Antibiotic Residues in Honey

INVESTIGATORS

Dr Sapna Johnson

Dr Nimisha Jadon

ADVISORS

Prof. H. B. Mathur

Prof. H. C. Agarwal

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CENTRE FOR SCIENCE AND ENVIRONMENT

41, TUGHLAKABAD INSTITUTIONAL AREA, NEW DELHI –110062
TEL: 91-11-2995 6110/5124/6394/6399
FAX: 91-11-2995 5879 EMAIL: cse@cseindia.org
WEBSITE: www.cseindia.org

POLLUTION MONITORING LABORATORY

INDIA HABITAT CENTRE, CORE-6A, FOURTH FLOOR
LODHI ROAD, NEW DELHI – 110003
TEL: 91-11-24645334/24645335
FAX: 91-11-24645334

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1. Pollution Monitoring Laboratory of CSE

The Centre for Science and Environment (CSE), a non-governmental organization based in New Delhi, has set up the Pollution Monitoring Laboratory (PML) to monitor environmental pollution. PML is an ISO 9001:2008 certified laboratory accredited by SWISO, CH-5610, Wohlen, Switzerland, conducting Pollution Monitoring and Scientific Studies on Environmental Samples. The Lab has highly qualified and experienced staff that exercise Analytical Quality Control (AQC) and meticulously follow what is called Good Laboratory Practices (GLP). It is equipped with sophisticated state-of-the-art equipments for monitoring and analysis of air, water and food contamination, including Gas Chromatograph with Mass Detector (GC-MS), Gas Chromatograph (GC) with ECD, NPD, FID and other detectors, High Performance Liquid Chromatograph (HPLC), Atomic Absorption Spectrometer (AAS), UV-VIS Spectrophotometer, Mercury Analyzer, Respirable Dust Sampler etc. Its main aim is to undertake scientific studies to generate public awareness about food, water and air contamination. It provides scientific services at nominal cost to communities that cannot obtain scientific evidence against polluters in their area. This is an effort to use science to achieve ecological security.

2. Introduction

Honey has the image of being a natural and healthy product. However, today honey is produced in an environment, polluted by different sources of contamination. The contamination sources can be environmental and apicultural ones. Environmental contaminants are pesticides, heavy metals, bacteria and radioactivity. These contaminants are present in air, water, soil and plants and are transported to beehives by bees. Contaminants from beekeeping practice includes acaricides used for parasitic mites (mainly *Varroa*) control, bee repellents used at honey harvest, pesticides for wax moth and small hive beetle control and antibiotics (Bogdonov, 2006).

Antibiotics are found in honey largely because they are used in apiculture for treatment of bacterial diseases. Oxytetracycline is commonly used to treat European foulbrood disease (EFB) and American foulbrood diseases (AFB) caused by *Paenibacilus* (Bacillus) *larvae* and *Streptococcus pluton* bacteria, respectively. However, there are now reports of tetracycline resistance in these bacteria because of its widespread use. Other antibiotics such as erythromycin, lincomycin, monensin, streptomycin, enrofloxacin etc. are also reportedly used in beekeeping.

The use of antibiotics in beekeeping is illegal in some EU countries. Moreover, there are no Maximum Residue Limits (MRLs) established for antibiotics in honey according to the

European Community regulations (Mutinelli, 2003), which means that honey containing antibiotic residues are not permitted to be sold.

The European Union (EU), on its part, regulates honey under the Council Directive 2001/110/EC. The standard for antibiotics in food (also referred to as Maximum Residue Limits or MRLs) is listed in Regulation (EU) No 37/2010 – it stipulates that each antibiotic must have an MRL before it can be used on a food-producing species. But there are no MRLs for antibiotics in honey -- which means the EU does not allow use of antibiotics for treatment of honeybees. For regulating residues of antibiotics in imported honey, the Union has set what are called RPAs, or 'Reference Points for Action'. RPAs are residue concentrations which are technically feasible to detect by food control laboratories. When an RPA is exceeded, the member state is obliged to reject the consignment. Till date, RPAs have been established in honey for substances such as chloramphenicol and nitrofurans. EU has also set a provisional MRL of 25 μg/kg or parts per billion (ppb) for oxytetracycline in honey.

Some countries, like Switzerland, UK and Belgium, have established Action Limits for antibiotics in honey, which generally lies between 0.01 to 0.05 mg/kg for each antibiotic group. Action Limits are the level of antibiotics in honey beyond which the sample is deemed non-compliant.

In the US, Canada and Argentina, preventive treatments with antibiotics are considered a routine procedure to prevent outbreaks of AFB. Consequently, various strains of *P. larvae* showing resistance to antibiotics, such as oxytetracycline-HCl (OTC), have been discovered in Argentina (Alippi, 2000) as well as in many areas of United States (Miyagi *et al*., 2000). The extensive use of antibiotics leads to an accumulation of residues in honey decreasing their quality and making their marketing more difficult (Fuselli *et al*., 2005). Antibiotic residues show a relatively long half-life and they may have direct toxic effects on consumers e. g., allergic reactions in hypersensitive individuals and disorder of the haemopoietic system, or cause problems indirectly through induction of resistant strains of bacteria (Tillotson *et al.*, 2006).

In last few years, there have been reports of antibiotic contamination in honey exported from India. The food and feed control authorities of the member states of the EU have found Indian honey contaminated with prohibited antibiotics like nitrofuran and chloramphenicol. Indian honey has also been found to be contaminated with tetracycline and streptomycin. In the US, consignments of Indian honey have been found to be contaminated with ciprofloxacin.

To promote the exports of honey, the Ministry of Commerce and Industries, Government of India has setup a Residue Monitoring Plan (RMP) to monitor the level of antibiotics, heavy metals and pesticides contamination in honey destined for exports. The Export Inspection Council (EIC) is responsible for implementing the RMP.

EIC has setup Level of Action limits (similar to standards) for antibiotics in exported honey. Sample found to be containing antibiotics beyond the Level of Action is deemed non-compliant. The monitoring result of RMP shows that a sizeable proportion of honey consignments destined for exports were contaminated with antibiotics. In 2007, about 28% of the samples tested did not meet the Level of Action for tetracyclines and 5.9% for sulphonamides. In 2008, 23.9% samples and 11.6% samples did not meet the Level of Action for tetracyclines and sulphonamides.

It is quite clear that India has setup an elaborate system to monitor the quality of honey exported to the EU and the US. However, there is no standard for antibiotics in honey for the domestic market. There is hardly any report on the antibiotic contamination of honey consumed within the country. Similarly, India also imports honey, but there is no standard to check the quality of honey being imported. This study was undertaken to fill this gap. The objective of this study, therefore, is to find out the level of antibiotics in honey samples available in the domestic market.

3. Honey

3.1 Definition

In Ayurveda honey is called as "Madhu". Its qualities are explained as follows.
"Vaatalam guru sheetam cha raktapittakaphapaham |
Sandhatru cchedanam ruksham kashayam madhuram madhu ||"

“It has sweetness (madhura rasa) with added astringent as end taste (Kashaya anu rasa). It is heavy (guru guna), dry (ruksha) and cold (sheeta). Its effect on doshas is as follows. It aggravates vata, scrapes kapha and normalizes pitta and rakta. It promotes healing process."

The Codex Alimentarius Commission defines honey as “'the natural sweet substance produced by honeybees from the nectar of flowers or from secretions coming from living organisms feeding on plants, that bees gather, transform and combine with specific ingredients, store and leave to ripen in the combs of the hive”.

According to the PFA Rules, 1955 under section A.07.03 honey is defined as:
“Honey means natural sweet substance produced by honey bees from the nectar of blossoms or from secretions of plants which honey bees collect, transform, store in honey combs for, ripening”

According to the Bureau of Indian Standards (BIS) specifications IS 4941:1994, honey is defined as “natural sweet substance produced by honey bees from the nectar of blossoms or from secretions of plants which honey bees collect, transform and store in honey combs for honey bees”. Further, honey is classified into three grades based on moisture content. It prescribes less than 20 per cent moisture for 'special grade', 20-22 per cent for 'grade A' and 22-25 per cent for 'standard grade'. According to the specifications, rubber honey belongs to medium grade (Grade A) with an average moisture content of 22 per cent.

3.2 Constituents of Honey

Honey is a mixture of sugars and other compounds. With respect to carbohydrates, honey is mainly fructose (about 38.5%) and glucose (about 31.0%), (National Honey Board, 2008) making it similar to the synthetically produced inverted sugar syrup which is approximately 48% fructose, 47% glucose, and 5% sucrose. Honey's remaining carbohydrates include maltose, sucrose, and other complex carbohydrates. Honey also contains tiny amounts of several compounds thought to function as antioxidants, including chrysin, pinobanksin, vitamin C, catalase, and pinocembrin. But it contains only trace amounts of minerals. The specific composition of any batch of honey depends on the flowers available to the bees that produced the honey (USDA, 2007).

Various ingredients of honey have helped it to become not only a sweet liquid but also a natural product with high nutritional and medicinal value. The medicinal quality, taste, texture, color, aroma of honey differs according to the geographical area and the species of plants from which it has been collected.

3.3 Uses

The main uses of honey are in cooking, baking, as a spread on breads, and as an addition to various beverages such as tea and as a sweetener in some commercial beverages. Honey can be used as instant energizer as it contains sugars which are quickly absorbed by our digestive system and converted into energy.

In Ayurveda honey is called as "Yogavahi", substance which has the quality of penetrating the deepest tissue. When honey is used with other herbal preparations it enhances the medicinal qualities of those preparations and also helps them to reach the deeper tissues. Honey is also used as a medicine because of its antioxidant and antibacterial properties.

3.4 Production and consumption

The annual world honey production is estimated at about 1.4 million tonnes (FAO, 2005). Asia is the largest producer of honey, accounting for about 40% of the global production. China is the largest producer of honey producing around 0.3 million tonnes annually.

Honey consumption in developing countries such as China, Argentina, India, Brazil and Egypt is estimated to be 0.1 to 0.2 kg per capita. Developed countries consume generally higher amounts. However, the per capita honey consumption does not follow the richness of the countries, as it is also determined by cultural influences. In the European Union, the biggest honey consumer is Greece with 1.8 kg per capita, followed by Germany with 1.5 kg, other EU countries like Italy, Spain, France and Hungary are in the intermediate range with 0.6-0.9 kg, while the UK is on the lowest end with 0.4 kg per capita annual consumption (Bogdanov, 2009).

India produces a total of 65,000 tonnes of honey every year (Indian Horticulture Database, 2009). Punjab, Haryana, Uttar Pradesh, Bihar and West Bengal are the major honey producing states. Among the southern states, Tamil Nadu ranks first in honey production followed by Kerala and Karnataka. Kashmir Apiaries Exports based in Doraha, Ludhiana, accounts for 40 per cent of the total organized sector honey production in India.

India exports about 25,000 tonnes of honey annually to more than 42 countries including the EU, the Middle East and the US.

3.5 Major brands of Honey in India

Currently, the domestic branded honey market is estimated at around Rs 250 crores. About 50% of honey is being used for religious and medicinal purposes. Dabur India Limited is the biggest player in the branded honey market and holds a share of over 75 per cent. The remaining share is taken up by brands like Baidyanath, Himani, Zandu, Mehsons, Himalaya and other smaller companies. Traditional players such as Khadi and Village Industries Commission (KVIC) and Himachal Pradesh Agro Industry Corporation (HPAIC) are also aggressively expanding their network.

Imported Brands

Market for imported honey is growing in India. Some imported brands available Capilano (Australia), Nectaflor (France), Darbo (Germany), Dana (Denmark), Lagnese (Germany), Hero (Switzerland) etc.

3.6 Beekeeping in India

India is a vast country with varied climates and ecological conditions ranging from tropical to sub-tropical in its southern, central and eastern regions, from sub-temperate to temperate along its north and north west and semi arid to desert conditions towards the west. The major geographical regions facilitating beekeeping development are classified into: 1) Southern peninsular region; 2) North east region; 3) Indo-Gangetic plains; and 4) Northern hill region (Thakar, 1976).

The geographical position of India and the related agro-climatic condition favor the growth of a wide variety of flora -natural and cultivated. The extensive area of forest and millions of acres of cultivated land sustains a large proportion of insects and honeybees. Due to diversity in flora, topography and activities of people, beekeeping and management is diverse. Beekeeping in India has been adapted to various ecosystems, socioeconomic profiles and habitat preferences.

India has four native species of honeybees and has also introduced exotic specie. The main harvest of honey is from following species (Thomas *et. al*, 2002)

- *Apis cerana* or the Asiatic honey bee (or the Eastern honey bee): *Apis cerana* beekeeping is practiced in India since time immemorial. It is a good pollinator, and has survival capacity due to the co-evolution of native floral sources, pests and predators accustomed to the same climatic conditions. Beekeeping with *Apis cerana* is largely practiced in Southern and Central India.
- *Apis dorsata* (the rock bee or giant bee): *Apis dorsata* contributes a large share of honey production in India. It is found in foothills of Himalayas and northern regions of the country. In central India in the forests and plains large number of *Apis dorsata* colonies are present from which the tribals collect large amount of honey. The Sunderban forests in West Bengal and Southern part of India are also rich in *Apis dorsata.*
- *Apis florea* (Little bee): It is common in central part of India, occurs in arid and desert region of extreme climates, and also in plains and forests. A large quantity of *Apis florae* honey is collected from the Kutch area of Gujarat.
- *Trigona irridipennis* (Dammar bee): It is common in all parts of the country. It is a very small bee and collects nectar from small flowers. Since the quantity of honey produced is small, these bees are not commercially used. It is a very important crop pollinator and the honey has repute in folk medicine.
- *Apis mellifera* (European honey bee or western honey bee): It is an exotic species imported initially to Punjab from Western Countries and has become popular among commercial beekeepers because of its higher honey yield. The *mellifera* gradually spreads to Himachal, Bihar, Uttar Pradesh, West Bengal and recently to Kerala, Karnataka and Maharashtra. Currently, largest amount of honey is produced from *Apis mellifera.* The EU allows imports of honey produced by *Apis mellifera* bees only.

3.7 Pests and Diseases of Honeybees

Honeybees are affected by protozoan, bacterial, viral and acarine diseases. There are a number of diseases which affect the honeybee in India. Of the major diseases which affect honeybee are the Acarine and Nosema diseases of the adult bees and the brood diseases of larval stages. Out these brood diseases, the European foul-brood disease and the Thai Sac-brood disease are common in India. Table 1 gives the diseases affecting the honeybees and the recommended practice of treatment.

Table 1: Major Diseases and Pests of Honey Bees

Type of Diseases	Disease	Organism	Treatment
PROTOZOAN	Nosema Disease	*Nosema apis.*	Fumigation of combs and hive parts with acetic acid or formalin Drug treatment of fumagillin is useful in controlling the infection. The drug is administered by giving a feed of 100 mg fumagillin per colony in 250 ml of sugar syrup for 10 days continuously.
BACTERIAL	European foul-brood	*Streptococcus pluton*	Antibiotics preferably terramycin oxytetracycline - terramycin is given dissolved in sugar syrup @ 100 mg of active terramycin in a litre of syrup. The terramycin syrup (freshly prepared) is fed every seventh day. The disease can also be controlled by fumigation with ethylene oxide. Quarantine is a must to prevent entry of any of the bee diseases.
	American foul-brood	*Bacillus larvae* White.	The disease can be controlled by total destruction of the diseased colony including the hive, frames, bees and honey or use of tylosin tartarate.
VIRAL	Thai Sac brood	Thai Sac-brood virus	Prevalent in South India and caused severe loss to bee keeping industry in 1990s. No effective method to control this disease is known as yet. Prevention is better than cure. It is better to isolate the infected colonies. Reported in Kerala, Karnataka and Tamil Nadu
ACARINE	Acarine Disease	Tracheal mites	Acaricide –Chlorobenzylate, Menthol
	Varroa	*Varroa* Mites	Fluvalinate and coumafos Thymol, oxalic acid , formic acid

Source: http://agritech.tnau.ac.in/apiculture/fe_api_pestanddiseases.html

4. Antibiotics

4.1 What are Antibiotics?

Antibiotics are medicines - therapeutically used to protect the health and welfare of humans and animals. It inhibits or abolishes the growth of microorganisms such as bacteria, fungi or protozoa.

The term antibiotic originally referred to any agent with biological activity against living organisms; however, ‘‘antibiotic” now refers to substances with antibacterial, anti-fungal, or anti-parasitical activity. There are currently about 250 different chemical entities registered for use in medicine and veterinary medicine (Kümmerer and Henninger, 2003).

4.2 Major Classes of Antibiotics

Antibiotics can be grouped by either their chemical structure or mechanism of action. They are often complex molecules which may possess different functionalities within the same molecule. Therefore, under different pH conditions antibiotics can be neutral, cationic, anionic, or zwitterionic. They are divided into different sub-groups such as ß-lactams, amphenicols, tetracyclines, macrolides, aminoglycosides, fluoroquinolones and others.

Beta lactams

β-Lactams are antibiotics that have a β-lactam ring nucleus with a heteroatomic ring structure, consisting of three carbon atoms and one nitrogen atom, used to treat bacterial infections by attacking the cell walls of bacteria. e .g penicillins, ampicillin, cloxacillin, amoxicillin

Amphenicols are a class of antibiotics with a phenylpropanoid structure. They function by blocking the enzyme peptidyl transferase on the 50S ribosome subunit of of bacteria e.g chloramphenicol, thiamphenicol,azidamphenicol and florfenicol

Tetracyclines are antibiotics with four (“tetra-”) hydrocarbon rings (“-cycl-”) derivation (“-ine”) defined as "a subclass of polyketides having an octahydrotetracene-2-carboxamide skeleton" used for treatment of bacterial brood diseases e.g. oxytetracycline, chlortetracycline, tetracycline

Macrolides are basic and lipophilic antibiotics with a 14 membered macrocyclic lactone ring linked via glycosidic linkages and are potent against wide variety of gram positive and

negative bacteria used for the treatment of infectious diseases in cattle, sheep, swine and poultry e.g. tylosine, erythromycin, lincomycin

Aminoglycosides consist of an aminocyclitol ring connected to two or more amino sugars linked via a glycoside link used for the treatment of bacterial brood diseases e.g streptomycin, gentamycin, neomycin, spectinomycin

Fluoroquinolones which have a fluorine atom attached to the central ring system, typically at the 6-position and are used as growth promoters e.g ciprofloxacin, ernofloxacin, norfloxacin.

Reliable and up-to-date data on antibiotic consumption (for both animals and humans) is not widely available.

4.3 Antibiotics authorized in Beekeeping

Beekeepers use antibiotics at relatively high doses, as therapeutic agents to treat clinical infections (bacterial brood diseases), or they may be administered at low, sub therapeutic doses as 'growth promoters'. Beekeeping with the use of antibiotics is less labour intensive and more profitable.

A list of products approved for use world-wide for fighting bee diseases is listed in Table 2. Acceptable Daily Intakes (ADIs) established either by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) or the Joint FAO/WHO Expert Committee on Food Additives (JECFA) are also indicated in the Table 2. These chemicals include Acaricides- Folbex VA (brompppropyllate), Perzin(coumafos), Apistan(Fluvalinate), Bayvarl(flumetrine) used against mites and antibacterial substances such as sulfonamides, tetracyclines, erythromycin tylosin and streptomycin used in the treatment of bacterial brood diseases. Maximum Residue Limits (MRLs) have been established for all food producing species for sulfonamides and tetracyclines but there are no MRLs for honey (Mutinelli, 2003).

Table 2 : List of Products approved in Apiculture

Substance	Major Application	Proprietary product	ADI (mg/kg bw per day)	
			JECFA	JMPR
Acrinathrine	Pesticide/Acaricide	Yes		
Amitraz (Apivar)	Pesticide/Acaricide	Yes		0–0.01
Bromopropylate	Pesticide/Acaricide	Yes		0–0.03
Chlorobenzilate	Pesticide/Acaricide	No		0–0.02
Chlortetracycline	Veterinary drug	No	0–0.003	
Coumaphos (Perizin) [a]	Pesticide	Yes		
Cymiazole hydrochloride (Apitol)	Pesticide/Acaricide	Yes		
Enilconazol (imazalil)	Pesticide/Acaricide	No		0–0.03
Erythromycin	Veterinary drug	No	0–0.0007	
Fenproximate	Pesticide	Yes		
Fipronil	Pesticide	No		0–0.0002
Flumethrin (Bayvarol)	Pesticide	Yes		0–0.004
Formic acid [b]	Veterinary drug	Yes	0–3	
Fumagillin	Pesticide	Yes		
Lactic acid [b]	Veterinary drug	No	Not limited	
Lincomycin hydrochloride	Veterinary drug		0–0.03	
Malathion	Pesticide	No		0–0.3
Menthol [b]	Veterinary drug	Yes	0–4	
Methyl bromide	Pesticide	No		
Monensin	Veterinary drug	No	0–0.01	
Oxalic acid	Pesticide	Yes		
Oxytetracycline	Veterinary drug	Yes	0–0.003	
Paradichlorobenzene	Pesticide	No		
Permethrin	Pesticide	Yes		0–0.05
Propargite	Pesticide			0–0.01
Rifampicin	Veterinary drug	No		
Spinosad	Pesticide	No		0–0.02
Streptomycin/dihydrostreptomycin	Veterinary drug	No	0–0.05	
Sulfathiazole	Veterinary drug	No	No ADI allocated	
Tau-fluvalinate (Apistan)	Pesticide	Yes		
Thymol [b]	Pesticide	Yes	Acceptable	
Tylosin tartrate	Veterinary drug	Yes	0–0.03	

a Temporary ADI withdrawn in 1980; no ADI allocated in 1990
b. Substances considered by many national authorities as generally regarded as safe

Source : Joint FAO/WHO Expert Committee on Food Additives. Meeting (70th : 2008: Geneva, Switzerland).Evaluation of certain veterinary drug residues in food : seventieth report of the Joint FAO/WHO Expert Committee on Food Additives.(WHO technical report series ; no. 954)

5. Regulations for Antibiotics in Honey

Honey is an important commodity which is traded internationally. For international trade, all member countries generally accept standards set by the Codex Alimentarius. However, individual countries also have their own separate standards. Following standards of antibiotics in honey were reviewed: Codex Alimentarius, EU, US, Canada, Australia and India. A comparison of these regulations is given in Table 3.

5.1 Codex

Codex Alimentarius standard for Honey (*Codex Stan 12- 1981 Rev 1 1987 Rev2 2001)* defines honey and lays down standards on its essential composition and quality (moisture content, sugar content, electrical conductivity etc.). The standard contains provisions relating to contaminants, hygiene, labeling and methods of analysis. CODEX STAN 12-1981 for honey in section 4.2, for residues of pesticides and veterinary drugs, states that the products covered by this standard shall comply with those maximum residue limits for honey established by the Codex Alimentarius Commission. Internationally agreed safety requirements of a number of veterinary medicines in food have been recommended by Joint FAO/WHO Expert Committee on Food Additives (JECFA) and adopted by Codex. **However, no Maximum Residue Limits (MRLs) have been set for antibiotics in honey or even proposed.**

5.2 The European Union (EU)

EU regulates honey under the Council Directive 2001/110/EC. The standard for antibiotics in food (also referred to as Maximum Residue Limits or MRLs) is listed in Regulation (EU) No 37/2010 – it stipulates that each antibiotic must have an MRL before it can be used on a food-producing species. But there are no MRLs for antibiotics in honey -- which means the EU does not allow use of antibiotics for treatment of honeybees.

EU has adopted detailed legislation on use of and monitoring for veterinary drugs. Council regulation 2377/90 places residues in animal products in 4 Annexes. Each pharmacologically active substance must have a Maximum Residue Limit (MRL) status before it can be registered for use in a food producing species. It covers 700 substances of which 200 are regulated by MRL.

- Annex I has the list of pharmacologically active substances for which a MRL has been fixed. No MRL for antibiotics in honey has been listed in Annex I.

- Annex II is the list of substances for which there is no need to set an MRL as they are unlikely to raise public health concerns, because any use in food-producing animals, especially the use in bees, is generally regarded as safe. Examples of such substances include formic acid, lactic acid, oxalic acid, thymol and menthol , fluvalinate, phenol etc.

- Annex III is the list of pharmacologically active substances for which a MRL cannot be set definitively but which may be given a provisional MRL for a defined period. A provisional MRL of 25 ppb has been set for Oxytetracycline in honey.

- Annex IV is the list of substances for which it appear no MRL can be set because they pose a risk to human health in whatever quantity and no exception for honey would be applicable. This list includes antibiotics like chloramphenicol, dimetridazole, metronidazole, nitrofurane including furazolidone, ronidazole.

Pharmacologically active substances not listed in Annexes I, II or III are not allowed to be used as veterinary drugs, if the animal is used for food production. According to Article 14 of the Council regulation 2377/90 “the administration to food-producing animals of veterinary medicinal products containing pharmacologically active substances which are not mentioned in Annexes I, II or III shall be prohibited within the Community….”. Standards for antibiotics in honey is not listed in Annexes I, II or III. This means that the use of antibiotics in honeybees is not permitted and antibiotics in honey are therefore considered “unauthorised substances”.

EU rules on setting of MRLs for pharmacologically active substances have been updated by Regulation (EC) No 470/2009. This legislation has, for the first time, introduced a mechanism for the extrapolation of MRLs from one species/food commodity to another. In addition the legislation elaborates the principles by which the European Commission can establish so-called "Reference Points for Action" (RPAs) for residues of pharmacologically active substances for which MRLs have not been (nor can not be) established. RPAs are residue concentrations which are technically feasible to detect by food control laboratories. In the event that the RPA is exceeded, the Member State is obliged to reject the consignment as it cannot be legally placed on the EU market (Article 23 of Regulation (EC) No 470/2009).

If a food control laboratory in an EU Member State unequivocally confirms and quantifies the presence of a substance at a concentration below the RPA (where an RPA has been established) in an imported consignment (i.e. the decision limit CCα as defined in Article 6 of Commission Decision 2002/657/EC has been exceeded), the Member State competent authority is obliged to permit the consignment to be placed on the market, however, it is also obliged to follow certain administrative procedures including, in some circumstances, informing the Commission services. Till date, RPAs have been established in honey for substances such as chloramphenicol and nitrofurans. EU has also set a provisional MRL of 25 parts per billion (ppb) for oxytetracycline in honey, chloramphenicol (0.3 ppb) and nitrofurans (1.0 ppb).

5.3 US

Residue limits (tolerances) for veterinary drugs, food additives and unavoidable contaminants in meat, poultry, and egg products, are set by the Food and Drug Administration (FDA). MRL for Veterinary drugs are found in Title 21, Part 556 (21 CFR 556). These tolerances are for the parent compound (the original chemical form of the compound given to the animal), or for the compound's metabolites (the chemical forms into which the compound is metabolized by the animal), or for a combination of parent plus metabolites. All tolerances are provided in units of parts per million (ppm). **There are no limits for veterinary drugs in honey which implies that it should be absent.**

Source: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=556

5.4 Australia

In Australia/New Zealand Food Standards Code (the Code) - **Standard 2.8.2 for Honey** defines honey and sets certain compositional requirements for the product. **Standard 1.4.2** Standard lists the maximum permissible limits for agricultural and veterinary chemical residues present in food. **Schedule 1 lists all of the agricultural and veterinary chemical limits in particular foods**. If a maximum residue limit for an agricultural or veterinary chemical **in a food is not listed in Schedule 1 there must be no detectable residues of that agricultural or veterinary chemical in that food**. Commodity and commodity groups which are referred to in this Standard are listed in Schedule 4 which specifies the part of the commodity to which the maximum or extraneous residue limit refers and honey is listed under Animal Food Commodity. **Australia has set MRL for only Oxytetracycline in honey at 300 ppb. Other antibiotics in honey is not allowed.**

Source: http://www.foodstandards.govt.nz

5.5 Canada

Health Canada's Veterinary Drugs Directorate (VDD) in agreement with the Canadian Food Inspection Agency (CFIA) has amended the joint Policy on Administrative Maximum Residue Limits/ Maximum Residue Limits (AMRLs/MRLs) for Veterinary Drugs in Food Products to include Working Residue Levels (WRLs) for antimicrobials used in honey. WRLs are recommended levels for drug residues in honey below which there is considered to be no undue risk to human health. The WRLs for honey have been derived by extrapolating lowest established AMRL/MRL values of antimicrobials that are approved for use in other food-producing animals. **Chloramphenicol and Nitrofuran antibiotics are banned in Canada. AMRL for Oxytetracycline is fixed at 300 ppb and WRL of Erythromycin is 30 ppb.**

Source: The Veterinary Drugs Directorate of Health Canada at http://www.hc-sc.gc.ca/vetdrugs-medsvet/mrl_oxytetracyclineletter_e.html

5.6 India

Honey is regulated by three standards at present; the Prevention of Food Adulteration (PFA) Act, the Bureau of Indian Standards (BIS) and the Agricultural Produce Grading and Marking Act

(Agmark). The export of honey is monitored by the Exports Inspection Council which has set 'Level of Action' for antibiotics in exported honey.

(a) PFA Act, 1954, which is mandatory, defines honey and lays down standards for its essential composition and quality (sucrose content, hydroxymethylfurfural, moisture content etc.) **but there are no standards for veterinary drugs/antibiotics in honey.**

(b) Bureau of Indian Standards has set standards for Extracted Honey in IS 4941:1994.This standard lays down specification for general requirements for 11 parameters (specific gravity, moisture, total reducing sugar, acidity, hydroxymethylfurfural etc.). **But there are no standards for antibiotics.**

IS 6695: 1998 – Honey Bees - Code for conservation and maintenance, in Annex B, Clause 3.2 has listed notifiable Honey Bee disease and their treatment. Terramycin (Oxytetracycline) is recommended for treatment of European Foul Brood disease. For American Foul Brood the treatment only specifies "Antibiotics'—no name is mentioned.

(c) AGMARK standard set up by the Directorate of Marketing and Inspection of the Government of India under the provisions of the Agricultural Produce (Grading and Marking) Act 1937 as amended in 1986. Honey is regulated under the Honey Grading and Marking Rules, 2008. This Rule gives specifications for quality of honey, method of packing, marking and labeling. Honey is classified into three grades - Special, Grade A and Standard – and their quality specifications is mentioned.
Schedule II (Grade designation and quality of Honey) of Honey Grading and Marking Rules, 2008 defines Honey as follows: "Honey" shall be obtained from the natural sweet substance produced by honey bees from the nectar of blossoms or from secretions of plants, which honey bees collect, transform and store in honey combs for ripening.

Clause (V) of Schedule II specifies that: Honey shall comply with restrictions in regards to Metallic Contaminants (rule 57), Crop Contaminants (rule 57 A), Naturally occurring toxic substances (rule 57 B), insecticides and pesticides residue (rule 65) and other food safety requirements as laid down under Prevention of Food Adulteration Rules, 1955 as amended from time to time for domestic purposes.

Clause (VI) of Schedule II specifies that: Honey shall comply restrictions in regards to heavy metals, pesticides and other food safety requirements as specified in Codex Alimentarius Commission or as per buyer's requirements for export purposes.
The Honey Grading and Marking Rules, 2008, therefore, links honey standards for domestic consumption to Prevention of Food Adulteration Rules, 1955 and for exported honey with Codex Alimentarius Commission or the standard of the importing country. Since Prevention of

Food Adulteration Rules, 1955 doesn't specifies any antibiotic standards for honey, under AGMARK also there is not standard for antibiotics in honey.

(d) Export Inspection Council: The Government of India, Department of Commerce (Ministry of Commerce and Industry), under the Export Inspection Council of India (EIC) Act monitors the quality of products exported from India. To promote the exports of honey, the Ministry of Commerce and Industries, Government of India has setup a Residue Monitoring Plan (RMP) to monitor the level of antibiotics, heavy metals and pesticides contamination in honey destined for exports. The Export Inspection Council (EIC) is responsible for implementing the RMP.

EIC has setup 'Level of Action' (similar to standards) for antibiotics in exported honey. Sample found to be containing antibiotics beyond the Level of Action is deemed non-compliant. The Level of Action for Chloramphenicol is 0.3 ppb, Nitrofurans 1 ppb, Tetracyclines (group) 10 ppb, Streptomycin 10 ppb and Sulphonamides (group) 20 ppb.

Table 3. Comparison of different regulation for Antibiotics in Honey

S. No.	Class	Antibiotic	Codex Alimentarius[1]	EU[2]	USA[3]	Australia[4]	Canada [5]	India- EIC[6]
1	Tetracycline	Oxytetracycline	No MRL	Provisional MRL- 25ppb	No MRL	300 ppb MRL[7]	300ppb AMRL[8]	10ppb
2	Amphenicol	Chloramphenicol	No MRL	No MRL RPA[9]-0.3 ppb	No MRL	No MRL	No MRL	0.3ppb
3	Macrolide	Erythromycin	No MRL	No MRL	No MRL	No MRL	100ppb-AMRL 30ppb –WRL[10]	No LOA[11]
4	Beta Lactam	Ampicillin	No MRL	No MRL	No MRL	No MRL	No MRL	No LOA
6	Fluoroquinolones	Enrofloxacin	No MRL	No MRL	No MRL	No MRL	No MRL	No LOA
		Ciprofloxacin	No MRL	No MRL	No MRL	No MRL	No MRL	No LOA

Notes:

1. FAO/WHO, 2008 Codex Alimentarius: Veterinary Drugs Residues in Food Maximum Residue Limits. Http://www.codexalimentarius.net/mrls/vetdrugs/jsp/vetd-q-e.jsp
2. EU- Http://www.emea.europa.eu/index/indexv1.htm. Veterinary medicines and Information technology Units Committee for Veterinary medicinal products
3. USA- Tolerances for residues of new animal drugs in food in Title 21, Part 556 (21 CFR 556). http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=556
4. Australia /New Zealand Food Standards Code http://www.foodstandards.govt.nz
5. Canada HC 2008a Health Canada WRLs in honey. http://hc-sc-gc.ca/dhp-mps/legislation/vet/pol/cfia-acia_amr-ram_table_e.htmll HC 2008 b Health Canada. Drugs& Health Products, Veterinary Drugs, Administrative Maximum Residue Limits (AMRLs)(MRLs) set by Canada and Maximum Residue limits available at http://www.hc-sc.gc.ca/dhp-mps/vet/mrl-lmr/mrl
6. According To Export Inspection Council of India's Residue Monitoring Plan (RMP) Honey 2010-2011
7. Maximum residue level (ppb or part per billion)
8. AMRL – is administrative MRL means that the scientific evaluation and decisions are complete and that regulatory process to publish this information is in progress. Once the regulatory process is complete the AMRL becomes an MRL
9. RPA – Reference point for action set by EU
10. WRL – Working Residue Levels. There are no MRLS for antibiotics in honey therefore WRLs are set. WRLs are recommended levels for drug residues in honey below which there is considered to be no undue risk to human health. The WRLs for honey have been derived by extrapolating from AMRL/MRLs for antimicrobials that are approved for use in other food-producing animals such as chickens, swine and cattle.
11. LOA- Level of Action- is the concentration of a drug residue in a sample at which it is deemed non-compliant.

6. Health Impacts

Antibiotics used in food animals can affect the public health because of their secretion in edible animal tissues in trace amounts usually called residues. For example, oxytetracycline (Saridaki-Papakonstadinou *et al*, 2006) and Chloramphenicol residues (Ortelli *et al*., 2004) have been found above the regulatory standards in honey. Some drugs have the potential to produce toxic reactions in consumers directly while some other are able to produce allergic or hypersensitivity reactions (Vellicer, 2004). For example, β-lactam antibiotics can cause cutaneous eruptions, dermatitis, gastro-intestinal symptoms and anaphylaxis at very low doses. Such drugs include the penicillin and cephalosporin groups of antibiotics (Paige *et al*., 1997). Indirect and long term hazards include microbiological effects, carcinogenicity, reproductive effects and teratogenicity. Microbiological effects are one of the major health hazards in human beings. Antibiotic residues consumed along with edible tissues like milk, meat, eggs and honey can produce resistance in bacterial populations in the consumers. These bacteria might then cause difficult-to-treat human infections. Certain drugs like 3-nitrofurans and nitroimidiazoles can cause cancer in human population. Similarly, some drugs can produce reproductive and teratogenic effects at very low doses consumed for a prolonged period of time.

6.1 Chronic health effects

Chronic health effects of some antibiotics detected in the present study are discussed below :

Oxytetracycline (Class: Tetracycline)

Oxytetracycline (OTC) is a broad-spectrum antibiotic used to treat a variety of infections and is also used as a growth promoter in animals. Symptoms of chronic exposure to oxytetracycline include blood changes (leucocytosis, atypical lymphocytes, lung congestion, toxic granulation of granulocytes and thrombocytopenia purpura). Liver injury and delayed blood coagulation may also occur. It can damage calcium rich organs such as teeth and bones and sometimes causes nasal cavities to erode. Children under 7 years of age may develop a brown discoloration of the teeth. Infants of mothers treated with OTC during pregnancy may develop discoloration of the teeth. Some other chronic effects of oxytetracycline includes increased sensitivity to the sun , wheezing and asthmatic attack. Toxicological studies indicate that this drug is not mutagenic, carcinogenic, or terratogenic. http://www.inchem.org/documents/jecfa/jecmono/v27je06.htm

Erythromycin (Class: Macrolides)

Erythromycin (ERY) is effective against many gram-positive organisms and is useful in the treatment of *staphylococcal* infections in animals and humans. Exposure to erythromycin (especially long courses at antimicrobial doses, and also through breastfeeding) has been

linked to an increased probability of pyloric stenosis in young infants a condition that causes severe vomiting in the first few months of life (Maheshwai, 2007).

Erythromycin is a reproductive hazard (terratogen) with chronic exposure. Cardiac malformation was observed in infants of women who had taken erythromycin in their early pregnancy (JECFA, 1997)

Enrofloxacin (Class: Fluoroquinolones)

Enrofloxacin (ENR) a fluroquinolone antibiotic which acts by inhibition of bacterial DNA gyrase Embryo lethality and terratogenicity of fluoroquinolone antibacterials in rats and rabbits has been suggested (Guzman *et al*., 2003). Chromosomal aberrations evaluated in cultures of human peripheral lymphocytes from eight healthy donors, exposed to the antimicrobial enrofloxacin or to its major metabolite ciprofloxacin suggested a genotoxic effect of enrofloxacin and ciprofloxacin (Gorla et al , 1999). It is also associated with increased photosensitivity. The Food and Drug Administration's Center for Veterinary Medicine has proposed to withdraw approval for use of the fluoroquinolone antimicrobial, enrofloxacin, in poultry based not on drugs direct toxicity but on potential for increasing human pathogen resistance. Source: http://www.fda.gov/cvm/Documents/baytrilDDL.pdf

Chloramphenicol (Class: Amphenicol)

Chloramphenicol (CAP) a bacteriostatic antimicrobial previously used in veterinary medicine. It has been found to be potentially carcinogenic, which makes it an unacceptable substance for use with any food producing animals, including honey bees. The United States, Canada, and the European Union (EU), as well as many other countries, have completely banned the usage of CAP in the production of food. Chloramphenicol is anticipated to be a human carcinogen and genotoxic from studies in humans. It is toxic to blood, kidney, liver. Repeated or prolonged exposure to Chloramphenicol can lead to target organ damage, bone marrow toxicity. The most serious effect of chloramphenicol is aplastic anaemia which is idiosyncratic (rare, unpredictable, and unrelated to dose) and generally fatal and could presumably be triggered by residues (Payne *et al*, 1999) Several reports document human fatalities resulting from ophthalmic preparations containing chloramphenicol, with total exposure dozes that could be achieved from food residues (Settepani,1984).

Ampicillin (Class: ß-lactam)

Ampicillin (AMP) is a penicillin derivative β-lactam antibiotic is widely used in cattle, swine, honey bees and poultry to treat infections and as feed or drinking water additives to prevent some diseases. Workers from an antibiotic-producing factory developed asthma and eosinophilia on inhalation of ampicillin and related substance (Davfes *et al,* 1974). Ampicillin may cause recurrent cholestatic hepatitis (Koklu *et al*, 2003). Repeated contact may cause

allergic reactions, asthmatic attack, exfoliative dermatitis, anemia, thrombocytopenia, thrombocytopenic purpura, eosinophilia, leukopenia, and agranulocytosis. http://www.druglib.com/druginfo/ampicillin/side-effects_adverse-reactions/.

6.2 Antibiotic Resistance

Antibiotic resistance is the ability of a microorganism to withstand the effects of an antibiotic. If even at a large dose, the antibiotic is not effective in treating an infection, then the microorganism that is responsible for the infection is declared as resistant to that antibiotic (Goosens, 2005).

Antibiotic resistance is a global public health concern today. The U.S. Centers for Disease Control and Prevention (CDC, 2000) has described antibiotic resistance as “one of the world’s most pressing health problems”, because “the number of bacteria resistant to antibiotics has increased in the last decade [and] … many bacterial infections are becoming resistant to the most commonly prescribed antibiotic treatments.” The World Health Organization (WHO) has identified antibiotic resistance as “one of the three greatest threats to human health.”

Its primary cause is long-term over-exposure to antibiotics through their use as medicines in humans, as well as in animals, horticulture and for food preservation. The types of antibiotics used in animals are frequently the same as, or closely related to, those used in humans. Factors influencing the development of resistance include drug concentration, duration of exposure, organism type, antimicrobial type and host immune status (WHO, 1997).

Antibiotic resistance in bacteria evolves via natural selection through random mutation. When a bacterium is exposed to an antibiotic it starts making changes in its DNA to withstand the effects of the medicine. Once it acquires a specific antibiotic-resistant gene, it quickly passes it on to its next generation. Nature has developed different systems for transfer of genes between bacteria (conjugation, transformation, transduction and transposition) and these mechanisms have proven effective in the promotion of resistant genes. If a bacterium carries several resistant genes, it is called multi-resistant or, informally, a superbug. When resistant bacteria are themselves pathogenic or can transfer their resistance genes to pathogenic bacteria, adverse health effects can result.

Adverse consequences of selecting resistant bacteria in animals include:

- the transfer of resistant pathogens to humans via direct contact with animals, or through the consumption of contaminated food or water;
- the transfer of resistance genes to human bacteria;
- an increase in the prevalence of resistant bacteria in animals;

- an increase in the incidence of human infections caused by resistant pathogens;
- and potential therapeutic failures in animals and humans.
- residues of antimicrobial agents in food of animal origin in excess of the agreed acceptable maximum residue levels (MRLs) may contribute to the generation of resistance in bacteria in humans.

Several WHO consultations and other expert bodies have identified links between antibiotic use in animals and the emergence of mainly food-borne bacteria which are resistant to important antibiotics which are used in treating infectious diseases in humans. In December 2003, an expert workshop was jointly convened by the Food and Agriculture Organization of the United Nations (FAO), the World Organization for Animal Health (OIE) and the World Health Organization (WHO) to make a scientific assessment of resistance risks arising from non-human use of antibiotics/antimicrobials. The workshop concluded that “there is clear evidence of adverse human health consequences due to resistant organisms resulting from non-human usage of antimicrobials. These consequences include infections that would not have otherwise occurred, increased frequency of treatment failures (in some cases death), and increased severity of infections”.

In recent years, more evidence has emerged of an association between use of antibiotic agents in food animals and antibiotic resistance among bacteria isolated from humans. An outbreak of human nalidixic acid-resistant *Salmonella typhimurium* DT104 infection in Denmark was traced to a pig farm. Another outbreak of the same infection, reported in the United Kingdom, was traced to a dairy farm where Fluoroquinolones had been used on the cattle a month before the outbreak. In the United States, there was a marked increase in the proportion of domestically acquired *Campylobacter* infections that were Fluoroquinolone-resistant, following the first approved use of Fluoroquinolones in food animals in 1995 (WHO, 2009).

The WHO, in fact, has recommended that antibiotics which are also licensed in human medicine should not be used any more as growth promoters in livestock. An EU resolution to this effect was put in place in 1999. Since then, studies from Denmark, Germany and Italy have shown a significant reduction in Vancomycin-resistant *Enterococci* isolations from poultry and poultry-derived food products. Some European member states (such as Denmark) have, with insignificant or no consequence either on disease rates in animals or on meat market prices, voluntarily suspended the use of all growth promoters irrespective of their human health importance.

The major challenge in combating antibiotic resistance lies in the development and implementation of methods for their prudent use. Attention also must be paid to the

development of mechanisms for safety assessments of antibiotics intended for 'food' animal use. There is a significant difference between 'traditional' chemical residue-based determination of safety of animal drugs and the determination of safety in the context of antibiotic resistance. Some 'methodologies' have been proposed for the latter, but none have been implemented so far.

7. Review of Literature

Antibiotic residues in honey have recently become a major consumer concern. It has become evident that residues of antibiotics in honey originate mostly not from the environment but from improper beekeeping practices. There are several international reports of antibiotic residues in honey samples, however there are very few reports of antibiotics in honey sold in domestic market in India. There are reports of tests conducted by Agricultural Processed Food Product Export Development Agency (APEDA) and EIC from 2005 onwards show high levels of antibiotics and heavy metals in honey exported from India to EU and US. In 2006, about 14 per cent samples were contaminated with tetracycline. In 2007-08, about 28 per cent samples were contaminated with this ame antibiotic. Of the 362 honey samples it tested in 2009-2010 by the EIC, 29.2 per cent samples had more than the prescribed limit of antibiotics and heavy metals (EIC documents).

Another consignment belonging to Lee Bee Impex, a big exporter based in Ludhiana in Punjab, was barred from entering the US market in 2007—the honey was found to have originated in China and had residues of fluoroquinolone.

According to the Alert Notices issued by FSA (Food Standards Agency) of UK in March 2003 on the contamination of Indian foods based on the tests at importing points Dabur Honey was contaminated with antibiotic Streptomycin (Mayande, 2007).

In the period 2000-2001, 248 samples of locally produced and imported honey were monitored for the presence of residues of veterinary drug residues. Streptomycin was detected in 4 out of 248, tetracycline in 2 out of 72, sulfonamides in 3 out of 72 samples. No residues of β lactam antibiotics and chloramphenicol were found. In imported honey samples streptomycin was detected in 51 out of 102 samples, tetracyclines in 29 out of 98 samples, sulfonamides in 31 out of 98 samples, chloramphenicol 40 out of 85 samples. For the streptomycin and tetracycline contamination, most cases involved the beekeeper admitting to having added foreign honey to his production (Reybroeck, 2003).

Of the 75 samples of honey obtained commercially in Switzerland, 34 which originated from Asian countries, 13 samples (17%) contained chloramphenicol residues. Concentration of chloramphenicol measured in honey between 0.4 and 6.0 μgkg^{-1}, with six samples containing

approximately 0.8–0.9 μgKg^{-1} (just below the Swiss limit) and two containing approximately 5 μgkg^{-1} (Ortelli *et al*, 2004).

Another study in which 251 honey samples produced across Greece were analysed by Liquid chromatography to detect tetracycline-derived residues. 29% of the samples had tetracycline residues. Majority of samples contained residues from 0.018-0.055 mg/kg of honey while some others had residues in excess of 0.100mg/kg (Saridaki-Papakonstadinou *et al*, 2006)

Centre for Food Safety (CFS) found that two of the 19 samples of honey collected for examination for antibiotics contained trace amounts of chloramphenicol, one brand of honey produced in Jiangxi (under batch number 20060424, with "best before" date 24.4.2008) and another brand produced in Zhuhai (with "best before" date 30.6.2008). Other antibiotics found in the honey samples in trace amount, namely streptomycin, sulfamethoxazole (a kind of sulfonamides) and ciprofloxacin (a kind of quinolone), they can normally be used in food animals. (CFS, 2006)

In February 2006, the Florida Department of Agriculture and Consumer Services reported that residues of two fluoroquinolones of concern, ciprofloxacin and enrofloxacin were found in honey samples that was traced back to a firm from China. The State subsequently, on August 14, 2006, FDA issued Import Alert No. 36-04 requiring detention without physical examination of honey due to presence of fluoroquinolones.
http://www.fda.gov/NewsEvents/Testimony/ucm110728.html

Nectar and Honey samples collected from bee hives during the peak flowering seasons of rubber (March to April) and banana (December to January) plantation crops in southern part of Tamil Nadu were analysed for antibiotic residues. Nectar and honey samples showed 4-17 and 11-29 µg/kg of streptomycin, 2-29 and 3-44 µg/kg of ampicillin and 17-34 and 26-48 µg/kg of kanamycin respectively (Solomon *et al*, 2006).
Out of the 3855 honey samples tested 1.7% samples were non compliant with the EU standards Antibiotic were detected in the honey samples in the range- Streptomycin 3 – 10,820µg/kg, Sulfonamides 5 – 4,592µg/kg, Tetracyclines 5 – 2,076µg/kg, Chloramphenicol 0.1 – 169µg/kg, Nitrofurans 0.3 - 24.7µg/kg, Tylosine 2 – 18µg/kg, Quinolones <1 - 504µg/kg (Diserens, 2007).

50 honey samples comprising chestnut, pine, linden and multiflower honeys collected from the hives in Southern Maramar region of Turkey were analysed for erythromycin residues by Liquid Chromatography-mass spectrometry using Electrospray ionization in the positive ion mode (LC-ESI-MS). Four of the honey samples were contaminated with erythromycin residues at concentrations ranging from 50 to 1776 ngg^{-1}. An erythromycin-fortified cake feeding assay was also performed in a defined hive to test the transfer of erythromycin

residue to the honey matrix. In this test hive, the residue level in the honey,3 months after dosing, was approximately 28 ngg^{-1}. (Gunes *et al* , 2008).

Another study aimed to assess oxytetracycline (OTC) residue levels in honey after treatment of honeybee colonies with two methods of application (in liquid sucrose and in powdered icing sugar. The samples of honey were extracted up to 12 weeks after treatment and following metal chelation and analysed by HPLC showed that the current method of application of Oxyteracyclin(Terramycin) in liquid form results in very high residue levels in honey with residues of 3.7 mg/kg, eight weeks after application(Thompson *et al* 2005).

Recently researchers have developed a method to simultaneously detect the presence of 17 antibiotics (macrolides, tetracyclines, quinolones, and sulfonamides) in honey samples taken from supermarkets while five were collected from various private beekeepers throughout Granada and Almería. The results of the study show that one of the commercial honey samples contained 8.6 μg/Kg, while another contained traces of sarafloxacin and residues of tylosin, sulfadimidine and sulfachlorpyridazine were found in the honey from one bee farm(Vidal *et al* 2009).

A total of 57 real royal jelly samples collected from beekeepers and supermarkets were analyzed for seven fluoroquinolones used in beekeeping, viz. ciprofloxacin, norfloxacin, ofloxacin, pefloxacin, danofloxacin, enrofloxacin, and difloxacin, were analysed by high performance liquid chromatography with fluorescence detection. Ofloxacin, ciprofloxacin, and norfloxacin, were detected in concentrations ranging from 11.9 to 55.6 ng/g in some royal jelly samplesmand difloxacin was found at concentrations of about 46.8 ng/g in one sample though it is rarely used in beekeeping (Zhou *et al* , 2009).

8.Sampling

A total of 12 branded honey samples were collected randomly from different shops in Delhi in the month of July 2009. 10 honey samples were from Indian companies and two were imported honey. Sample details and related information is given in Annexure I. The samples were analyzed at PML during 2009-2010.

9. Materials and Methods

Each honey sample was analyzed in triplicate for 6 antibiotics of 5 major classes using High Performance Liquid Chromatography (HPLC) with Diode Array detector (DAD) and Fluorescence Detector (FLD). Published methods were used for the extraction and clean up and validated at PML.

9.1 Equipments

• HPLC Agilent technologies (1100 series) equipped with DAD, FLD, and Post Column Derivatization unit.
• HPLC Column: Zorbax ODS column, C18 – 5 µm, 4.6mm x 250mm
• HPLC Column: Zorbax Eclipse XDB column, C8- 3 µm, 4.6mm x 150mm
• Vacuum manifold
• Solid Phase extraction Cartridges – SampliQ OPT 3 ml, 60 mg cartridges, SampliQ OPT 6 ml, 150 mg cartridges and SampliQ C18 endcapped, 3 mL tubes, 500 mg cartridge
• 250 µL syringe from Hamilton Co.
• Syringe filters 4mm syringe filter 0.45 µm, PFTE
• Vortex
• Sonicator
• Centrifuge of Remi equipments
• Nitrogen evaporator, with 50^0C water bath

9.2 Chemicals

All the solvents used (acetonitrile, methanol and ethyl acetate) were HPLC grade. Other reagents used for the analysis like Formic acid, Acetic acid, Potassium di-hydrogen phosphate, di-Potassium hydrogen phosphate, Ammonia, Sodium Hydroxide, Potassium Hydroxide, Sodium Chloride etc. were of analytical grade and purchased from Merck Ltd. Water used was HPLC grade (Milli-Q).

9.3 Glassware

All the glassware used - beaker, volumetric flask, conical flask, funnel, pipettes, sample tubes, centrifuge tubes etc. - were cleaned with detergent and 10% nitric acid and rinsed thoroughly with distilled water before use.

9.4 Standards

Antibiotic reference standards were obtained from Sigma chemicals USA. The purity of the reference standards used is as follows:

- Oxytetracycline hydrochloride: 98.1%
- Chloramphenicol palmitate: 97.9%
- Ampicillin trihydrate: 99.8%
- Erythromycin A-dihydrate: 95.5%
- Enrofloxacin: 99.9%
- Ciprofloxacin: 99.9%

9.5 Sample extraction, Clean up and Analysis

9.5.1 Oxytetracycline (Class: Tetracyclines)

a. **Standard solutions and reagents preparation**

Stock standard solution (100 μg/mL)

Accurately weighed 2.5 mg of oxytetracycline into 25 mL volumetric flask; dissolved in methanol and diluted to volume. Stock solutions were stored at 4ºC for about 1 month.

Working standard solutions (5-100 ng/mL)

Working standard solutions (5-100ng/mL) were prepared from stock standard solution by appropriate dilution with 0.01M oxalic acid in a 10 mL volumetric flask. The solution was mixed thoroughly and prepared fresh every day.

10mM oxalic acid

1.26 g oxalic acid dihydrate dissolved in water in 1L volumetric flask and diluted to a final volume with water and mixed well by inversion and prepared daily.

McIlvaine buffer-pH 4.0

Mix 1L of 0.1M citric acid with 625 mL of 0.2M anhydrous Na_2HPO_4 in a 2L volumetric flask and pH adjusted to 4.

Na_2EDTA-McIlvaine buffer solution (0.1M)

To 1.625 L McIlvaine buffer add 60.5 g disodium EDTA dihydrate and mixed until dissolved. Prepared weekly.

b. Sample preparation

Extraction by liquid-liquid extraction (LLE)

5 g sample of honey was dissolved in 20 mL of 0.1 M Na_2EDTA-McIlvaine buffer at pH 4. The solution was vortexed for 5 minutes, filtered and made ready for SPE clean-up procedure (Pagliuca et. al., 2002).

Clean-up by solid phase extraction (SPE)

After extraction, sample was loaded on a SampliQ OPT 3 ml (60 mg) cartridge previously conditioned with 1 mL methanol and 1 mL water. The SPE cartridge was then washed with 10 mL water. Finally, the sample was eluted with 1 mL ethyl acetate directly in sample tube. After evaporating the solvent at 40ºC under nitrogen stream, the residues were reconstituted with 1 mL HPLC mobile phase (Pagliuca *et. al*., 2002).

c. Analysis

The sample was analysed by (Pagliuca et. al., 2002) with some modifications using HPLC equipped with DAD detector and Zorbax Eclipse XDB C8 3 μm (150 x 4.6 mm I.D.) column at 30ºC in isocratic conditions with mobile phase; aqueous oxalic acid (0.01M), acetonitrile and methanol (70:15:15). The flow rate was 1.0 mL/minute. The DAD detector monitored the eluent at 360 nm and measured spectra from 200 to 400 nm. The sample injection volume was 100 μL. Retention time of oxytetracycline was 2.7 minutes.

9.5.2 Chloramphenicol (Class: Amphenicol)

a. Standard solutions and reagents preparation

Stock standard solutions (100 μg/mL)

Accurately weighed 2.5 mg of chloramphenicol into 25 mL volumetric flask and dissolved in acetonitrile and diluted to volume. Stock solutions were stored at 4ºC for about 1 month.

Working standard solutions (5-100 ng/mL)

Working standard solutions (5-100 ng/mL) were prepared everyday from stock standard solution by the appropriate dilutions with acetonitrile and water (20:80 ratio) in 10 mL volumetric flasks.

b. Sample preparation

Extraction by liquid-liquid extraction (LLE)

5 g honey sample was weighed into 50 mL capped centrifuge tube (Zhao & Ball 2009). This was followed by addition of 5 mL of water and vortexed for 3 minutes to mix the sample thoroughly. 5 mL of ethyl acetate was then added to the centrifuge tube. The tube was tightly capped and vortexed for 5 minutes. The tube was then centrifuged at 3,200 rpm for 5 minutes. The upper organic layer was carefully transferred to another tube using disposable pipettes. This process was repeated twice and supernatant was combined and evaporated to dryness with a controlled nitrogen flow drier at 50ºC and reconstituted into 5 mL of water, vortexed and sonicated to completely dissolve the residue. The sample was then ready for SPE clean up.

Clean-up by solid phase extraction (SPE)

For SPE clean up, SampliQ OPT 3 mL (60 mg) cartridges were preconditioned with 3 mL methanol and then equilibrated with 5 mL of water (Zhao and Ball, 2009). The 5 mL sample extract was then loaded to cartridge and passed through the cartridge slowly (0.5 mL/min). The SPE cartridges were rinsed with 5 mL water twice. The entire effluent was discarded. Full vacuum was applied to the cartridge for 3 minutes to completely dry the resin. Finally, the residues were eluted with 5 mL of 20:80 methanol/ethyl acetate (2.5 mL x 2) at a rate of 1

mL/min. The eluent was collected into clean tubes and dried under nitrogen stream at 50ºC. The residue was reconstituted in 1 mL of 20:80 Acetonitrile/water. The sample was vortexed and sonicated to completely dissolve the residue in the tubes.

c. Analysis

The sample was analysed by HPLC equipped with DAD detector using a Zorbax Eclipse XDB C8 3 μm (150 x 4.6 mm I.D.) column in gradient conditions in gradient conditions given below, with mobile phase - Acetonitrile (A) and Water–pH (8.5) (B) adjusted with 0.01% ammonia (Zhao & Ball, 2009). The column temperature was maintained at 30ºC.

Time (min)	Acetonitrile (A)	Water(pH 8.5 adjusted with 0.01% ammonia) (B)	Flow (ml /min)
0	20	80	0.5
0.5	20	80	0.5
7	100	0	1.0
8	100	0	1.0
10	80	20	0.5
12	80	20	0.5

The DAD detector monitored the eluent at 280 nm and measured spectra in UV range. The sample injection volume was 100 μL. The retention time of Chloramphenicol was at 8.7 minutes.

9.5.3 Ampicillin (Class: β-Lactam)

a. Standard solutions and reagents preparation

Stock standard solutions (100 μg/mL)

Accurately weighed 2.5 mg of ampicillin into 25 mL volumetric flask and dissolved in acetonitrile-water (1:1) and diluted to volume. Stock solutions were stored at 4ºC for about 1 month.

Working standard solutions (5-100 ng/mL)

Working standards (5-100 ng/mL) were prepared everyday from stock standard solution by appropriate dilutions with acetonitrile – water (1:1) in 10 mL volumetric flask.

25mM Phosphate buffer (KH_2PO_4)

Accurately weighed 3.4 g of KH_2PO_4 into 1L volumetric flask and dissolved in water and the pH was adjusted to 3 with phosphoric acid and diluted to volume.

Extraction solution-0.1M phosphate buffer (pH-9.2)

Accurately weighed 13.8 g of sodium di-hydrogen orthophosphate monobasic; monohydrate, into a 1L volumetric flask and dissolved in water and adjusted to pH 9.2 with drop-wise addition of 10N NaOH and diluted to volume with water.

Sodium chloride solution (2%)

Accurately weighed 20 g of NaCl and dissolved in water, transferred to a 1L volumetric flask and diluted to volume with water.

b. Sample preparation

Extraction by liquid-liquid extraction (LLE)

5 g of honey sample was weighed into 50 mL capped centrifuge tube (Wang, 2004). 20 mL of extraction solution was added and the centrifuge tube was capped tightly and vortexed for 5 minutes until honey was completely dissolved. The tubes were then centrifuged at 4,000 rpm for 10 minutes at room temperature to remove particles from solution and thereby avoid plugging the SPE cartridge. After centrifugation the upper layer was carefully transferred to another tube using disposable pipettes. The sample was then ready for SPE clean up.

Clean-up by solid phase extraction (SPE)

For SPE clean up, SampliQ C18 endcapped, 3 mL tubes, 500 mg cartridges were preconditioned sequentially with 10 mL methanol, 10 mL water, 10 mL NaCl solution (2%) and then equilibrated with 2 mL of extraction solution (Wang,2004). The sample extract was then loaded on cartridge and passed through the cartridge slowly under vacuum. The SPE cartridges were then rinsed with 5 mL of water. The entire effluent was discarded. Evacuated the cartridge to dryness by applying full vacuum for 5 minutes. Finally eluted with 3 mL of acetonitrile at a flow rate of 1-2 mL/min under vacuum in a 5 mL test tube. The eluent was evaporated to dryness using nitrogen evaporator at 40ºC-50ºC under a stream of nitrogen. The residue was reconstituted in 1 mL of Acetonitrile : Water (1:1 ratio). The sample was vortexed and sonicated to completely dissolve the residues in the tube.

c. Analysis

The sample was analysed by HPLC equipped with DAD detector using a Zorbax ODS C18 5 μm (250x4.6 mm I.D.), at room temperature (25ºC) in isocratic conditions with 25 mM KH_2PO_4 (pH-3) and acetonitrile (70:30 ratio) mobile phase (Huber & Onigbinde, 2002). The flow rate was 0.8 mL/min. The DAD detector monitored the eluent at 204 nm and measured spectra in UV range. The sample injection volume was 100 μL. The retention time of ampicillin was 2.6 minute.

9.5.4 Enrofloxacin and Ciprofloxacin (Class:Fluoroquinolones)

a. Standard solutions and reagents preparation

Stock standard solutions (100 μg/mL)

Accurately weighed 2.5 mg each of enrofloxacin and ciprofloxacin separately into 25 mL volumetric flasks and dissolved in acetonitrile : acetic acid in water (2%) (16:84 ratio) and diluted to volume. Stock solutions were stored at 4ºC for about 1 month.

Working standard solutions

Working standard solutions of ciprofloxacin (10 -100 ng/mL) and enrofloxacin (5 - 50 ng/mL) were prepared everyday from stock standard solutions by appropriate dilutions with acetonitrile: acetic acid in water (2%) (16:84) in 10 mL volumetric flask .

Formic acid solution (0.1%)

1 mL of pure formic acid was added into a 1L volumetric flask and diluted to a final volume with water and mixed well by inversion.

Acetic acid solution (2%)

Placed 20 mL of acetic acid in 1L volumetric flask and diluted to volume with water.

b. Sample preparation

Extraction by liquid-liquid extraction (LLE)

2.5 g of honey sample was weighed into 50 mL centrifuge tube. 5 mL water was added and the tube was vortexed for 1 minute until all of the honey dissolved (USFDA Method 2006). Thereafter, 10 mL of acetonitrile and 200 μL acetic acid was added to the sample. The centrifuge tube was capped tightly and vortexed for approximately 30 seconds. 2 g of NaCl was added and the centrifuge tube was again vortexed for 15 seconds. The tube was then centrifuged at 2,400 rpm for 5 minutes and upper organic layer was carefully transferred to another tube using disposable pipettes. This process was repeated twice and supernatant was combined and passed through sodium sulfate cartridge. Sample was then evaporated to dryness with a controlled nitrogen flow at 50ºC before being reconstituted into 1 mL of acetonitrile : 2% acetic acid in water (16:84 ratio), vortexed and sonicated to completely dissolve the residue.

c. Analysis

The sample was analysed by HPLC equipped with Fluorescence (FLD) detector (Verdon *et. al*., 2005) using a Zorbax ODS C18 5 μm (250x4.6 mm I.D.), at room temperature (25ºC) in gradient conditions given below. The flow rate was 1 mL/min. The FLD detector monitored the eluent at excitation wavelength - 295 nm and emission wavelength - 500 nm. The sample

injection volume was 100 μL. The retention time was 5.4 minutes for ciprofloxacin and 6.6 minutes for enrofloxacin.

Time (min)	0.1% Formic acid (A)	Acetonitrile (B)	Flow(ml /min)
2	20	80	1.0
12	90	10	1.0

9.5.5 Erythromycin (Class: Macrolides)

a. Standard solutions and reagents preparation

Stock standard solutions (1000 μg/mL)

Accurately weighed 25 mg of erythromycin into 25 mL volumetric flask and dissolved in methanol and diluted to volume. Stock solutions were stored at 4ºC for about 1 month.

Working standard solutions (0.1-10 μg/mL)

Working standard solutions (0.1 – 10 μg/mL) were prepared everyday from stock standard solution in a 10 ml volumetric flask by appropriate dilutions with methanol-water (1:1 ratio).

20mM Phosphate buffer (K_2HPO_4)

Accurately weighed 3.48 g of K_2HPO_4 into 1L volumetric flask dissolved in water and diluted to volume.

0.1M phosphate buffer (pH-8)

Accurately weighed 13.8 g of monobasic sodium- phosphate (monohydrate); dissolved in water and adjusted to pH 8 with drop-wise addition of 10N NaOH. Transferred to a 1L volumetric flask and diluted to volume with water.

Sodium chloride solution (2%)

Accurately weighed 20 g of NaCl, dissolved in water. Transferred to a 1L volumetric flask and diluted to volume with water.

b. Sample preparation

Extraction by liquid-liquid extraction (LLE)

2.5 g of honey sample was weighed into 50 mL capped centrifuge tube. 20 mL of 0.1 M phosphate buffer (pH-8) was then added and the tube was capped tightly and vortexed for 5 minutes until honey was completely dissolved. The tube was then centrifuged at 4,000 rpm for 10 minutes at room temperature to remove undissolved particles from solution and thereby avoid plugging the SPE cartridge. After centrifugation the upper layer was carefully transferred to another tube using disposable pipettes (Wang, 2004).

Clean-up by solid phase extraction (SPE)

For SPE clean up, SampliQ OPT 6 mL (150 mg) cartridges were preconditioned sequentially with 10 mL methanol, 10 mL water, 10 mL NaCl solution (2%) and then equilibrated with 2 mL of 0.1M phosphate buffer (pH 8.0) (Wang, 2004). The sample extract was then loaded on cartridge and passed through the cartridge slowly under vacuum with a flow rate of 1 mL/min. The SPE cartridge was then rinsed with 5 mL of water at a flow rate of 2 mL/min, followed by 5 mL of 40% methanol in water with the same flow rate and the entire effluent was discarded. The cartridge was then dried by applying full vacuum for 5 minutes. Finally, the erythromycin residues were eluted from the cartridge with 5 mL of methanol at a flow rate of 1-2 mL/min under vacuum in 15 mL tube. The eluent was evaporated to dryness using nitrogen evaporator at 40ºC- 50ºC under a stream of nitrogen. The residues were reconstituted in 1 mL of Methanol: Water (1:1). The sample was vortexed and sonicated to completely dissolve the residue in the tubes.

c. Analysis

The sample was analysed by HPLC equipped with DAD detector (Civitareale *et. al.*, 2004) using a Zorbax ODS - C18 5 μm (250x4.6mm I.D.), at room temperature (25ºC) in isocratic conditions with 20 mM K_2HPO_4 and acetonitrile (30:70) as mobile phase. The flow rate was 1.4 mL/min. The DAD detector monitored the eluent at 210 nm and measured spectra in UV range. The sample injection volume was 100 μL. The retention time of erythromycin was 4.9 minutes.

10. Results and Discussion

Pollution Monitoring Lab (PML) tested 12 honey samples – 10 Indian and 2 Imported samples purchased from Delhi Market for the presence antibiotic residues. Six antibiotics from five classes – oxytetracycline (tetracycline), chloramphenicol (amphenicol), ampicillin (β-Lactam), enrofloxacin and its metabolite ciprofloxain (fluoroquinolones) and erythromycin (macrolides) - were analysed with HPLC-DAD/FLD .

Validation The HPLC- DAD/FLD methods were tested for repeatability and reproducibility to determine accuracy and precision for all the 5 classes analysed. The performance characteristics for the HPLC DAD/FLD methods with respect to method specific validation requirements are summarized in Table 4. Validation study was carried out at three different concentrations and a limit of quantification (LOQ) was established for all the five classes.

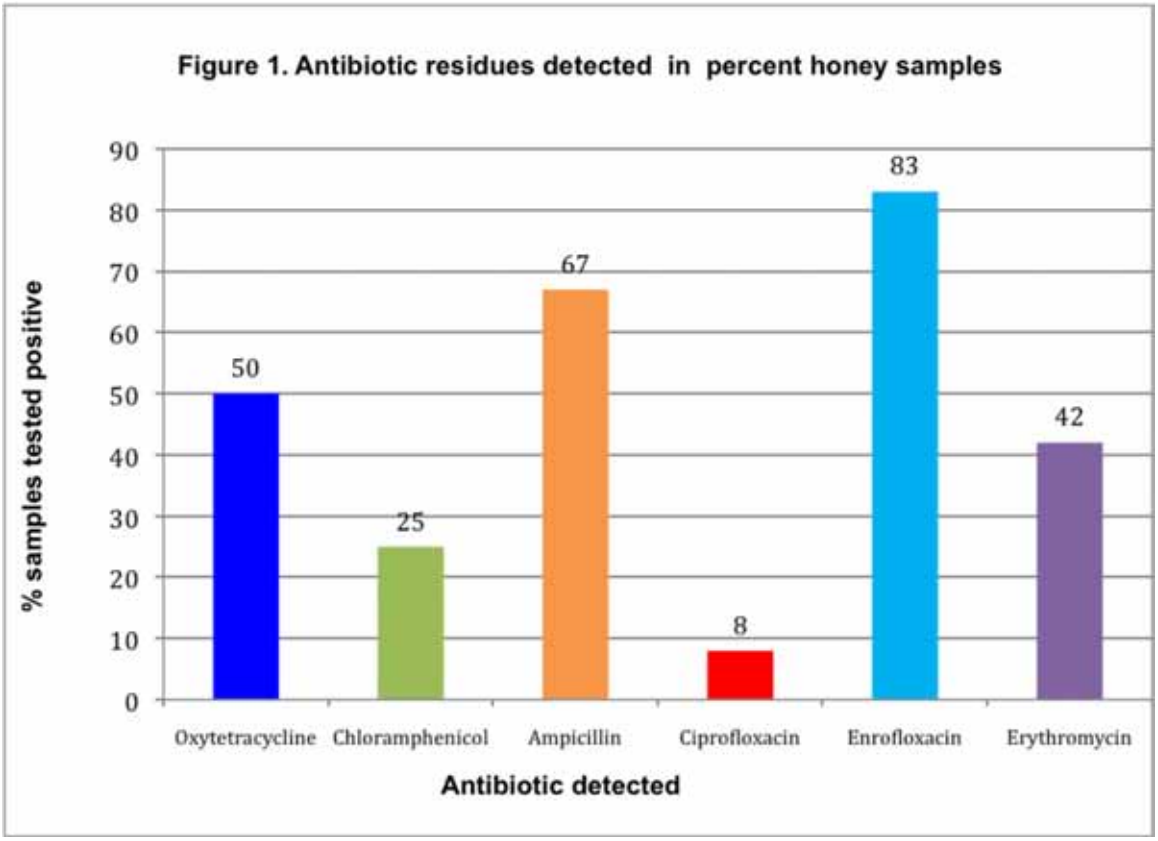
The calibration curves prepared at five different concentrations were obtained using the linear least squares regression procedure of the peak area versus the concentration. The linearity

of calibration curve for - oxytetracycline, chloramphenicol, ampicillin, ciprfloaxacin, enrofloxacin, erythromycin was good with the correlation coefficients (r^2) is above 0.995 for 3 calibration curves, prepared on different days. The recovery and repeatability of the method were evaluated by the analysis of spiked samples with oxytetracycline, chloramphenicol, ampicillin, ciprfloaxacin, enrofloxacin, erythromycin at three different concentrations The limit of detection for oxytetracycline, chloramphenicol, ampicillin, ciprfloaxacin, enrofloxacin, erythromycin in honey was 1.45, 0.87, 1.38, 2.55, 1.31, 17 μg/kg. Recoveries were more than 70% with relative standard deviation (RSD) of <10% for all the antibiotics (Table 4).

Table 4. Validation of analytical methods for antibiotic residues in Honey using HPLC DAD/FLD.

Class	**Tetracycline**	**Amphenicol**	**β-Lactam**	**Fluoroquinolone**	**Fluoroquinolone**	**Macrolide**
Antibiotic	**Oxytetracycline**	**Chloramphenicol**	**Ampicillin**	**Ciprofloxacin**	**Enrofloxacin**	**Erythromycin**
R_t (min)	2.7	8.7	2.6	5.4	6.7	4.9
Calibration Curve	5-100 ng/mL	5-100 ng/mL	5-100 ng/mL	10-100 ng/mL	5-50 ng/mL	0.1-10 μg/mL
Regression Equation	Y= 0.179x+0.024	Y= 0.286x+0.464	Y= 0.515x+1.721	Y= 3.134x+29.46	Y= 4.662x+27.73	Y= 7.595x+0.447
Linearity (R^2)	0.997	0.999	0.996	0.998	0.999	0.999
LOD (μg/kg)	1.45	0.87	1.38	2.55	1.31	17
LOQ (μg/kg)	4.82	2.92	4.60	8.55	4.36	58
Recovery (%)	86	78.71	75.22	70.78	90.8	80.53
RSD	2.24	1.47	1.22	0.3	0.2	0.2

Each sample was analysed in triplicate. At least one control (matrix blank) was run with every set of sample. No interference was encountered from the controls or honey fortified with oxytetracycline, chloramphenicol, ampicillin, ciprfloaxacin, enrofloxacin, erythromycin. The antibiotics detected in the honey samples were identified on the basis of retention time of reference standard peaks within (+/- 0.25 minutes) (Table 4) and the identity was confirmed by spiking the sample extract with known concentrations of standard at 2 different levels. Antibiotics detected in the different Indian and imported honey samples are given in (Annexures II & III).



Oxytetracycline, a tetracyclines antibiotic, most commonly used against bacterial foul brood diseases by beekeepers was detected in 50% of the honey samples (6/12) (Figure 1). The Level of Action set by Export Inspection Council, India for tetracyclines is 10 μg/kg. Oxytetracycline was detected in the range of 27.1 to 250.4 μg/kg in the 12 honey samples analysed. The average concentration detected in Dabur Honey was 91.3 μg/kg (9 times), Patanjalis Pure Honey was 27.1μg/kg (2.7 times), Khadi honey was 250.4 μg/kg (25 times), Gold honey was 57.7 (5.7 times) higher than the Level of Action set by EIC.

Interestingly, Oxytetracycline was also detected in imported brands -the average concentration of Oxytetracycline in Capilano's pure and natural honey from Australia was 151 μg/kg 15 times the EIC standard, but within the Australian standard of 300 μg/kg for Oxytetracycline in honey, in Nectaflor Natural Blossom Honey from Switzerland was 112 μg/kg -11 times the EIC standard

Chloramphenicol, a broad spectrum antibiotic, and a potential carcinogen banned from use in food producing animals, including honey bees in Canada, the United States, the European Union and other countries. It was detected in 25% of the honey samples (3/12) in the ranged from 3.6 - 4.4 μg/kg. The highest level of 4.4 μg/kg was detected in Gold Honey manufactured by Vardhaman Food & Pharmacetuticals, which is 15 times higher than the Level of action of 0.3 μg/kg set by Export Inspection Council, India for Chloramphenicol. Chloramphenicol was also detected in both the imported samples at a level of 3.6 μg/kg in Capilano's honey (Australia) and at a level of 3.7 μg/kg in Nectaflor Natural Blossom Honey

(Switzerland), 12 times higher than the EIC standard. Results from different laboratories showed that a great part of Chinese honey and also of honey from various countries, contains chloramphenicol in quantities greater than the EU regulatory standard of 0.3 µg/kg (Reybroeck, 2003; Ortelli *et al*., 2004).

Ampicillin a β-lactam antibiotics, widely used in veterinary medicine for the treatment and prevention of bacterial diseases was detected in 67% of honey samples (8/12). The average concentration of ampicillin detected in honey samples was in the range of 10.1-614.2 µg/kg. The average concentration of ampicillin in Umang honey was 208.1µg/kg. Highest level of ampicillin was detected in Nectarflor Natural Blossom Honey (Switzerland) at a concentration of 614.2 µg/kg. There is no level of action given by EIC for β lactam class. Ampicillin, is therefore, an unauthorized and illegal substance in honey.

In a study from Tamil Nadu, India Ampicillin examined in honey collected during peak flowering seasons of rubber (March and April) and banana (December and January) was detected in the range of 3-44 µg/kg (Solomon *et al*. 2006).

Enrofloxacin, a synthetic antibacterial belonging to fluoroquinolone class approved to treat bacterial infections in cattle, but in no other food animal. Use of fluoroquinolones to treat any honey bee disease is considered to be an unapproved drug by FDA. Enrofloxacin was detected in 83% of the samples -10 out of 12 samples analysed. The average concentration ranged from 10.9 to 144.8 µg/kg, the highest amount being present in Capilano's Pure & Natural Honey (Imported brand).The average concentration of enrofloxacin detected in domestic samples was- Dabur Honey -88.7 µg/kg, Himalaya forest Honey - 63.8 µg/kg and Patanjalis Pure Honey - 75.1 µg/kg and Umang Honey 122.1 µg/kg.

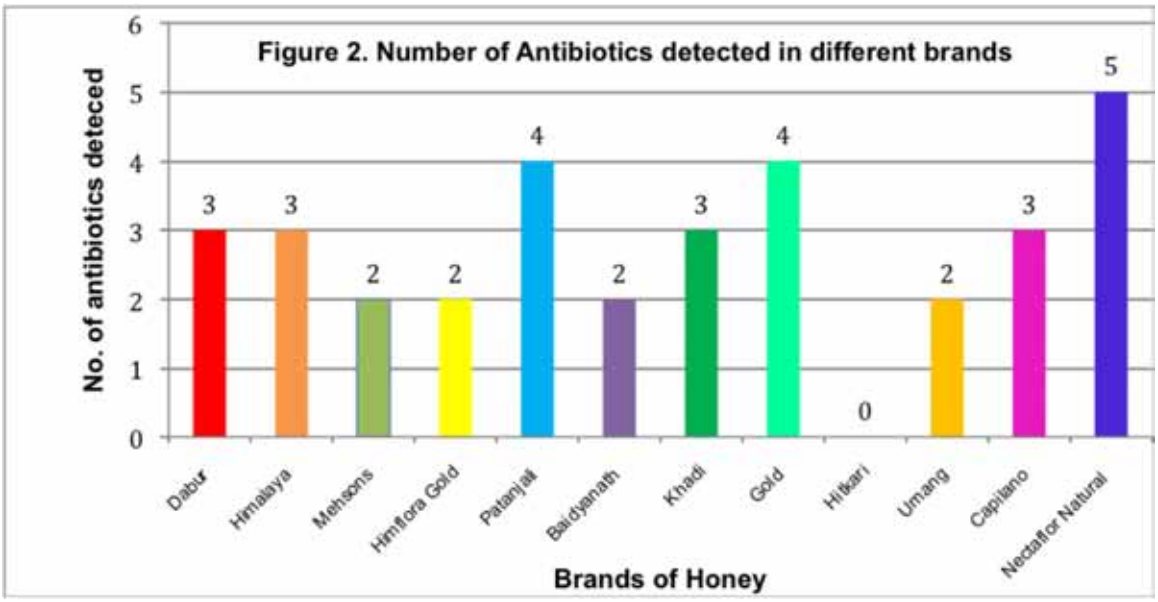
Ciprofloxacin, a metabolite of enrofloxacin (derived by enrofloxacin dethylation) which has been restricted to use in medicine, was found in only 1 out of 12 samples (8%) at a concentration of 19.9 µg/kg in Baidyanath Wild Flower Honey. No standard.

Erythromycin an important macrolide widely used to protect honey bees from bacterial diseases was detected in 42 % of the samples in 5 out of 12 samples. It was detected at a range of 69.7 to 280.3 µg/kg, the highest being in Nectaflor Natural Blossom Honey (Switzerland). The average concentration detected in Himalaya Forest Honey was 69.7 µg/kg, Mehsons Pure Honey was 85 µg/kg, Patanjali Pure Honey was 186 µg/kg and Gold honey was 231.3µg/kg. There is no level of action given by EIC for Macrolide class. There are reports of erythromycin residues in honey where 8% of the honey samples were found to be contaminated with erythromycin at a concentration ranging from 50 -1776 µg/kg (Gunes,

2008). Vidal *et al*. (2009) reported presence of upto 8.6 μg/kg erythromycin in three out of 16 samples of honey in Granada & Almeria.

11. Conclusions

It is clear from the results that 11 out of the 12 samples of honey analyzed were non compliant with EIC standard for honey to be exported for antibiotics. Multiple antibiotics were detected in all domestic and imported brands of honey tested-except Hitkari Honey of Hitkari Pharmacy, Delhi was found to be free of antibiotics. Highest number of antibiotics - 5 out of 6 were detected in imported Nectaflor Natural Blossom Honey, followed by Patanjalis Pure honey which had 4 antibiotics. The number of antibiotics in other brands was 3 each in Dabur, Himalaya Forest and Khadi Honey, 2 each in Mehsons Pure Honey, Himflora Gold, Umang Honey and Baidyanath Wild flower Honey. No antibiotic was detected in Hitkari Honey. Three antibiotics were detected in imported brand from Australia (Capilano Pure and Natural Honey) (Figure 2).



One reason for this could be the prevalent practice whereby honey is collected from different sources and then pooled before being packed and distributed for sale. Widespread contamination of different components of environment by antibiotics has been reported including milk, eggs, meat and honey etc. (Khaskheli, 2008; Schneider, 2001; Gunes 2008). The concentrations detected in the present study honey samples are low and not likely to cause any acute effect, chronic health effects cannot be ruled out. There is a need to regulate and monitor the level of antibiotics in honey as continuous long term exposure to low levels of antibiotics could in due course of time lead to antibiotic resistance in pathogenic bacteria making their treatment difficult.

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Annexure I: Details of the samples analysed for antibiotic residues

S. No.	Code	Brand Name	Company	Date of manufacturing	Expiry Date	Batch No.	Remarks
1	001	Dabur Honey	Dabur India Ltd. Vill. Billanwali Lavana P.O. Baddi Distt. Solan H.P. - 173205	Mar-09	Best before 18 months from date of packing	BD0822	-
2	002	Himalaya Forest Honey	The Himalaya Drug Company, Makali, Bangalore - 562123	Mar-09	Best before 2 years from date of packing	N0290050	No sugar added and preservative
3	003	Mehsons Pure Honey	Mehsons (India) Ltd., Kamla Bhawan, Madari Gate, Bareilly - 243003	Nov-08	Best before 18 months from date of packing	1475	Agmark honey special grade, awarded for purity & quality, gold medal london
4	004	Himflora Gold	Food Max E-45, Sec-8, NOIDA (UP)	Apr-09	Best before 18 months from date of packing	SH/001	-
5	005	Patanjali Pure Honey	Patanjali Ayurved Ltd., D - 38, Industrial Area, Haridwar, Uttarakhand - 249401	Jun-09	Best before 12 months from date of packing	PH - 021	-
6	006	Baidyanath Wild Flower Honey	Shree Baidyanath Ayurved Bhavan Pvt. Ltd., Gwalior Road Jhansi (U. P.)	Jan-09	Best before 18 months from date of packing	3	-
7	007	Khadi Honey	Khadi Gram Udyog Sewa Samiti, Distt. Madhyapura, Bihar	Jun-09	Best before 12 months from date of packing	A - 25	Agmark honey grade-A
8	008	Gold Honey	Vardhman Food & Pharmaceuticals, Plot No. 3/59, HSIDC Ballabgarh, Faridabad, Haryana	10-Jan-09	Best before 18 months from date of packing	109	Agmark honey grade-A
9	009	Hitkari Honey	Hitkari Pharmacy, WZ - 322, Skur Pur Village, Delhi - 110034	24-Mar-09	Best before 18 months from date of packing	2-2009	Agmark honey grade-A
10	010	Umang Honey	Udyog Bhartiya Registered KVI society C-9, R. P. Singh Delhi 7	May-09	Best before 18 months from date of packing	32009	Agmark honey grade-A
11	011	Capilano Pure & Natural Honey	Capilano Honey Ltd., 399 Archerfield Road, Richlands - 4077, Australia	5-Feb-08	4-Feb-11	Not Mentioned	-
12	012	Nectaflor Natural Blossom Honey	Blossom Honey, A6293 Narimpex AG, Biel, Switzerland	5-Jan-09	4-Jan-12	L61811	Imported & Marketed By: L-Comps & Impex Pvt. Ltd.. 182/63, Industrial Area, Chandigarh, Month of Import Apr - 09

Annexure II: Antibiotic residues in Honey samples in ppb (µg/Kg ± SD)

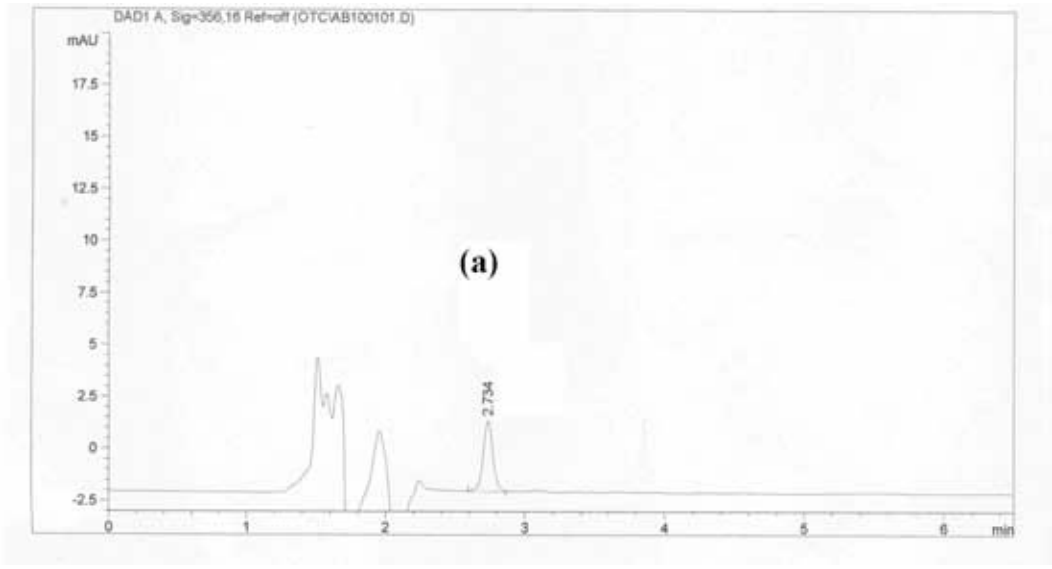
		Antibiotic tested	**Oxytetracycline**	**Chloramphenicol**	**Ampicillin**	**Enrofloxacin**	**Ciprofloxacin**	**Erythromycin**	**No. of antibiotics detected**
S. No.	**Code**	**Level of Action (LOA) - Export Inspection Council, India (µg/Kg)**	10	0.3	No LOA	No LOA	No LOA	No LOA	
1	001	Dabur Honey	91.3 ±4.6	ND	26.6 ±0.4	88.7±0.7	ND	ND	3
2	002	Himalaya Forest Honey	ND	ND	23.8 ±1.3	63.8±1.2	ND	69.7±8.23	3
3	003	Mehsons Pure Honey	ND	ND	ND	58.3±1.0	ND	85.0±7.84	2
4	004	Himflora Gold	ND	ND	35.5 ±1.9	37.7±0.2	ND	ND	2
5	005	Patanjali Pure Honey	27.1±1.4	ND	30.5 ±0.7	75.1±0.2	ND	186.0±3.43	4
6	006	Baidyanath Wild Flower Honey	ND	ND	25.2 ±0.3	ND	19.9±0.2	ND	2
7	007	Khadi Honey	250.4 ±3.6	ND	10.1±0.2	10.9±0.1	ND	ND	3
8	008	Gold Honey	57.7±10.6	4.4±0.2	ND	34.3±0.7	ND	231.3±11.09	4
9	009	Hitkari Honey	ND	ND	ND	ND	ND	ND	0
10	010	Umang Honey	ND	ND	208.1 ±1.1	122.1 ±0.3	ND	ND	2
11	011	Capilano Pure & Natural Honey	150.8±6.3	3.6±1.4	ND	144.8±1.8	ND	ND	3
12	012	Nectaflor Natural Blossom Honey	112.0±10.0	3.7±0.4	614.2 ±2.5	56.1±0.6	ND	280.3±4.95	5

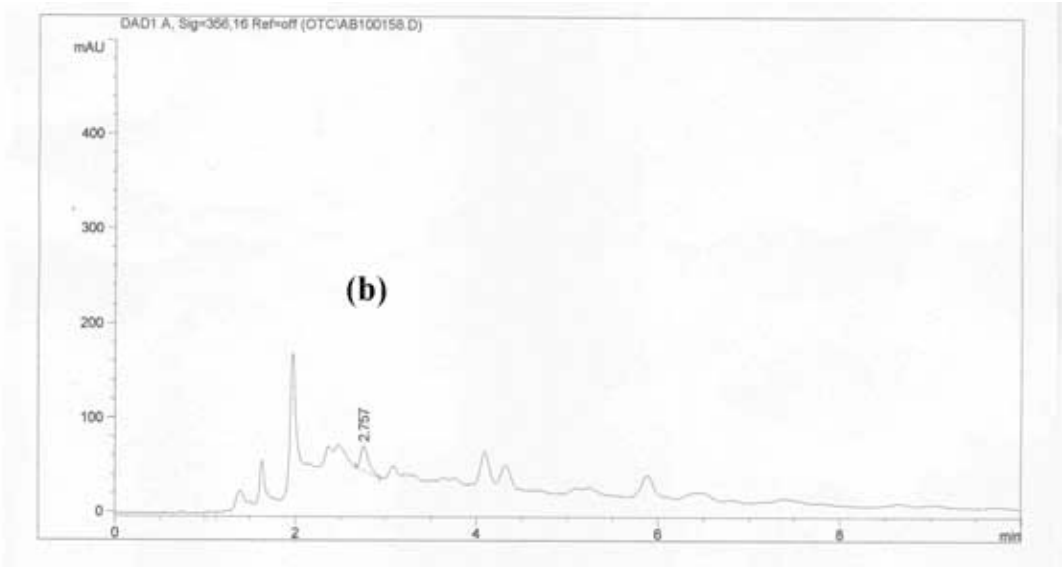
Notes:
1. Analysis carried out in triplicate
2. SD -Standard deviation
3. ND = not detected; the values of antibiotics in honey is in microgram per kilogram (µg/kg), also referred to as parts per billion (ppb)

Annexure III Antibiotic residues in Honey samples

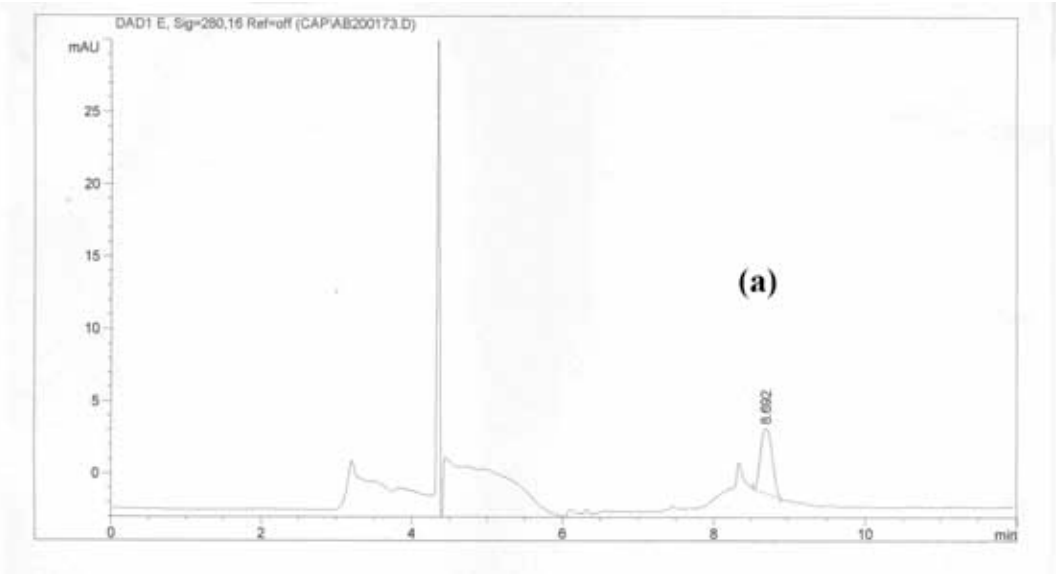
Antibiotic	Oxytetracycline	Chloramphenicol	Ampicillin	Ciprofloxacin	Enrofloxacin	Erythromycin
Level of Action (LOA) - Export Inspection Council, India (µg/Kg)	10	0.3	No LOA	No LOA	No LOA	No LOA
Samples analysed	12	12	12	12	12	12
No. samples tested positive	6	3	8	1	10	5
% of samples above LOA	50	25	67	8	83	42
<LOD	6	9	4	11	2	7
0-100 (µg/kg or ppb)	3	3	6	1	8	2
100-200 (µg/kg or ppb)	2	0	0	0	2	1
>200 (µg/kg or ppb)	1	0	2	0	0	2

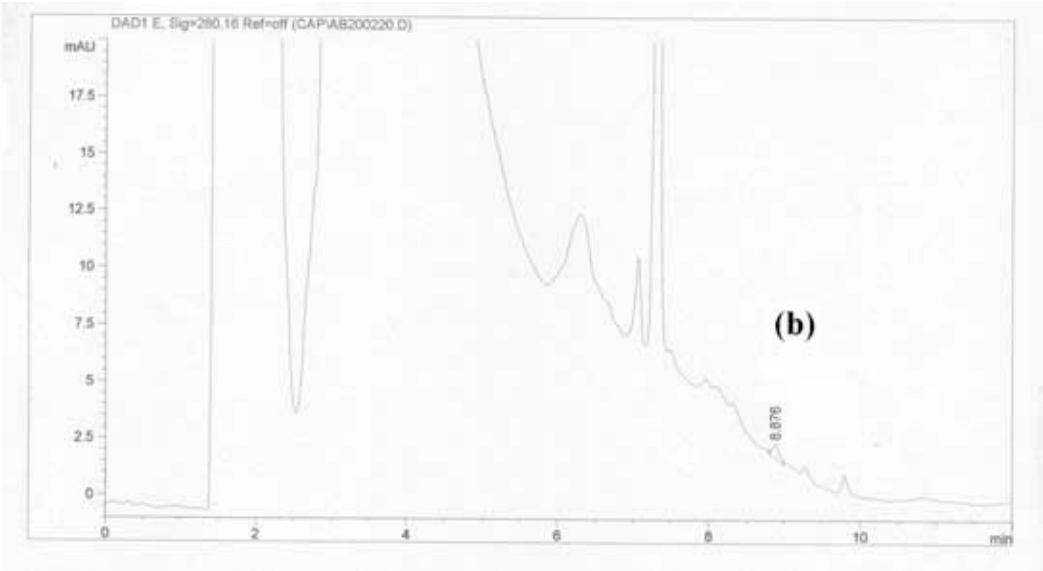
Note: LOD – Limit of detection (µg/Kg)



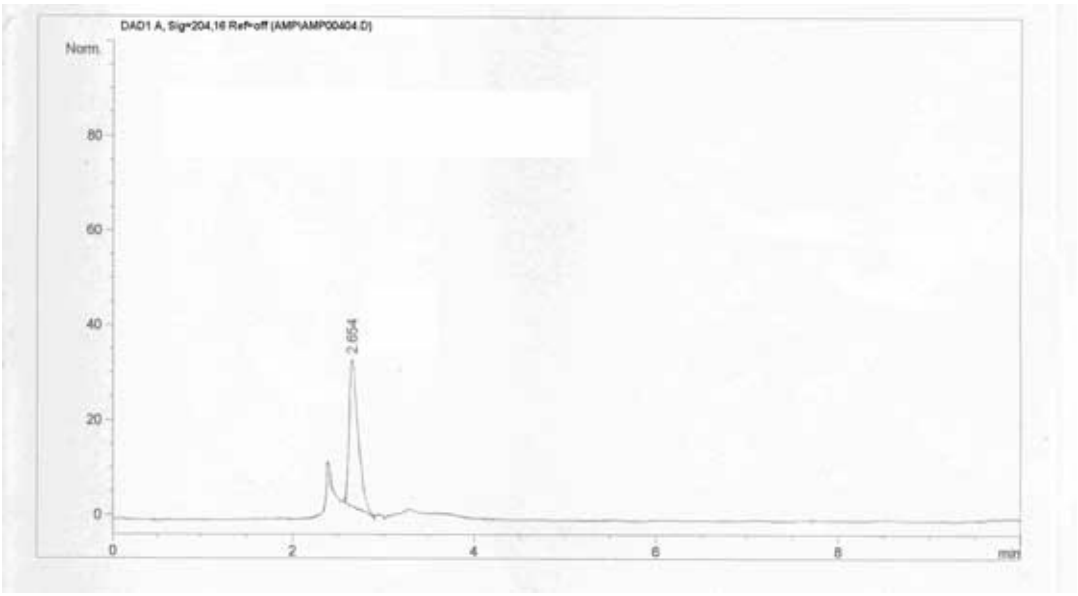


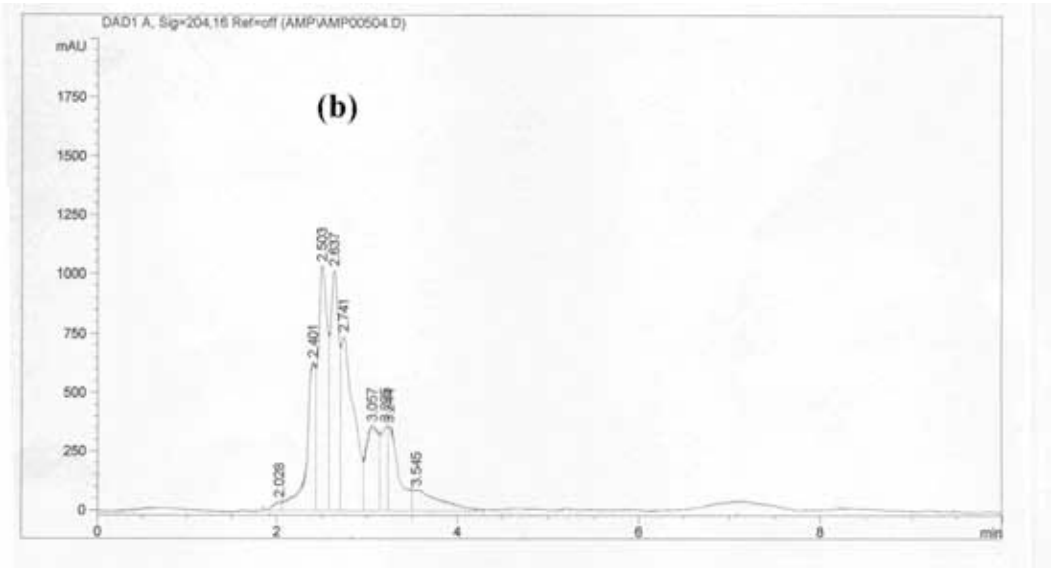
Figures 1: *HPLC Chromatogram of (a) Oxytetracycline standard 100 ng/mL (b) Oxytetracycline detected in Honey sample (007)*



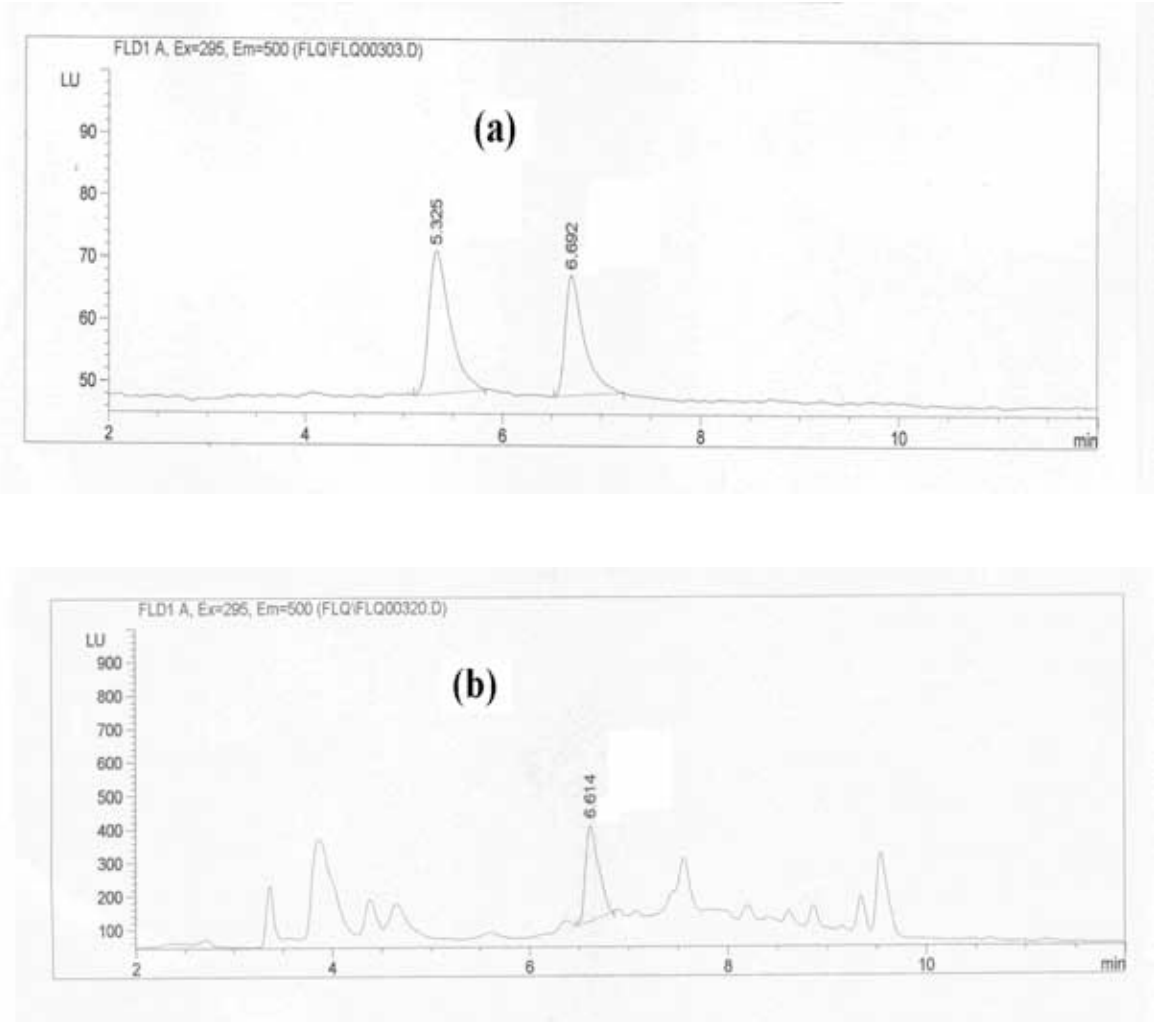


Figures 2: *HPLC Chromatogram of (a) Chloramphenicol standard 100 ng/mL (b) Chloramphenicol detected in Honey sample (012)*

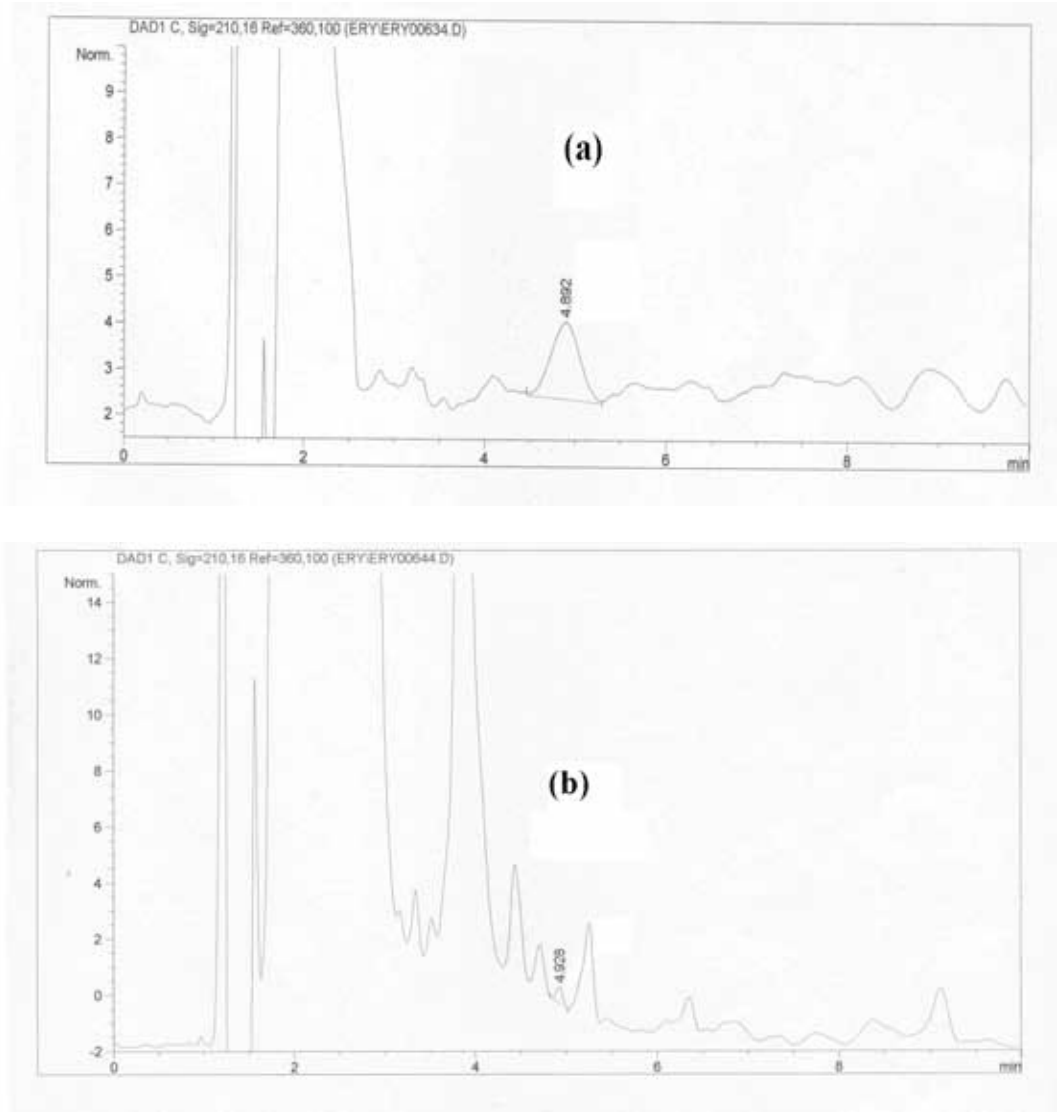




Figures 3: *HPLC Chromatogram of (a) Ampicillin standard 200 ng/mL (b) Ampicillin detected in Honey sample (007)*



Figures 4: *HPLC Chromatogram of (a) Enrofloxacin (50 ng/mL) & its metabolite ciprofloxacin standard (100 ng/mL) (b) Enrofloxacin detected in Sample (011)*



Figures 5: *HPLC Chromatogram of (a) Erythromycin standard 5 μg/mL (b) Erythromycin detected in Honey sample (005)*