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Interaction of Some Plant Extracts with Some Antibiotics against *Salmonella* from Chickens

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A B S T R A C T

Multidrug-resistant bacterial strains are becoming a serious problem. Therefore, the application of natural antimicrobial agents from plant extracts combined with antibiotics to overcome this problem is of major importance. The antimicrobial activity of five plants (Rosemary, Marjoram, Mint, Dill and Neem) methanol extract prepared by ultrasonic-assisted (UAE) combined with antibiotics (amoxicillin, doxycycline, gentamicin and difloxacin) against 41 *Salmonella* poultry isolates was tested using *in vitro* methods. The interactions between plant extracts and antibiotics are known to be either additive or synergistic or antagonistic. The mean zones of inhibition (mm) and the minimum inhibitory concentration (MIC) of plant extracts and of antibiotics and combination between them was determined. The total phenolic content (TPC) and the antioxidant activity (DPPH·) of plant extracts was evaluated. Methanol extracts had high total phenolic compounds which used as a source of natural antioxidants. The results revealed that synergistic effects appear in rosemary with amoxicillin and gentamicin and difloxacin, dill with doxycycline and gentamicin, also neem with amoxicillin and doxycycline. Synergistic activity against Gram-negative bacteria demonstrated that extracts could be a source of bioactive substances with a broad spectrum of antibacterial activity especially when combined with antibiotics. In addition, extracts are potential safe sources of bioactive compounds, antioxidants, antibacterial agents which might be applied in different foods and pharmaceutical products.

Introduction

Antimicrobial resistance is one of the most common serious threats facing poultry industry as it can transfer to other pathogenic bacteria, causing a compromise in the treatment of severe infections (Enayat *et al.*, 2013; Stefanovic and Comic, 2012). This problem has encouraged scientists to search for new alternatives to antibiotics (CDC, 2013).

Gram-negative bacteria are more resistant to antibiotics than the Gram-positive bacteria due to the permeability barrier provided by the cell wall or to the membrane accumulation mechanism (Mounia *et al.,* 2010). To overcome this problem, some medicinal plants, as source for multidrug resistance inhibitors (Eze *et al.,* 2013), were utilized in combination with antibiotics *in vitro* as antimicrobial agents.

The MIC is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism by overnight incubation, usually reported as mg/L (Delaquis *et al.,* 2002). It represents a monitor for resistance to antimicrobial agents and is carried out by broth dilution methods (Handa *et al.,* 2008).

Nowadays, to overcome environmental pollution caused by plant residues, numerous studies focused on recovering, recycling of plant residues as it has potential biological effects (Cioffi *et al.,* 2009; Gavaric *et al.,* 2015).

About99% of plant residues after extraction are rich with secondary metabolites and bioactive compounds including natural antioxidants and phenolic compounds which play an important role in protection against infection, preventing oxidation and degenerative diseases (Singleton *et al.,* 1965; Valko *et al.,* 2006; Zhao and Gao, 2014).

This study was carried out to evaluate the interaction of some plant extracts with some antibiotics against *Salmonella* from chickens and to determine MIC for each antibiotic and plant extracts by using DAA method to detect the effect of interaction between antibiotics and plant extracts.

Materials and Methods

Bacterial strains

Standard strain (ATCC)

The tested *Salmonella* were provided from the culture collections of the Microbiological Department, National Research Center (NRC) Dokki, Giza, Egypt.

Field strain

41 isolates out of 120 diseased poultry samples which isolated from different poultry farms in Dakahlia governorates (Mahtet Elsalam, Mahtet El-Aml, Tawonya) and in Sharkia governorates (Gamsa, Sherbin, Elsalehia project).

Plants

Plant materials

Five plant including rosemary (*Rosmarinus officinalis*) leaves, marjoram (*Origanum majorana*) leaves, mint (*Mentha spicata*) leaves, dill (*Anethum graveolens*) seeds, neem (*Azadirachta meliaceae*) leaves were obtained from Faculty of Agriculture, Zagazig University (Egypt).

Preparation of the ultrasonic-assist methanol (80%) extract

Extraction was performed by ultrasound to overcome (time-solvent) consuming and increase extraction efficiency according to Betancount (2008).

Isolation and identification of the suspected bacteria

Research Institute Zagazig lab, 41 poultry samples were subjected to biochemical identification as described by Harley and Prescott (2002).

Antimicrobial susceptibility testing

Disk Diffusion Method

This was performed according to guidelines set by the Clinical Laboratory Standards Institute CLSI (2010). The diameters of the zones of inhibition were measured in millimeter and classified as resistant, intermediate or sensitive. The assay was repeated using plant extract alone, antibiotics alone or combination between them by disk diffusion method to detect the effect of ten standard antibiotic discs and five selected extracts (Oxoid®) against *Salmonella* according to Bauer *et al.,* (1966).

Minimal inhibitory concentration (MIC)

The isolated strains matched the 0.5 McFarland standard (1.5 × 10^5CFU mL^{-1}) and results of antibiotics and/or extracts showed no visible bacterial growth were considered as MIC and interpreted with recommendations of the National Committee for Clinical Laboratory standards Lorian (1996), Adam *et al.,* (1998) and Dorman and Deans (2000).

Evaluation of the combined activity of antibiotics and extracts using Decimal Assay for Additivity (DAA)

The evaluation was performed as described by Sanders *et al.,* (1993) to detect end point for additivity so that interactions greater or less than additivity defined as synergism and antagonism respectively.

Determination of total phenolic compounds (TPC)

TPC was measured using UV spectrophotometer according to Škerget *et al.,* (2005) using Folin-Ciocalteu reagent. The results were expressed as mg gallic acid equivalents (GAE) per gram of dry weight (mg GAE g-1 DW) using a calibration curve and the yield of extracts (g/100g).

Antioxidant DPPH˙ radical-scavenging activity

The ability of extracts for electron donation was measured by bleaching of the purple colored solution of DPPH· (2,2-diphenyl-1-picrylhydrazyl) to the yellow color as described by Gulcin *et al.,* (2004). The color intensity varies according to the amount of oxidant in the sample. The absorbance of this color was measured spectrophotometrically at 530 nm (Dikilitas *et al.,* 2011).

Results and Discussion

The study focused on the incidence of *Salmonella* in a total of 120 samples that were aseptically collected from visceral organs, as samples revealed 41 *Salmonella* out of 120 specimens with percentages of (34.2%) respectively in Table 1.

For further identification of Gram-negative isolates, biochemical tests such as IMViC were used under standard conditions which discussed in Table 2.

Salmonella showed negative results with Indole and V.P and positive result with Citrate and M.R.

Antimicrobial susceptibility testing showed the highest sensitivity rate of *Salmonella* strains that recorded to fluorophenol, cefotaxime and colistin (29, 18, and 14%, respectively) of sensitive strains and the highest intermediate rate was recorded to colistin, difloxacin a gentamicin (26, 21, and 18%, respectively) of intermediate strains and the highest resistant rate was recorded to erythromycin, amoxycillin and doxycycline (33, 22, and 20%, respectively) as shown in Table 3.

The clear zones around four antibiotic discs indicated organism's inability to survive in the presence of the test antibiotic antibacterial activity of natural antimicrobial agents (Rosemary, Marjoram, Peppermint, Dill and Neem) with the lowest concentration had a 10, 15, 12, 15 and 14 mm, respectively.

On the other hand, antibiotics (amoxicillin, doxycycline, gentamycin, difloxacin) exhibited different I.Z from 14 to 15 mm for amoxycilin, 0-16 mm for doxycycline,13 mm for gentamycin and 18-23 mm for difloxacin against field isolated *Salmonella* in Table 4.

In this study, every 4 antibiotics and 5 plant extracts were subjected to a broth macrodilution assay and after 24 h, observation of *Salmonella* bacterial growth to determine the MIC values.

The result of minimum inhibitory concentration on field strain is compared with their results on standard strain as rosemary (0.5 µg on field and 0.25 µg on standard), peppermint (32 µg on field and 8µg on standard), majoram (8µg on field and 4 µg on standard), dill (4 µg on field and 1 µg on standard), neem was (64 µg on field and 8 µg on standard) as shown in Table 5.

The result of minimum inhibition concentration of antibiotics on field strain is compared with their results on standard strain as AML was (0.5µg on field and 0.125µg on standard), INN was (0.25µg on field and 0.06µg on standard), DO was (1µg on field and 0.5µg on standard), GN was (2 µg on field and 0.25µg on standard) shown in Table 6.

Antimicrobial activities of methanol extracts in combination with antibiotics on selected *Salmonella* isolates as Interactions lead to antagonistic, additive and synergistic, as additive observed when the combined effect is equal to the sum of the individual effects, antagonism is observed when the effect of one or both compounds is less when they are applied together then synergism is observed when the effect of the combined substances is greater than the sum of the individual.

Synergistic effect between plant extracts and antibiotics was evaluated by comparing the size of inhibition zone of plant alone and antibiotics alone on *Salmonella.*

The results revealed that synergistic effects appeared in rosemary with amoxicillin at ratio (7:3) and gentamicin with ratio (7:3) and difloxacin at ratios (7:3) and (6:4), while dill and doxycycline at ratio (7:3), also majorana and gentamicin with ratio (5:5), finally neem with amoxicillin at ratio (5:5) and doxycycline at ratio (5:5) as shown in Table 7.

Table.1 Number of *Salmonella* isolates obtained from various specimens collected from chicken localities in Sharkia and Dakahlia governorates

Locality		**No. of cases**	***Salmonella***
Dakahlia Farms	Private farms (MahtetElsalam,MahtetElAml,Tawonya)	19	**6**
	Private farms (Gamsa, Sherbin)	18	**12**
Sharkia Farms	Farms in Sharkiagovernate Elsalehia project	29	**15**
	Private farms in Sharkiagovernate	13	**8**
Total		**120**	**41 (34.2%)**

Table.2 Biochemical characteristics of isolated bacteria by the IMViC results of some species

Specie	Indole	Methyl red	Voges-Proskauer	Citrate
Escherichia coli	Positive	Positive	Negative	Negative
Klebsiella spp.	Negative	Negative	Positive	Positive
Salmonella spp.	Negative	Positive	Negative	Positive
Shigella spp.	Negative	Positive	Negative	Negative
Proteus mirabilis	Negative	Positive	Negative	Positive
Citrobacterfreundii	Negative	Positive	Negative	Positive

Table.3 Antimicrobial susceptibility of *Salmonella* spp. (n=79) by agar disc diffusion method

Antimicrobial agent	Trade name	S	I	R
Amoxicillin	AML	6	13	22
Colistin	CT	14	26	1
Difloxacin	DIF	9	21	11
Doxycyclin	DO	5	16	20
Gentamycin	GN	13	18	10
Erythromycin	E	--	8	33
Flurophenicol	F	29	4	8
Cefotaxime	CTX	18	4	19
Streptomycin	S	10	9	22

Table.4 Diameter of I.Z (mm) of antibiotics and extracts as well as combination on *Salmonella*

	Inhibition zone (mm)								
Salmonella	Plant alone	Antibiotics alone				Combination			
		Amoxy	**Doxy**	**Genta**	**Diflo**	**Amoxy**	**Doxy**	**Genta**	**Diflo**
Rosemary	10	14	---	13	18	19	---	17	23
Majoram	15	15	13	13	18	15	---	13	18
Peppermint	12	14	---	12	20	10	---	13	20
Dill	15	14	16	13	19	18	18	13	18
Neem	14	14	15	13	18	15	18	13	18

Table.5 Antibacterial Activity of plants extract by Minimal Inhibitory Concentration (MIC) on *Salmonella* spp

	***Salmonella* isolate**		
Plant	*Salmonella*		MIC (µg/mL)
Rosemary	Field	S.10	0.5 ±0.41
	Standard strain		0.25 ±0.04
Pepermint	Field	S.10	32 ±3.27
	Standard strain		8 ±1.63
Majoram	Field	S.10	8 ±0.82
	Standard strain		4 ±0.41
Dill	Field	S.10	4 ±1.25
	Standard strain		1 ±0.82
Neem	Field	S.10	64 ±2.49
	Standard strain		8 ± 0.82

Table.6 Antibacterial Activity of antibiotics by Minimal Inhibitory Concentration (MIC) on *Salmonella* spp

	***Salmonella* isolates**		
Antibiotic	***Salmonella***		**MIC µg / ml**
AML	**Field**	S.10	0.5
	Standard strain		**0.125**
INN	**Field**	S.10	0.25
	Standard strain		**0.06**
DO	**Field**	S.10	1
	Standard strain		**0.5**
GN	**Field**	S.10	2
	Standard strain		**0.25**

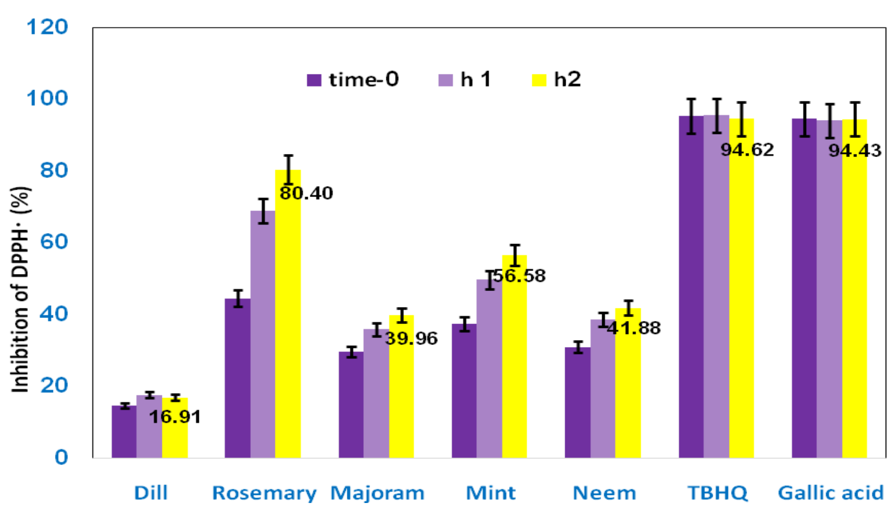
Table.7 Combination activity of antibiotics with extracts using DAA

Salmonella						
Plant extracts	Antibiotics	DAA			MIC	Effect
		AB	E	DAA	AB alone	
Rosemary	a) Amoxycillin	7	3	0.125	0.5	Synergy (S)
	b) Gentamicin	7	3	0.06	2	Synergy (S)
	c) Difloxacin	7	3	0.06	0.25	Synergy (S)
		6	4	0.06	0.25	
Oregano	a) Gentamicin	5	5	1	2	Synergy (S)
Dill	a) Doxycycline	7	3	0.125	1	Synergy (S)
Neem	a) Amoxycillin	5	5	0.06	0.5	Synergy (S)
	b) Doxycycline	5	5	0.5	1	

Table.8 Total phenolic compounds (mg gallic acid/g extract) in UAE and MAW extracts

Plant	**Extract yield**	**% Extract**	**TPC (mg GAE/g extract)**
Dill	2.04	10.2	**36.96 ±0.81**
Rosemary	2.68	13.4	**186.25 ±1.23**
Marjoram	3.24	16.2	**119.38 ±0.83**
Mint	2.53	12.65	**143.45 ±1.22**
Neem	4.96	24.8	**39.38 ±0.83**

Figure.1 DPPH· radical scavenging activity of plant extracts, TBHQ and gallic acid



The results proved that extracts contained a high amount of total phenolic compounds that showed high antioxidant activity as rosemary with DPPH activity 80.4% then followed by mint, neem, marjoram and dill extracts with respective data 56.58%, 41.88%, 39, 96% and 16.91%, respectively which shown in Table 8 and Figure 1.

Salmonellosis is considered to be the major bacterial disease in poultry industry worldwide. Kabir (2010) and Markov *et al.,* (2009) reported that out 95% of *Salmonella* are ingested through food and the most common sources of infection are meat and meat products.

Biochemical tests such as IMViC with recoded results agreed with Hendriksen (2011). Antimicrobial susceptibility testing on clinical veterinary *Salmonella* strains shows results not similar to Boyen *et al.,* (2010) and contrast with Dong *et al.,* (2014) but it similar to Gonzales *et al.,* (1998) and agree with Sallam *et al.,* (2014). On the other hand this pattern similar to other clinical veterinary *Salmonella* strains as reported by Threlfall *et al.,* (1996).

The plant extracts had varying degrees of growth inhibition against *Salmonella*. ZI of rosemary had an average of 10 mm and similar to Smith *et al.,* (1998) with who reported ZI of 9.3 mm but not agree with Busatta *et al.,* (2008), while majoram had ZI at an average of 15 mm which in parallel with Chan *et al.,* (2012). In addition, peppermint had ZI at an average of 12 mm which in fair correlation with Pattnail *et al.,* (1997) and Sabahat *et al.,* (2006) who reported ZI at an average of 11.78 mm. Dillhad ZI of 15 mm which agree with Mohammad (2017) who reported ZI of 15 mm, finally neem had ZI of 14mm which not agree with Maragathavalli *et al.,* (2012). The synergistic effect was evaluated by comparing the size of the inhibition zone in plates containing plant extracts and in control plates without plant extracts.

On the other hand, the clear zones around each antibiotic discs indicate the extent of the test organism's inability to survive in presence of the test antibiotic with different IZ on *Salmonella* (14-15mm) around amoxicilin and in parallel with Ramanauskiene *et al.,* (2004), also (13-16 mm) around doxycycline that agrees with Moodi Helal *et al.,* (2016); while 13 mm around gentamycin as not confirmed with Andrea *et al.,* (2009).

The synergistic interaction determined between plant extracts and antibiotics in this study revealed that dill has the highest effect on Gram-negative microorganisms and this contrast with Ljiljana *et al.,* (2016) who reported that dill had the highest effect on Gram-positive. Also, Bakkali *et al.,* (2008) who reported that dill extract is significant for animal pathogens as well as for food protection.

Phenolic substances have been shown to be responsible for the antioxidant activity of plant materials (Kim *et al.,* 2011).

The high antioxidant activity has been positively correlated with the concentration of phenolic compounds in extracts, wherein rosemary and mint had high phenolic compounds with respective values of 186.25 and 143.45 mg GAE extract. On the other hand, rosemary and mint extracts had the strongest scavenging activity of DPPH· free radical with 80.4% and 56.58% against synthetic antioxidant (TBHQ) with 94.62% and gallic acid. Our obtained results were very close to data reported by Bryngelsson *et al.,* (2002), and Sun *et al.,* (2007).

In conclusion synergistic activity by antibiotic and extracts against Gram-negative bacteria demonstrated that plants can be a source of bioactive substances with a broad spectrum of antibacterial activity especially when combined with antibiotic. In addition, the methanol extracts have high total antioxidant and phenolic compounds which could be used in pharmaceutical products as a source of natural antioxidants. More research is required to investigate the synergistic capacity of plants with antimicrobial activity.

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