24]</sup>.

**Corresponding Author:**

**Mohammed Radi Obaid**

High School, Ministry of  
Education Babel Arrangement  
Directorate, West Bengal, India

Tetracycline has classified their activity as broad-spectrum and minimum cost, tetracycline (TC) involving oxytetracycline, chlortetracycline and doxycycline are generally utilized in prevention and treatment many bacteria and mycoplasma as well as large virus from side and feed additive from another side as growth promoter (Yuann *et al.*, 2022) [30]. Anethole is one of the aromatic ether compounds that appear the most predominate and effective substances in some herbs, especially in the anise of fruits and fennel (Abbasi *et al.*, 2022) [11]. The importance of anethole stems from its several activities, which include aromatic, antibacterial, antioxidant, and characteristics with a highly sweet flavor. Therefore, anethole is commonly applied in different medical, nutritional, and industrial applications (Pascual-Villalobos *et al.*, 2020) [23]. Eugenol has a potent antioxidant effect, which was considered one of the most essential properties that act to reduce free radical and improvement public health as well as immunity against many disease (Jasim *et al.*, 2019) [13]. Eugenol is highly effective in reducing free radicals compared to many other synthetic compounds (Al-Ahbabi *et al.*, 2016) [3]. There are many medical uses for eugenol as an ant proliferative for cancer cells, as well as its role in alleviate the adverse effects of many chemical drugs for tumors, which was accompanied by an block of the proliferation of cancer cells by up to 50% during the inhibition of expression of certain genes that have an effect on division of cell (Hamdoon *et al.*, 2020) [9]. Hydroxytyrosol (HT) is extracted from herbal plant olive oil and has many medical application such as anti-cancer and anti-inflammatory activities, immune stimulant and antioxidant (Paraskeuas *et al.*, 2017) [22].

The liver microsomes include the CYP3A marker, which may prevent the production of reactive oxygen species. Numerous studies have examined the effects of various olive oil phenols and related test compounds on the suppression of 6 $\alpha$ -androstenedione hydroxylase activity (a CYP3A marker) in human liver microsomes. Athukuri and Neerati (2017) [5] reported that olive oil derivatives as oleuropein and hydroxytyrosol and other structurally similar compounds has some influence on in rat liver microsomal enzymes (CYP3A and CYP2C11). The current study aimed to use of active substances of plant origin produced from anise, cloves and olive oil with anti-bacterial activity and high effectiveness in inducing liver enzymes responsible for the process of excreting drugs and toxins, especially antibiotics, and removing them from the poultry body, thus making them suitable for human consumption.

## Material and Methods

### Materials

Trans-Anethole (C<sub>10</sub>H<sub>12</sub>O) purchased from Tokyo Chemical AMIRCA Industry (TCI America) (100g/100ml), Tokyo, Japan, CAS No. 4180-23-8, concentration > 98%, Molecular weight 148.21. Eugenol (C<sub>10</sub>H<sub>12</sub>O<sub>2</sub>) was purchased from CHEM CRUZ Biotechnology company, Inc. (100g/100ml), Dallas, USA, CAS No 97-53-0, concentration > 98%, molecular weight 164.20. Hydroxytyrosol 98%, purchased from Shenzhen Dieckmann Tech Co., Ltd

### Experimental Chickens

This study is carried out in poultry Farm, which belongs to the Hamza Agricultural High School, in Al-Medhatiyah sub-district / Babil Governorate. The research was conducted in a semi-closed hall divided into four partitions and its dimensions were 3 x 2 m<sup>2</sup>, divided by wooden partitions, wire

mesh and independent doors. Saw dust with a height of 10-8 cm and equipped with all breeding requirements that included feeders, manholes and gas incubators. Lohman brown chicken 8 weeks. The chicken farm had air conditioning, which kept the ambient temperature close to 25 C and the relative humidity between 45 and 65%. The light cycle (16 hours of light/8 hours of dark) was the same as that used in the commercial farm. Throughout the experiment, no clinical symptoms of a disease were discovered.

### Experimental design

In this study, sixty chickens chosen according to weight and healthy condition with an approximate weight of 1800-2000 gram were selected and used, randomly distributed into four equal groups, they dosed the antibiotic tetracycline (250 mg / liter) for 3 days (Yousif and Jwher, 2021) [29]. First group as negative control, second given orally eugenol (150 mg per kg), third group received orally anethol (100 mg per kg (16), and fourth group orally given hydroxytyrosol (50mg per kg). These compounds were administrated only on the 4<sup>th</sup> and 5<sup>th</sup> day from experiment. On the 6<sup>th</sup> day all poultry were slaughtered and samples were taken from the thigh, liver and chest to evaluate the tetracycline residues.

### Working solution

Na<sub>2</sub>EDTA-McIlvaine (pH 4) as a buffer solution of was ready by dissolving 15000 mg of disodium hydrogen phosphate, 3.72 g of EDTA in deionized water, 13000 mg of monohydrate of citric acid and as well as complete solution to 1 L.

2.5. Extraction procedures sample meat was isolated to extracting tetracycline residues from chicken samples (thigh and liver) where 5 000 mg sample were used: chicken meat placed in a 25mL tube, 20ml buffer from Na<sub>2</sub>EDTA-McIlvaine has pH 4 was transfer, and centrifuged at 5000 for 20 min. The supernatant was loaded with methanol (5mL) and Milli-Q water (10 ml). The solvent was removed using oven 45 c. PTFE filter (0.45 m) was used for residues filtration and freezing until HPLC detection (Moghadam *et al.*, 2018) [19].

### Chemicals and reagents

Tetracycline-HCl (TC) were purchased from TCI japan. Methanol and acetonitrile HPLC grade type from Merck company. others chemical were purchased from china such as citric acid monohydrate and disodium hydrogenphosphate.

Every day, a phosphate buffer solution with a pH of 2.5 was made by combining 0.01 M H<sub>3</sub>PO<sub>4</sub> and 0.1 M Na<sub>2</sub>HPO<sub>4</sub> (adding a few drops to correct the pH). The mobile phase for the HPLC is composed of phosphate buffer (0.01 M, pH 2.5) and 20% acetonitrile.

Chicken Cytochrome P450 3A4 (CYP3A4) ELISA Kit Catalogue Number:SL0208Ch was purchased from sunlong biotechnology.

### Standard solutions

standard Stock solution of each TC drug was done by dissolving 10 mg of the TC in 10 ml of methanol to obtain a final concentration of 1 mg /ml A and stored at 20 C and were stable for at least 4 weeks.

### Chromatographic separation of TCs

A Waters Acquity UPLC system (Waters Corporation, Milford, MA, USA) that included a binary flow solvent administration system, an online degasser, and an automatic

sampler was used for the analysis. The detector was a triple quadrupole mass spectrometer manufactured by applicable biosystems (shimadzu, Japan). The column was a Waters ACQUITY™ UPLC BEH C18 2.1 mm 50 mm, 1.7 m size (Waters, Milford, MA, USA). The tetracycline was separated by program of a gradient elution. Mobile phase was done by acetonitrile (80:20, v/v) and phosphate buffer (0.01 M, pH 2.5), a flow-rate was 1.0 mL per min at room temperature. The injected sample at volume was 10-μ. The method was cycle tim 10 min per injection (Malar *et al.*, 2020) [15].

**Evaluation of CYP3A4 activity**

The activities of Cytochrome P450 3A4 was measured using sunlong kit (China). absorbance measuring was determined by tecan microplate reader from SunRise, Vienna, Austria).

**Statistical analysis**

The data of each group was listed as the mean plus SE. A one-

133-way test of variance was applied followed by competed randomized design. At  $p < 0.05$ , variations were appear statistically significant (Michel *et al.*, 2020) [17].

**Results**

Figure (1, 2 and 3) recorded highly a significant values in the tetracycline residues in the liver, thigh , and chest muscle to recorded mean value ( $74.25 \pm 8.5$  ,  $35.6 \pm 6.4$  and  $18.5 \pm 2.53$  μg/ g) respectively in poultry group that received only tetracycline, where the chicken that received tetracycline and hydroxytyrosol showed significant reduction in concentration of antibiotic in the liver and thigh and chest muscle (  $37.75 \pm 14.3$  and  $24 \pm 2.6$  and  $10 \pm 2.4$  μg/ g). While, chickens that received Anethole and Eugenol that revealed highly a significant reduction of tetracycline residues in liver, thigh and chest muscle ( $10.25 \pm 3.28$ ,  $2.25 \pm 0.75$ ,  $1.75 \pm 2.04$  μg/g) and ( $6.75 \pm 1.8$  ,  $2.7 \pm 1.11$  and  $0.95 \pm 0.03$  μg/ g) respectively when compared with other treated groups.

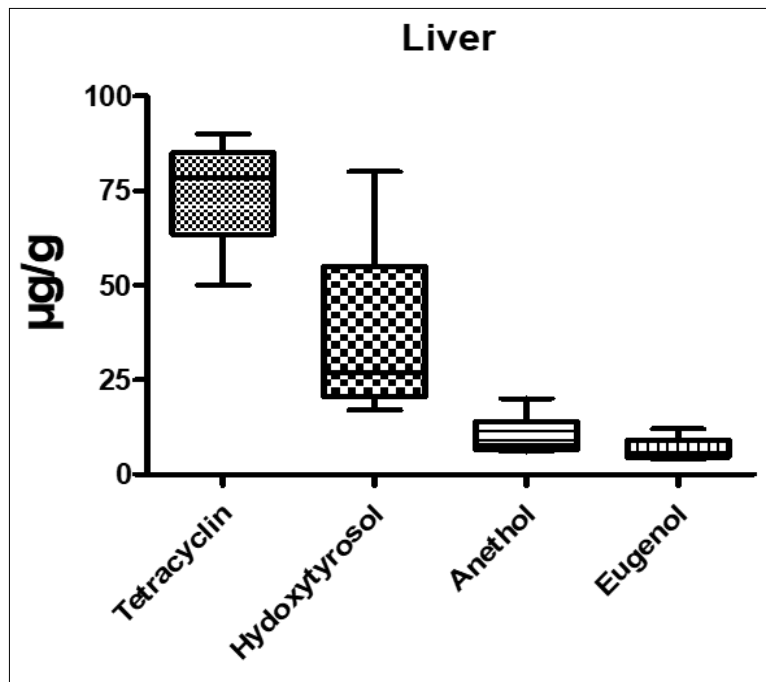


Fig 1: Tetracycline concentration in liver tissue of chickens after received hydroxytyrosol, anethole and eugenol

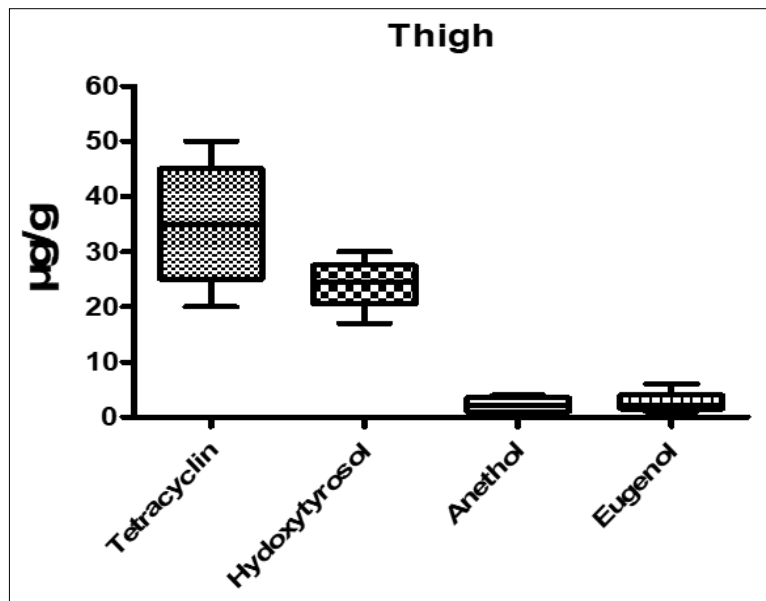
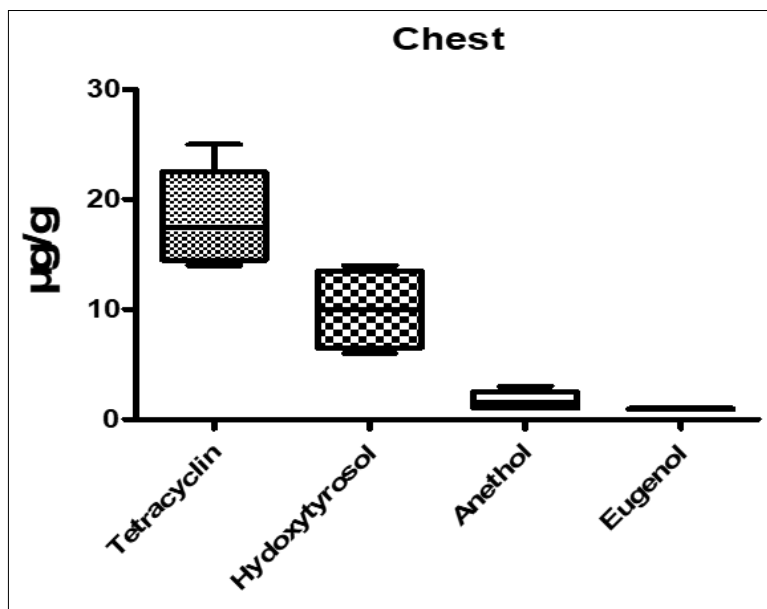
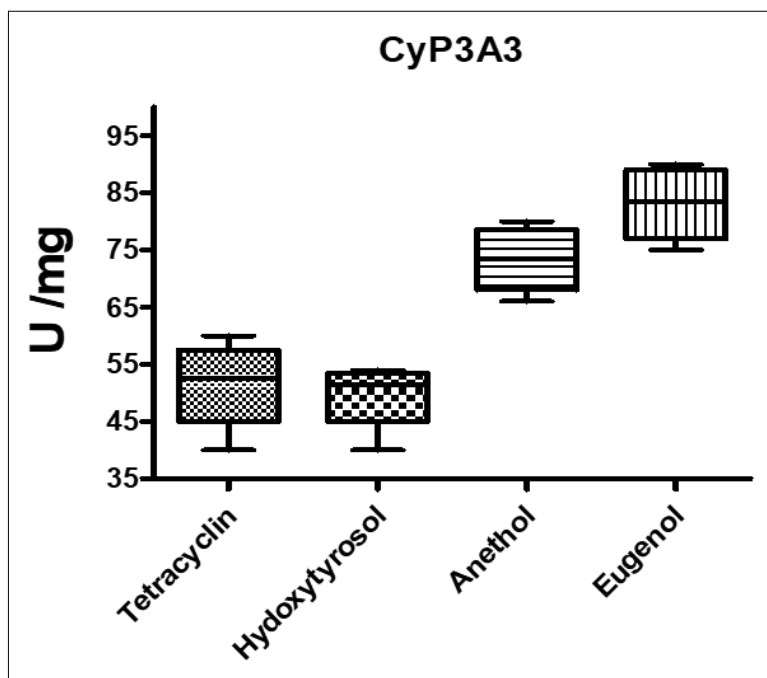


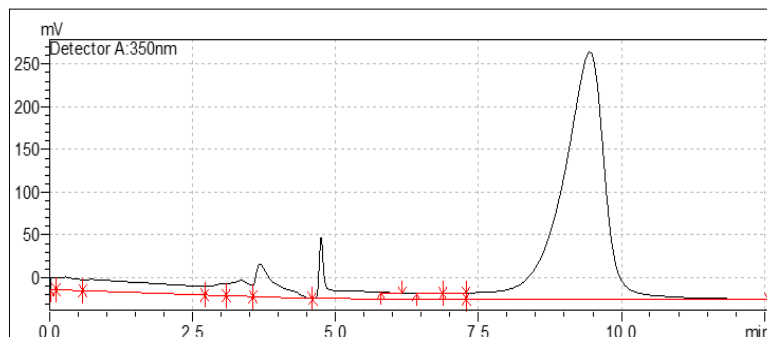
Fig 2: Tetracycline residues in thigh tissue of chickens after received hydroxytyrosol, anethole and eugenol



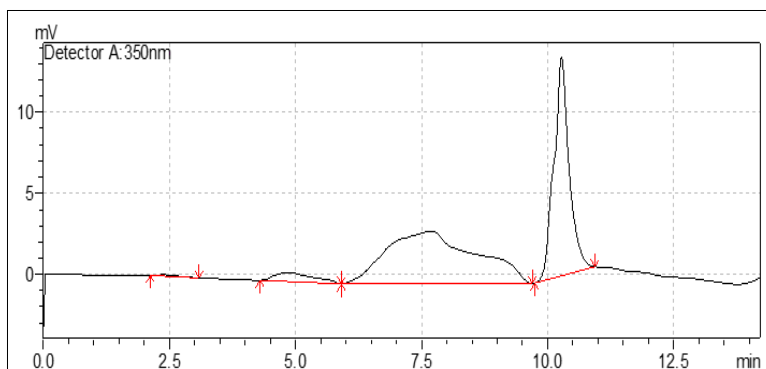
**Fig 3:** Tetracycline concentration in chest tissue of chickens after received hydroxytyrosol, anethole and eugenol



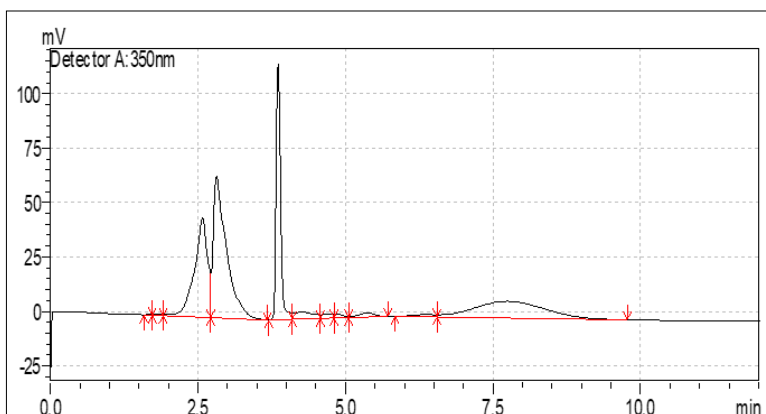
**Fig 4:** Tetracycline concentration in Cyp3A4in liver tissue of chickens after received hydroxytyrosol, anethole and eugenol



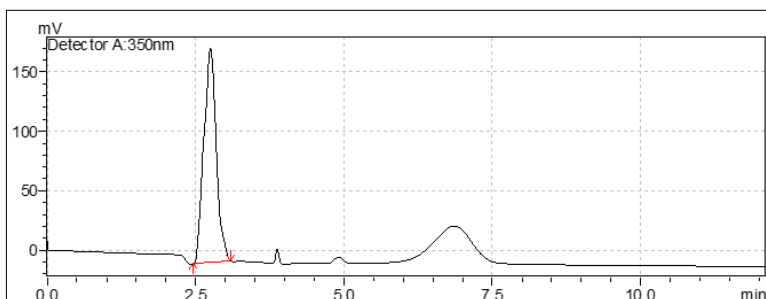
**Fig 5:** HPLC retention time of standard tetracycline



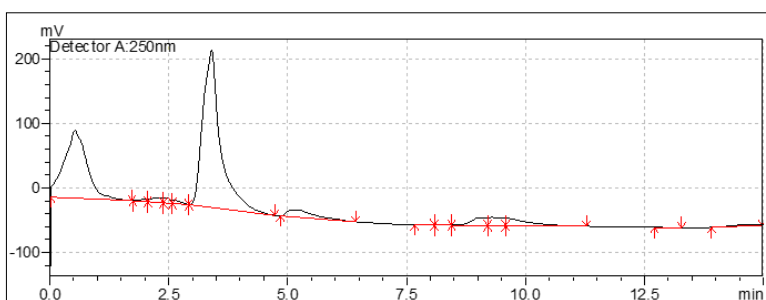
**Fig 6:** HPLC retention time of tetracycline in liver tissue



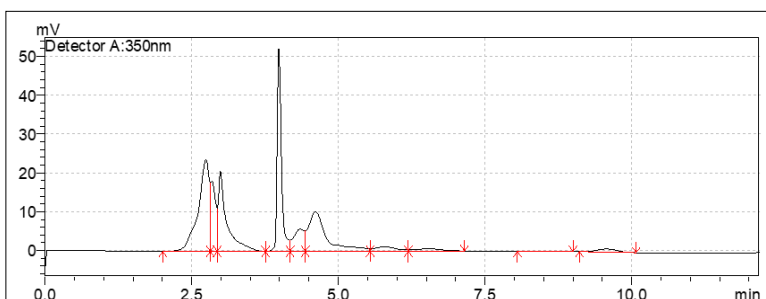
**Fig 7:** HPLC retention time and area under curve of tetracycline of thigh tissue after received Euglenol.



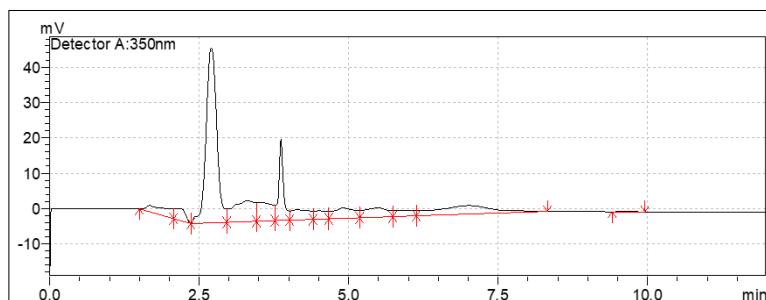
**Fig 8:** HPLC retention time and area under curve of tetracycline of liver tissue after received Euglenol .



**Fig 9:** HPLC retention time and area under curve of tetracycline of liver tissue after received hydroxytyrosol from tissue of liver



**Fig 10:** HPLC retention time and area under curve of tetracycline of thigh tissue after received hydroxytyrosol



**Fig 11:** HPLC retention time and area under curve of tetracycline of liver tissue after received hydroxytyrosol in liver

Poultry meat and eggs are important foods to meet the nutritional needs of an

The difference was no a significant at level CYP3A activity in chicken that received Tetracycline ( $51.2 \pm 4.26$  U/mg) and that received hydroxytyrosol ( $49.25 \pm 3.1$  U/mg). While a significant increase of CYP3A4 activity was recorded in groups that received Anethole and Eugenol ( $73.2 \pm 3.19$  and  $83 \pm 3.51$  U/mg) respectively figure (4). Figure 5: HPLC retention time of standard tetracycline, Figure 6: HPLC retention time of tetracycline in liver tissue, Figure (7 and 8) HPLC retention time and area under curve of tetracycline of thigh and liver tissue after received Eugenol .in addition to , Figure( 9 and 10) HPLC retention time and area under curve of tetracycline of liver and thigh tissue after received hydroxytyrosol. HPLC retention time and area under curve of tetracycline of liver tissue after received Anethole in liver ( Figure 11).

## Discussion

Poultry meat and eggs are important foods to meet the nutritional needs of an ever-increasing population. However, the high production of poultry necessitates the use of many medicines, such as antibiotics, as preventive and curative means to ensure rapid growth and improvement of animal's health. However, the improper and unwise use of these antibiotics leads to the accumulation it and their toxic and harmful metabolites in the meat and eggs of the treated birds which affects the health of the consumer by induce allergic reactions and transmission of antibiotic-resistant microbial infection and many side effects on the vital organs of the body. The identification of Peak in the chicken sample was depend on value of retention time tetracycline standard was recorded at 9.38 minutes. This retention time was applied for three peak identify in the chromatogram of the meat sample. The residues tetracyclin in meat chicken samples were calculated depend on retention time, i.e. 9.38 minutes, Moreover the retention time in current study was concord with agreement with who reported with that showed retention time at same area (Hamdoon *et al.*, 2020) [9]. Cytochrome P450 3A4 (abbreviated CYP3A4) is an important enzyme in the body, mostly found in the intestine and liver. A tetracycline one of substrate of this enzyme that responsible of metabolism of many drugs.

Current study showed that chicken received tetracycline and hydroxytyrosol showed significant reduction in residues of tetracycline in the liver and thigh and chest indicating moderate excretion to tetracycline. Furthermore the liver tissue homogenate showed that hydroxytyrosol has negative effect on CYP3A4. Cytochrome P450 (CYP) 3A4 is abundant isoform in liver and responsible for metabolizes nearly about 60% of the therapeutic drugs. This character provides CYP3A4 more susceptible to reversible and irreversible inhibition. The olive oil has major phenolic compounds involve oleuropein, hydroxytyrosol and tyrosol are likely

involved in the inactivation of CYP3A4 (Joven *et al.*, 2013) [14]. Eugenol is one of active compound of clove that has Anti-inflammatory, anti-carcinogenic properties, block 5-lipoxygenase activity and in turn LTC<sub>4</sub> production in human (Ullah *et al.*, 2023) [27]. It also alleviates LPS-induced COX-2 expression leading to block PGE<sub>2</sub> production. Eugenol considered major constituents, it involve (60–75%) of clove compounds strongly activated the CYP3A4 promoter (Butt *et al.*, 2013) [7]. Induction of CYP450 has role in detoxifying of drugs and poisoned materials. Antioxidant enzymes from plants and endogenous has advantage for cancer prevention (Almashhadany, 2020) [4]. In normal intestinal cells; in cell with cancer state block proliferation by minimize the high oxidative state cancer cells need for proliferation (Raghavenra *et al.*, 2006) [25]. Induction of CYP3A4 might be beneficial, since an enhanced tetracycline metabolizing system and eliminate antibiotics accumulation (Miyata *et al.*, 2022) [18]. On the other hand, many xenobiotics up-regulate CYP3A4 or inhibit it activity leading to reduce ability to metabolize toxicants. Clove, and its essential oil has important role in poultry via ameliorate growth rate by boost the intestinal microbial inhabitance (Abdullah *et al.*, 2021, Hussein *et al.*, 2019) [2]. The meat quality very important guide in poultry meat palatability could be increased by adding plant source of antioxidants like clove has potent antioxidant due to high content of phenolic acid that increase from meat palatability. In the present study, tetracycline residual a significantly reduced in chicken received eugenol and anethole (Wieczyńska and Cavoski, 2018) [28]. Recent studies showed that volatile oils extracted cinnamon and cumin cloves as natural source of antioxidants that aid in remove of hepatotoxicity result from expose to cyclophosphamide by normalize of liver enzyme and regenerate of liver tissue and return changes in activities of internal antioxidant enzymes like SOD, GST, CAT, and glutathione peroxidase) as well as inhibit the expression of CYP 2E1 and 3A4 (D'Angelo *et al.*, 2020) [8].

## Conclusion

The present study concluded that hydroxytyrosol has moderate activity to clear the tetracycline residual from chicken mean of thigh, chest and liver without effect on CYP3A4. A significant highly inducer activity of CYP3A4 was recorded with using of eugenol and anethole that might be was responsible of metabolism and excretion of tetracycline residual. That will give promise in future for safely use in improvement meat quality and control antibiotic residual for control antibiotics resistance by bacteria

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**Ethical:** This clinical studies in has been approved according to Ethics Commission at NO:533FD2 specific for College

Veterinary Medicine.

**Conflict interest:** In this study was no any competing interest.

**Author's contribution:** Study design and conception were performed by Mohammed Radi Obaid. Materials preparations and procedural works were done by mohammed abed Al-Hussein. Data statistically analysis were done by Raed Hussein Salih. The first draft manuscript was written by Adnan Mansour jasim reviewed the Draft. Every author checked and approved the final manuscript.

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