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Correlation between Antibiotics and Herbal Extracts against Multidrug Resistant *Gallibacterium anatis* Isolated from Layer Chickens

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Abstract

Herbal extracts are potential safe sources of bioactive compounds, antioxidants, antibacterial agents which might be applied in different foods and pharmaceutical products. This study aimed to detect the minimal inhibitory concentrations for antibacterial agents and herbal extracts, Using DAA method (decimal assay for additivity) to determine interaction effect between combination of herbal extracts and antimicrobial agent methods and pathogenicity test of isolated *G. anatis*. The total incidence of *G. anatis* was 30% in the examined infected layer chickens. By using antibiotic sensitivity test, the retrieved strains were resistant to doxycycline (98.3%), amoxicillin (95.8%) and gentamycin (95%). But susceptible to erythromycin (95.8%), florfenicol (90%), sulfamethoxazole- trimethoprim (57.5%) and difloxacin (44.17%). The minimum inhibitory concentration of the methanolic herbal extract of rosemary leaves was 64 µg/ml and for *Eucalyptus* globules was 256 µg/ml, while for Cinnamon was 1024 µg/ml. The current study revealed that rosemary gave the best results in combination with amoxicillin at ratios 9:1 and 7:3. Moreover, combination with doxycycline gave acceptable results at ratios 9:1, 8:2 and 7:3 but with florfenicol gave good results at ratios 9:1 and 8:2. Moreover, with erythromycin gave good results at ratios of 9:1 and 8:2. While *Eucalyptus* globules mixed with four antibiotics at ratios of 9:1and 8:2 gave the best results with amoxicillin, but for doxycycline was at three ratios (9:1, 8:2 and 7:3). After intra nasal (IN) experimental infection of chicks with *Gallibacterium anatis* strains, majority of chickens exhibited dullness, depression, decreased feed and water intake, and respiratory signs. Treatment was effective by DAA method by combination between doxycycline and rosemary at ratio 9:1. In addition, combination of amoxicillin and rosemary, between erythromycin with rosemary, and florfenicol with rosemary at 9:1 were very effective.

KEYWORDS
Gallibacterium anatis, Poultry, MIC, Antimicrobial sensitivity test, DAA.

INTRODUCTION

The worldwide emergence of multiple drug resistance (MDR) and the increased awareness of the role of antimicrobial use in veterinary field (O'Neill, 2015). Antibiotics are extensively used in veterinary field, both to prevent and treat diseases; in many countries they are also added to commercial animal feeds as antimicrobial growth promoters (Pagel and Gautier, 2012).

Due to this problem of resistance against antibiotics, attention is now being shifted towards using biologically active components isolated from plant species (herbs) for treatment, as they have antifungal and antibacterial activities (Souza *et al*., 2005; Maiyo *et al*., 2010).

The Minimum Inhibitory Concentration (MIC) is the lowest concentration of an antimicrobial that showed no visible growth of a microorganism by overnight incubation, usually reported as mg/L (Delaquis *et al*., 2002). It represents a monitor resistance to antimicrobial agents and done by broth dilution methods (Handa *et al*., 2008). Although *Gallibacterium anatis* infection that was treated with antimicrobials, there were non-responsive cases and recurrence (Bojesen *et al*., 2011; Singh *et al*., 2016; El-Adawy *et al*., 2018; Hess *et al*., 2020).

Medicinal and pharmacological research has applied in popularity over the last few decades. Because of their limited side effects and fewer complications, researchers focused more on herbs in herbal medicine (Boyd *et al*., 2019).

The therapeutic power of rosemary has been determined in various assay types based on either MIC or MBC. In this regard, Sienkiewicz *et al*. (2013) demonstrated the antibacterial activities of rosemary (*Rosmarinus officinalis*, L). It was reported that it suppresses microbial growth by essential oils which presented as MIC and antibiotic susceptibility which carried out using disc diffusion.

The inhibitory effect of rosemary is due to the action of rosmarinic acid, rosmaridiphenol, carnosol, epirosmanol, carnosis acid, rosmanol and isoromanol. They bind with the cell membrane, causing changes in genetic material and nutrients, altering the transport of electrons, causing leakage of cellular components and producing changes in fatty acids. In addition, it also produced an interaction with cell membrane that produced the loss of membrane functionality and its structure (Fung *et al*., 1977).

Antimicrobial effect of *Eucalyptus* is that the herbal medicine, it is increasingly used to treat colds, chest pain, and coughs. *Eucalyptus* leaf extracts used to cure influenza, chest infections, and skin rashes and has been used to fight inflammation (Musyimi and Ogur, 2008).

The therapeutic power of *Eucalyptus* globules leaves was evaluated and the methanolic extract was obtained to be more effective in comparable to the aquous extract. The watery extract of the leaves, explaining its weak antibacterial effect when comparing with the methanolic extract (Hashemi and Davoodi, 2012).

The major component of Cinnamon is cinnamaldehyde which have antimicrobial effects on microorganisms, as it inhibits cell

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wall formation, cell membrane function, and specific enzyme activities (Shreaz *et al*., 2016). The current work evaluated the interaction of some herbal extracts with some antibiotics against *G. anatis* from diseased layer chickens and determine the minimal inhibitory concentration (MIC) for each antimicrobial agents and herbal extracts by decimal assay for additivity (DAA) method to detect effect of interaction between antibiotics and plant extracts.

MATERIALS AND METHODS

Animal Ethics

All procedures were completed in compliance with the applicable laws. The Suez Canal University in Egypt's Animal Ethics Review Committee approved of all methods and handling of birds , under an approval number of 2019031.

Plants

The plant materials used in the current work, were rosemary leaves, *Eucalyptus* globules leaves and Cinnamon which were grown in Egypt. These plants were collected from the Faculty of Agriculture, Zagazig university in Sharkia governate, Egypt.

Bacterial suspension

Gallibacterium anatis bacterial inoculums was prepared by creating bacterial suspensions in saline solution (0.85% NaCl) adjusted to a turbidity equivalent to 0.5 McFarland (1.5×10^8 CFU/ml).

Preparation of the herbal extract

Plant samples (rosemary, *Eucalyptus* and Cinnamon) were cleaned out of other contaminated plants and the fresh plant was collected, air dried away from sunlight then they driedin an oven at 40°C and ground to a fine powder in a mill. Ten grams of the ground material were extracted using 100 mL methanol (80%) by ultrasonic at power 140 watt (40 KHz) at 40°C for 30 min. Combined filtrate was evaporated in a rotatory evaporator below 40°C. The residue was freeze-dried. The dried extract was stored at -20°C until further use (Soaad and Ramesa, 2012).

Tryptic soya broth medium (Oxoid)

It was used for cultivation of culture for determination of the minimal inhibitory concentration (MIC) of antimicrobial agents and herbal extracts.

Antimicrobial agents included amoxicillin, doxycycline, florfenicol and erythromycin were used for determination of the minimal inhibitory concentration.

Minimal inhibitory concentration (MIC)

The isolated strain matches the 0.5 McFarland standards (1.5×10^8 CFU µg/ml) and results of antimicrobial agents and herbal extracts showed no visible bacterial growth considered as MIC and interpreted with recommendations of the Clinical Laboratory standards (2018).

Determination of MIC of herbal extract by tube dilution method (Awoyinka et al., 2007)

Herbal extracts of rosemary, *Eucalyptus* and Cinnamon which identified by using serially diluted (2-fold) plant extracts that showed no visible bacterial growth were considered as MIC and interpreted with recommendations of the National Committee for Clinical Laboratory standards (Adam *et al*., 1998).

Evaluation of the combination between the antimicrobial agents and herbal extracts by Decimal Assay for Additivity (DAA)

The evaluation was carried out as minimal inhibitory concentration (MIC) value determined by detection the tube which present before the tube with turbidity as the MIC tube that has the lowest concentration of antibiotics with plant extract to inhibit growth of microorganism (Irith *et al*., 2008).

Stock solutions of both antimicrobial agents and herbal extract were prepared, each contained 1024 µg/ml. These stocks mixture contained 9 parts of antimicrobial agents and 1part of herbal extract, 8 parts of antimicrobial agents and 2 parts of herbal extract and 7 parts of antimicrobial and 3 parts of herbal extract. Double fold serial dilutions for each combination were prepared to detect MIC against the isolated strain. The obtained concentrations were ranged from 0.5 to 1024 µg/ml (Sanders *et al*., 1993).

Experimental infection with Gallibacterium anatis strains in layer chickens (El-Hamid et al., 2016)

One hundred day-one old chicks were separated into five groups, and they were provided with food and water. Groups 1–4 included 80 birds that were inoculated intranasally (IN) with *G. anatis* strains at a dose of 0.5 ml/bird (10^8 CFU/ml).

Experimentally infected birds were observed for 10 days post infection (dpi). Group 5, which served as control positive included 20 birds. The chicks were examined for symptoms of infection such as tracheal rales, nasal discharge, watery eyes and wheezing. The clinical signs from all the chickens in all groups were observed regularly. Morbidity and mortality were recorded. Postmortem lesions were also carefully examined in the dead chickens.

RESULTS

Erythromycin and florfenicol showed intermediate results against *G. anatis*. The minimal inhibitory concentration of the methanolic herbal extract of rosemary leaves was (64 µg/ml), and it was 256 µg/ml for *Eucalyptus* globules, while for Cinnamon it was >512µg/ml.

Rosemary showed the best results in combination with amoxicillin against resistant strain of *Gallibacterium anatis* by DAA method was 8 µg/ml at ratio 9:1, 8 µg/ml at ratio 8:2 and at ratio 7:3 was 16 µg/ml. in addition, combination with doxycycline gave acceptable results at ratios 9:1, 8:2 and 7:3. But combination of florfenicol with rosemary using intermediate strain gave good results at ratios 9:1 and 8:2. Moreover in combination with erythromycin using intermediate strain gave good results at ratios 9:1 and 8:2 as revealed in Table 1.

As shown in Table 2, *Eucalyptus* globules in combination with four antibiotics against intermediate strain of *G. anatis*. Antibiotic/ plant extract ratio (9:1) was 16 µg/ml, 16 µg/ml at 8:2 gave the best results with amoxicillin. But for doxycycline was 8 µg/ml at three ratios (9:1, 8:2 and 7:3). In addition, combination with florfenicol gave acceptable result at ratio 8:2 was 8 µg/ ml as shown in Table 3.

As revealed in Table 4, combination of Cinnamon with antibiotics against intermediate strain of *Gallibacterium anatis* re-

vealed no enhancement effect on the isolated *G. anatis*.

Pathogenicity test of isolated G. anatis

After intra nasal experimental infection of layer chickens with *G. anatis* isolates. Whole chickens exhibited respiratory signs in the form of rales, coughing, sneezing, conjunctivitis, and teary eyes at the 2nd dpi with a decreased water and food intake at the 4th dpi. During the postmortem inspection, the trachea and lung showed severe tracheitis and pneumonia. But others showed mild tracheitis and congested lungs. Air sacculitis was only seen in chickens experimentally infected. All was positive from tracheal swabs which mean that the bacterial re-isolation was possible following IN infection, mainly from the trachea and lung.

DISCUSSION

Infectious diseases are considered a health concern accounting for most of the global disease burden measured in terms of Disability Adjusted Life Year (Noah and Fidas, 2000). One of the main causes of this problem is the widespread of acquired bacterial resistance to antibiotics in such a way that the world is facing today (Chopra, 2000) in the form of not only epidemics, but also pandemics of antibiotic resistance (Chanda and Rakholiya, 2011; Osman *et al*., 2012).

The usual use of antibiotics resulted in the appearance of resistant bacteria and antibiotic residues in meat and other food commodities, which pose a significant threat to public health and the environment (Jazi *et al*., 2018).

The plant extracts had different synergistic ability to suppress the growth of microorganisms depending on the method of extraction. Plants antimicrobial activity have been found to be enhancers in antimicrobial properties in combination with standard drugs to increase the effect of that drug (Rakholiya and Chanda, 2012). Drug combination between different antibiotics and bioactive plant extracts is a novel concept and could be beneficial (synergistic or additive interaction) or deleterious (antagonistic or toxic outcome) (Mhanna and Adwan, 2008). Plant scientists have become interested in phytochemical studies due to the emergence of new and sophisticated techniques. These techniques were crucial in the search for additional phytochemical raw materials resources (Shakeri *et al*., 2012). Determination of combination effect between antimicrobial agents and herbal extracts by comparing the minimal inhibitory concentration of antimicrobial agents alone and MIC of herbal extract alone and combination between them on *G. anatis* isolates.

Eucalyptus Eucalyptus It has been known that the most beneficial ways to decrease the antimicrobial resistance is by gaining antibiotic activity through the synergistic action of the antibacte-

Table 1. Combination effect of antimicrobial agents and rosemary.

Antimicrobial resistance	Amoxicillin + Rosemary			Doxycycline+ Rosemary			Florfenicol + Rosemary			Erythromycin+ Rosemary		
	Resistant 32 µg/ml			Resistant 32µg/ml			Intermediate 16 µg/ml			Intermediate 16 µg/ml		
Antibiotic/ plant extract	9:01	8:02	7:03	9:01	8:02	7:03	9:01	8:02	7:03	9:01	8:02	7:03
	A/E	A/E	A/E	A/E	A/E	A/E	A/E	A/E	A/E	A/E	A/E	A/E
MIC (µg/ml)	8	8	16	8	8	16	8	8	16	8	8	16

A/ E: Antibiotic/ plant extract

Table 2. Synergistic effect of antibiotics and *Eucalyptus* globules.

Antimicrobial resistance	Amoxicillin+ *Eucalyptus*			Doxycycline+ *Eucalyptus*			Florfenicol+ *Eucalyptus*			Erythromycin+ *Eucalyptus*		
	Resistant 32 µg/ml			Resistant 32 µg/ml			Intermediate 16 µg/ml			Intermediate 16 µg/ml		
Antibiotic/ plant extract	3/8	1/3	2/7	3/8	1/3	2/7	3/8	1/3	2/7	3/8	1/3	2/7
	A/E	A/E	A/E	A/E	A/E	A/E	A/E	A/E	A/E	A/E	A/E	A/E
MIC (µg/ml)	16	16	16	16	16	32	16	8	16	16	16	16

A/ E: Antibiotic/ plant extract

Table 3. Combination effect of antibiotics and Cinnamon.

Antimicrobial resistance	Amoxicillin +Cinnamon			Doxycycline+ Cinnamon			Florfenicol+ Cinnamon			Erythromycin+Cinnamon		
	Resistant 32 µg/ml			Resistant 32 µg/ml			Intermediate 16 µg/ml			Intermediate 16 µg/ml		
Antibiotic/ plant extract	9:01	8:02	7:03	9:01	8:02	7:03	9:01	8:02	7:03	9:01	8:02	7:03
	A/E	A/E	A/E	A/E	A/E	A/E	A/E	A/E	A/E	A/E	A/E	A/E
MIC (µg/ml)	>32	>32	>32	>32	>32	>16	>16	>16	>16	>16	>16	>16

A/ E: Antibiotic/ plant extract

Table 4. Therapeutic treatment of experimentally infected chicks.

	Control (+ve)		Doxycycline+ Rosemary		Amoxicillin+ Rosemary		Erythromycin + Rosemary		Florfenicol + Rosemary	
	20 birds		20 birds A/E (Rosemary) 9:01		20 birds A/E (Rosemary) 9:01		20 birds A/E (Rosemary) 9:01		20 birds A/E (Rosemary) 9:01	
	No.	%	No.	%	No.	%	No.	%	No.	%
Morbidity	20/20	100	2/20	10	0	0	0	0	0	0
Mortality	6/20	30	0/20	0	0	0	0	0	0	0

A/ E: Antibiotic/ plant extract

rial substances from synthesized and natural agents (Stefanovic and comic, 2012). After intranasal experimental infection of one day old chicks with *Gallibacterium anatis* isolates, most chickens showed severe respiratory signs as rales, coughing, sneezing, conjunctivitis, and teary eyes. During the postmortem inspection the tracheal and lung lesions was severe tracheitis and pneumonia, these data were matched with Bojesen *et al*. (2004) and Shaw *et al*. (1990) who mentioned that at least some strains of *Gallibacterium* possess a pathogenic effect.

In this study, there was little mortalities appeared in chicken group due to the importance of stress factors predisposing like impaired host immunity, co-infections and environmental factors as bad ventilation, overcrowdings and climatic changes leading to the penetration of *Gallibacterium* organism into the mucosa of the respiratory tract then into the systemic circulation causing mortalities as supported by Bojesen *et al*. (2003) and Neubauer *et al*. (2009).

Re-isolation of *Gallibacterium anatis* from the experimental chickens, all was positive from tracheal swabs till 10 dpi as shown before with Paudel *et al*. (2013) who mentioned that bacterial re-isolation was possible following IN infection, mainly from the trachea, and lung.

CONCLUSION

Rosemary at ratio of 9:1 can be used for therapeutic treatment of experimentally infected chicks in combination with different antibiotics (doxycycline, erythromycin, florfenicol and amoxicillin).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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