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Antibacterial activity of bile on *Escherichia coli* and *Salmonella* spp isolated from faeces, urine and blood.

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ABSTRACT

The antibacterial activity of bile on *Escherichia coli* and *Salmonella* spp isolated from faeces, urine and blood was determined in this study. Bile from three animal origin (cow ,goat and chicken) was extracted using alcohol and water and their antibacterial activity tested using the agar well diffusion method at different concentrations . The crude extracts exerted the highest inhibition zone diameter within the range of 20mm-49mm and 38mm-48mm against *Salmonella* spp and *Escherichia coli* respectively, followed by the alcoholic extract with inhibition zone diameter in the range of 6mm-35mm and 7mm-33mm against *Salmonella* spp and *Escherichia coli* respectively. While the aqueous extract exhibited the least activity in the range of 5mm-25mm and 7mm- 25mm against *Salmonella* spp and *Escherichia coli* respectively. The MIC of the bile extracts which was determined using the tube macrodilution method and the MBC showed that bile have both bactericidal and bacteriostatic activity on the test organisms. The findings of this work has shown that bile contains some substances that can exert inhibitory effect on bacteria hence can be employed in the treatment of diseases caused by these groups of bacteria.

Keywords: Bile, Antibacteria, Feaces, Urine, Blood,Farm animals.

1. INTRODUCTION

The digestive system typically combats potentially pathogenic microbe through the production of several bactericidal agents along the tract. Some of these bactericidal agents are gastric secretions, hydrochloric acid and bile (Megan *et al* .,2009). Bile, a yellow/green aqueous solution of organic and inorganic compounds whose major constituent include bile acids, bile alcohols, phosphatidylcholine and a pigment bilverdin is an end product of cholesterol metabolism in vertebrates (Hofmn *et al.*,2008).

Cholanology, the science of bile salts, despite being of research interest for than a century continues to be a rapidly emerging field in medicine and biology, several questions that puzzled scientists about the role of bile salts in clinical medicine have been recently answered. For example, a clear understanding of the molecular biology of bile salts biosynthesis, transcriptional and translational regulation of hepatic export pump (BSEP, ABCB11) is necessary for the

development of new therapies. New discoveries involving the role of bile salts in disorders have been made in the field (Samrat and Uday,2004).

A plethora of studies on actions of bile on a variety of pathogenic and commensals bacteria have identified membranoytic effects as well as DNA –induced damage on these bacteria. Begley *et al* (2005) reviewed studies on the effects of bile on the integrity of the bacterial membrane. The bacteriocidal properties of bile *in vivo* as a result of their surfactant properties that renders them powerful solubilizers can never be overemphasized (Inagaki *et a*l.,2006). Determining the effect that bile salts have on the integrity of bacterial membrane mainly has been investigated through molecular analysis studying the regulation of membrane proteins in the presence of bile salts(Riuz *et al.*,2009). Analyzing the regulation of gene encoding outer membrane proteins, efflux pumps and cell membrane biosynthesis enzymes has indicated that bile salts interact with bacterial cell membranes (Nikado *et al.*,2008, Rince *et al.*,2003, Riuz *et al.*, 2009).

Bile alters the fatty acid composition as well as the ratio of membrane proteins to phospholipid resulting in an altered cell surface structure in bacteria such as *Lactobacillus reuteri* and *Bifidobacterium animalis* (Riuz *et al.*,2007,Taranato *et al*.,2003). In contrast, molecular studies on the mechanisms of bile salt tolerance by certain microbes indicated presence of unusual proteins buttressed the fact that bile salts are strong antimicrobial agents.

Bile – induced damage on *Eschericha coli, Salmonella enterica* and some Gram positive enteric bacteria have been studied by Megan *et al.*,(2009), though the studies showed that some strains resist the antimicrobial effect of bile salts while improving their pathogenicity in contrast to the strong bacteriostatic actions against some strains.

The results of many other investigations including electron microscopy showing cells exposed to bile becoming shrunken and empty as well as enzyme assays confirming leakage of intracellular materials implies that bile alters membrane integrity and permeability(Begley *et al.*,2009). These studies showed that exact outcome of action of bile on cell membranes is determined by concentration of bile, type and structure of bile imposing the stress and membrane architecture and composition. In addition to affecting membrane characteristics, bile can have numerous other effects on bacterial cells as reviewed by Begley *et al.*,(2005) including disturbing macromolecule stability, alteration of the conformation of proteins resulting in their misfolding or denaturation and,oxidative stress through the generation of oxygen free radicals. The intracellular dissociation of bile salts may impose a low pH stress on cells and the movement of ions may have osmotic effects. As bile can chelate calcium and iron, the presence of bile inside a cell may result in low intracellular calcium and iron concentrations. It is evident that bile represents a plethora of challenges to a bacterial cell. The multifaceted nature of the stress it imposes is reflected in the diverse function of genes that play a role in resistance to bile(Begley *et al.*,2005).

Bile is a biological detergent composed of multitude of components including proteins, ions, pigments, cholesterol, phospholipids and various bile salts.(Inagaki *et al*,2009). The deleterious effects of bile on cell can be broadly classified into two based on their action on cell membrane and cell internal environment (Perez and Briz,2009) : The bile acid-induced cell membrane injury and bile aid- induced cell death pathway. Studies conducted by Perez and Briz,(2009) showed that bile acids cause membrane damage by the swelling and oxidation mechanisms.

Experiments using human and rat cells buttressed these facts.(Perez and Briz,2009). The basic principle underlying dissolution is “like dissolves like” and bile like every other amphipathic

substance possesses both polar and non polar ends which are hydrophilic and hydrophobic respectively and just like every other detergents follows two main steps in exhibiting membranolytic activity; partitioning of the detergent molecule in the membrane and solubilization of the membrane. (Garidel *et al*,2007). This is achieved by the interaction of polar ends of both components as a result, the membrane integrity is distorted due to loosening of it's components and influx of substances from the external environment causing swelling and eventually lysis and death of the cell (Garidel *et al*,2007).

Hydrophobic bile acids impair respiration and electron transport in mitochondria. These compounds decrease the activities of several enzyme complexes involved in the electron transport chain such as complexes i,iii and iv where as complex ii is not affected.

These bile acid induce membrane permeability to protons, either by acting as protonophores or by disruption of the structural organization of membrane components, these compounds can stimulate the generation of reactive oxygen species leading to a depletion of antioxidant defense including total mitochondrial glutathione contents and the concentration of substance involved in electron transport chain such as ubiquinone-9 and ubiquinone-10.

(Perez and Briz,2009).

Furthermore, bile acid concentration within the cell can result in cell injury and death through mechanisms of apoptosis and necrosis in low and high concentrations respectively.

These are the proposed mechanisms by which bile exerts their membranolytic and cytotoxic effects(Perez and Briz,2009).

2. MATERIALS AND METHODS

2.1 BILE SAMPLE COLLECTION

The bile samples used in this are crude bile extracts from chicken collected at Michael Okpara University of Agriculture,Umudike poultry farm while the cow and goat bile were collected at slaughter farms Olokoro and Ahiaeke respectively. After collection, they were placed in a cellophane bag and taken to the laboratory.

2.2 BILE EXTRACTION

Bile from the different animals; cow, goat and chicken were extracted aseptically using sterile syringes and needles by puncturing the gallbladders of these animals and aseptically dispensed into sterile specimen collection bottles. The bile was extracted immediately after the animals were slaughtered and stored at room temperature.

2.3 PREPARATION OF EXTRACT CONCENTRATION

Three different bile preparations were used; crude bile extract, aqueous bile and alcoholic bile solutions.

2.4 ISOLATION OF TEST ORGANISMS

The test organisms used in this study are the *Enterobactericeae* isolated from urine, faeces and blood samples. Specimen of urine, faeces and blood were each inoculated onto Eosin-Methylene-Blue agar, Salmonella-Shigella agar and MacConkey agar respectively by streaking using sterile wire loop, immediately after collection from student with urinary tract, gastrointestinal tract and

typhoid fever infections, the culture media were incubated at 37°C for 24 hours and growth observed after incubation.

2.4 BILE ACTIVITY ASSAY

2.4.1 PREPARATION OF STANDARD INNOCULUMS OF TEST ORGANISMS

From the confirmed colonies, inoculums were obtained by diluting 0.1ml of the test organisms in 0.9 normal saline which was emulsified and 9mls of water added to the emulsion.

2.4.2 ANTIBACTERIAL SUSCEPTIBILTY TESTING

A 0.1ml inoculums suspension each was swabbed uniformly on different plates containing 20ml of solidified Mueller-Hinton agar and allowed to air dry for 5 minutes. Two wells of 6mm in diameter were made using a sterile cork borer on the agar plates for each of the concentration and agar plugs removed with sterile forceps. The different bile concentrations: 1000mg/ml, 500mg/ml, 250mg/ml, 200mg/ml of the crude extract and 100mg/ml, 50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml,3.125mg/ml of the aqueous and alcoholic solutions of the three bile origin were introduced into the wells labeled properly and allowed to stand for some minutes at room temperature. Controls were set up. The plates were incubated at 37°C for 24 hours. After incubation, plates were observed for inhibition zone diameter around the wells and the inhibition zone diameter were measured using a transparent metre rule and recorded in millimetres.

2.4.3 DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC) USING THE MACRODILUTION METHOD

Three sets of test tube racks loaded with eighteen sterile test tubes each were set up. These were divided into sections and labeled for the aqueous, alcoholic and crude bile extract of each bile

origin(cow, goat and chicken). 9mls of sterile Mueller-Hinton broth was added to each test tube aseptically and 1ml of the different bile concentrations added into each test tube in accordance with the labeling followed by addition of 0.1ml of the test inoculums. The tubes were corked with non-absorbent cotton wool and incubated at 37ºC for 24 hours. Controls were set up without the bile. After incubation, the tubes were checked for turbidity which indicated growth of organism. MIC was defined as the lowest sample concentration showing no turbidity

(Cheesebrough,2010)

2.4.4 DETERMINATION OF MINIMUM BACTERICIDAL CONCENTRATION (MBC)

The MBC was determined by sub-culturing growth from the non-turbid tubes onto a nutrient agar plate by streaking using a sterile wire loop. The plates were incubated at 37ºC for 24 hours and checked for growth at the elapse of 24hours. The least concentration showing no growth on the agar was recorded as the MBC (Cheesbrough,2010).

3. RESULTS AND DISCUSSION

3.1 Results

The antibacterial activities of the different bile origin at the different concentrations against the test organisms using the well diffusion assay is presented in table 2-4. Inhibition zone diameter below 5mm were ignored. From the results, bile from cow exerted most antibacterial activity against the isolates in each of the three different phases; aqueous and alcoholic and crude, in the range of 8mm-48mm inhibition zone diameter against *Salmonella* spp and in the range of 7mm-49mm against *Escherichia coli* followed by the goat bile in the range of 7mm-33mm inhibition

zone diameter against *Salmonella* spp and 7mm-35mm against *Escherichia coli*. The bile from chicken origin exerted the least antibacterial activity in the range of 7mm-30mm inhibition zone diameter against *Salmonella* spp and 5mm-29mm against *Escherichia coli.*

Table 4 and 5 shows the MIC and MBC of the different bile origin against the isolates. For the aqueous bile extracts, the test tubes containing the cow bile showed turbidity in the tubes containing the 6.25mg/ml and 3.125mg/ml and no turbidity in the tubes of concentrations 100mg/ml down to the 12.5mg/ml against the *Salmonella* spp. The bile from the goat and chicken showed turbidity in the tubes of concentrations of 12.5mg/ml down to 3.125mg/ml and no turbidity in tubes of concentrations of 100mg/ml to 25mg/ml against *Salmonella* spp. Thus MIC was recorded at concentrations of 12.5mg/ml for the cow bile and 25mg/ml for both the goat and chicken bile against *Salmonella* spp. On sub-culturing of the non turbid tubes, MBC was recorded at 25mg/ml concentration for cow bile and at 50mg/ml concentration for both the goat and chicken bile against *Salmonella* spp. Against *Escherichia coli*, the cow bile recorded MIC at 25mg/ml and MBC at 50mg/ml concentrations. while the goat and chicken bile both recorded MIC and MBC at 25mg/ml and 50mg/ml concentrations respectively. For the alcoholic bile extract, the tubes containing different concentrations of the bile from cow and goat showed no turbidity and thus both recorded MIC at concentration of 3.125mg/ml. while for the chicken bile, concentrations of 6.25mg/ml and 3.125mg/ml showed turbidity while the rest showed no turbidity and MIC was recorded at 12.5mg/ml concentration against *Salmonella* spp, on sub-culturing the non turbid tubes MBC was recorded at 12.5mg/ml c0ncentration for cow bile and 25mg/ml concentration for both goat and chicken bile against *Salmonella* spp. Against *Escherichia coli* similar results were also obtained for the different concentrations of the alcoholic bile extracts of the three bile origin as regards the MIC. On sub-culturing the non

turbid tubes, the MBC was recorded at 6.25mg/ml concentration for the cow bile, 12.5mg/ml concentration for the goat and 25mg/ml concentration for the chicken bile against *Escherichia coli*. The tubes containing crude extracts of the different bile origin all showed no turbidity and no growths on plates against the test organisms after sub-culturing, and thus all recorded MIC and MBC at 200mg/ml concentration.

3.2 DISCUSSION

A plethora of studies have shown that the antibacterial activity of bile can never be overemphasized. Bile is strongly cytotoxic and membranolytic according to different molecular and cellular analytical studies of bile at different concentrations. Bacterial infectious disease represents an important cause of morbidity and mortality worldwide coupled with the increasing rate of resistance of pathogenic bacteria to already available antimicrobials. Therefore, the development of new antimicrobial agents for the treatment of bacterial infections is of increasing interest. Bile the most dreaded part of animals slaughtered for food because of it's bitter, pungent condemning taste can be made useful in pharmacology, by employing it's antibacterial effect for therapeutic purposes.

The main objective of this study was to evaluate the ability of bile extracts to inhibit the growth of the two *Enterobacteriaceae* (*Salmonella* spp and *Escherichia coli*) whose natural habitat is the intestine. Though counter studies show that these group of microbes have the ability to resist the deleterious effects of bile *in vivo*. This study employed the service of both organic and inorganic solvents at different concentrations in exploring the antibacterial effects of bile *in vitro*. In this experiment, the well diffusion method was used to measure the antibacterial effects of bile at different concentrations which was recorded when the inhibition zones diameter was greater

than or equal to 5mm. The crude extracts exerted the highest inhibition zone diameter 20mm-49mm and 38mm-48mm against *Salmonella* spp and *Escherichia coli* respectively, followed by the alcoholic extract with inhibition zone diameter within the range of 6mm-35mm and 7mm-33mm against *Salmonella* spp and *Escherichia coli* respectively. The aqueous extract exhibited the least activity in the range of 5mm-25mm and 7mm- 25mm against *Salmonella* spp and *Escherichia coli* respectively.

Generally, the bile from the cow showed the highest potency with inhibition zone diameter of 49mm followed by the bile from goat at 35mm while the chicken bile recorded the least potency with inhibition zone diameter of 30mm. The difference in the antibacterial activities of the bile extracts from the three different origin (cow, goat and chicken) may be due to the structural difference in the bile constituents.(Hofmann *et al.*,2010). Also, the difference in their antibacterial activity may be attributed to the system of rearing. The chicken bile was gotten from Michael Okpara University of Agricultue, Umudike poultry farm, where strict intensive farming is the modus operandi. These chickens are fed with feeds incorporated with many additives and antibiotics. By antibiotics selection pressure mechanism, the *Enterics*, both commensals and pathogens in a bid to survive the pressure presented by both the bile and the range of antibiotics in the feeds *in vivo* might have modified the bile rendering it less effective against the test organisms (also *Enterics*) *in vitro.* The goat bile was gotten from Ahiaeke slaughter house where local goats under semi-intensive farming is predominant. The cow from Olokoro slaughter is under strict extensive farming and feeds on wide range of herbs, grasses and many organic feeds with little or no synthetics, this might be responsible for its increased activity against the isolates than the bile from the other two animals.

Comparison of the alcoholic and the aqueous bile extracts showed from the results that the alcoholic extract has greater effect in inhibiting the test organisms than the aqueous extracts which may be due to the fact that alcohol is the better solvent for extraction than water ("like dissolves like" in the principle of dissolution). Also alcohol itself has antibacterial activity against bacteria.

The activities of the bile tends to decrease with decrease in concentration and is in agreement with the studies conducted by Inagaki *et al* .,(2006) on "Regulation of antibacterial defense in the small intestine by the nuclear bile acid receptor" and Hofman *et al*.,(2006) on "How bile confer gut mucosal protection against bacteria" which suggested that high concentration of bile acids restrict the population size of microbes in the intestine.

For the MIC, the least concentrations of the extracts of the three bile origin that showed no turbidity in the tubes was recorded as the MIC. The MIC value of the aqueous extract of the three bile origin against the isolates of *Escherichia coli* was recorded at 25mg/ml concentration . While it is 12.5mg/ml concentration for cow and 25mg/ml concentration for both goat and chicken against *Salmonella* spp. For the alcoholic extract, the cow and goat bile recorded MIC at 3.125mg/ml concentration against each of the isolates, while the chicken bile recorded MIC at 12.5mg/ml concentration against the two genera. The determination of the MBC of the bile extracts which is the lowest concentration of the non turbid tubes that showed no growth after sub-culturing showed different results indicating that at certain concentrations, the bile exerted bacteriostatic and bactericidal effects on the test organisms. The MBC was observed to be 25mg/ml concentration for the cow and 50mg/ml concentration for both goat and chicken bile in aqueous solution while the alcoholic extract showed MBC at 12.5mg/ml concentration for cow and 25mg/ml concentration for both goat and chicken against *Salmonella* spp . Against

Escherichia coli, the MBC was recorded at 50mg/ml concentration for aqueous extract of the three bile origin while the alcoholic extracts recorded MBC at 6.25mg/ml,12,5mg/ml and 25mg/ml concentrations for the cow, goat and chicken bile respectively.

CONCLUSION

On basis of the antibacterial assay of this study, the alcoholic extract exhibited more potent inhibitory effect than the aqueous extract and bile of cow origin exhibited the highest inhibitory effect when compared to the bile from the goat and chicken. The field of cholanology therefore should extend to cholanopharmacology which will encourage the use of bile in combating infections caused by pathogenic enteric bacteria. It can also be concluded that only very small amount of bile is required to inhibit the growth of these bacteria and may reduce the deleterious effects of bile on human cells, as it also exerts its membranolytic and cytotoxic effects on human cells. This study of therapeutic potentials of bile should also extend to other pathogenic organisms and also explore the possibility of synergistic activity with other antimicrobial.

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TABLE 1: ANTIBACTERIAL ACTIVITY OF AQUEOUS BILE SOLUTION FROM COW, GOAT AND CHICKEN AGAINST THE ISOLATES

CONCENTRATION (mg/ml)	INHIBITION ZONE DIAMETER (MM)			ISOLATES
	COW	GOAT	CHICKEN	
100	30 29	25 25	18 18	*Salmonella* spp *Escherichia coli*
50	26 23	20 19	17 15	*Salmonella* spp *Escherichia coli*
25	21 18	17 16	10 8	*Salmonella* spp *Escherichia coli*
12.5	17 13	15 13	11 9	*Salmonella* spp *Escherichia coli*
6.25	12 10	10 9	11 10	*Salmonella* spp *Escherichia coli*
3.125	8 7	7 7	7 5	*Salmonella* spp *Escherichia coli*

TABLE 2: ANTIBACTERIAL ACTIVITIES OF ALCOHOLIC EXRACTS FROM COW, GOAT AND CHICKEN AGAINST *THE* ISOLATES

CONCENTRATION (mg/ml)	INHIBITION ZONE DIAMETER(MM)			ISOLATES
	COW	GOAT	CHICKEN	
100	35 33	30 29	23 21	*Salmonella* spp *Escherichia coli*
50	30 28	27 28	19 18.5	*Salmonella* spp *Escherichia coli*
25	25 21	23 21	18 16	*Salmonella* spp *Escherichia coli*
12.5	20 18	19 20	15 13	*Salmonella* spp *Escherichia coli*
6.25	15 13	11 10	10 9	*Salmonella* spp *Escherichia coli*
3.125	11 9	9 8	7 6	*Salmonella* spp *Escherichia coli*

TABLE 3: ANTIBACTERIAL ACTIVITIES OF CRUDE EXTRACTS FROM COW, GOAT AND CHICKEN AGAINST THE ISOLATES

CONCENTRATION (mg/ml)	INHIBITION ZONE DIAMETER(MM)			ISOLATES
	COW	GOAT	CHICKEN	
1000	48 49	33 35	30 29	*Salmonella* spp *Escherichia coli*
500	41 43	29 32	28 26	*Salmonella* spp *Escherichia coli*
250	41 39	27 27	21 23	*Salmonella* spp *Escherichia coli*

200	38 36	23 25	21 20	*Salmonella* spp *Escherichia coli*

TABLE (4a): MNIMUM INHIBITORY CONCENTRATION (MIC) AND MINIMUM BACTERICIDAL CONCENTRATION (MBC) OF THE COW BILE EXTRACTS AGAINST *SALMONELLA* SPP.

EXTRACT	CONCENTRATION (mg/ml)	TURBIDITY IN TUBES	GROWTH ON PLATE	MIC (mg/ml)	MBC (mg/ml)
AQUEOUS	100 50 25 12.5 6.25 3.125	- - - - + +	- - - +	12.5	25
ALCOHOLIC	100 50 25 12.5 6.25 3.125	- - - - - -	- - - - + +	3.125	12.5
CRUDE	1000 500 250 200	- - - -	- - - -	200	200

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TABLE (4b) :MINIMUM INHIBITORY CONCENTRATION(MIC) AND MINIMUM BACTERICIDAL CONCENTRATION(MBC) OF THE GOAT BILE EXTRACTS AGAINST *SALMONELLA* SPP

EXTRACT	CONCENTRATION (mg/ml)	TURBIDITY IN TUBES	GROWTH ON PLATES	MIC (mg/ml)	MBC (mg/ml)
AQUEOUS	100 50 25 12.5 6.25 3.125	- - - + + +	- - +	25	50
ALCOHOLIC	100 50 25 12.5 6.25 3.125	- - - - - -	- - - + + +	3.125	25
CRUDE	1000 500 250 200	- - - -	- - - -	200	200

TABLE (4c): MINIMUM INHIBITORY CONCENTRATION(MIC) AND MINIMUM BACTERICIDALCONCENTRATION(MBC) OF THE CHICKEN BILE EXTRACTS AGAINT *SALMONELLA* SPP

EXTRACT	CONCENTRATION (mg/ml)	TURBIDITY IN TUBES	GROWTH ON PLATES	MIC (mg/ml)	MBC (mg/ml)
AQUEOUS	100 50 25 12.5 6.25 3.125	- - - + + +	- - +	25	50
ALCOHOLIC	100 50 25 12.5 6.25 3.125	- - - - + +	- - - +	12.5	25
CRUDE	1000 500 250 200	- - - -	- - - -	200	200

TABLE (5a): MINIMUM INHIBITORY CONCENTRATION (MIC) AND MINIMUM BACTERIAL CONCENTRATION (MBC) OF THE COW BILE EXTRACTS AGAINST *ESCHERICHIA COLI*

EXTRACT	CONCENTRATION (mg/ml)	TURBIDITY IN TUBES	GROWTH ON PLATES	MIC (mg/ml)	MBC (mg/ml)
AQUEOUS	100 50 25 12.5 6.25 3.125	- - - + + +	- - +	25	50
ALCHOLIC	100 50 25 12.5 6.25 3.125	- - - - - -	- - - - - +	3.125	6.25
CRUDE	1000 500 250 200	- - - -	- - - -	200	200

TABLE (5b): MINIMUM INHIBITORY CONCENTRATION (MIC) AND MINIMUM BACTERICIDAL CONCENTRATION (MBC) OF THE GOAT BILE AGAINST *ESCHERICHIA COLI*

EXTRACT	CONCENTRATION (mg/ml)	TURBIDITY IN TUBES	GROWTH ON PLATES	MIC (mg/ml)	MBC (mg/ml)
AQUEOUS	100 50 25 12.5 6.25 3.125	- - - + + +	- - +	25	50
ALCHOLIC	100 50 25 12.5 6.25 3.125	- - - - - -	- - - - + +	3.125	12.5
CRUDE	1000 500 250 200	- - - -	- - - -	200	200

TABLE (5c): MINMUM INHIBITORY CONCENTRATION (MIC) AND MINIMUM BACTERICIDAL CONCENTRATION (MBC) OF THE CHICKEN BILE AGAINST *ESCHERICHIA COLI*

EXTRACT	CONCENTRATION (mg/ml)	TURBIDITY IN TUBES	GROWTH ON PLATES	MIC (mg/ml)	MBC (mg/ml)
AQUEOUS	100 50 25 12.5 6.25 3.125	- - - + + +	- - +	25	50
ALCOHOLIC	100 50 25 12.5 6.25 3.125	- - - - + +	- - - +	12.5	25
CRUDE	1000 500 250 200	- - - -	- - - -	200	200

KEY: + = Turbidity

- = No turbidity