Interaction of some plant extracts with some antibiotics against *E.coli* from chickens

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A B S T R A C T

Multidrug-resistant bacterial strains are becoming a serious problem, therefore, application of newer antimicrobial agents, as the plant extracts as a natural antimicrobial source combined with antibiotics as tried to overcome this problem. *In-vitro* methods as the Decimal Assay for Additivity (DAA) is being applied to define the end point for additivity, as the interactions are known on a mechanistic basis to be either additive or synergistic or antagonistic. The present study was carried out to evaluate the antimicrobial activity of five plant extracts prepared by the ultrasonic-assisted methanol extract (UAE) namely *Rosmarinus officinalis* L. (Rosemary), *Origanum majorana L.* (Marjoram), *Mentha spicata* L.(Mint), *Anethum graveolens* L.(Dill) and *Azardirachta indica* L.(Neem);combined with antibiotics like amoxicillin, doxycycline, gentamycin and difloxacin against 120 isolates from poultry, among them 79 isolates were belonging to *E.coli* by using agar diffusion method. The mean zones of inhibition (mm) of methanol plant extracts and antibiotics were determined at different concentrations; also the minimum inhibitory concentration (MIC) of the plant extracts or the antibiotics or combination between them was determined by the twofold dilution method; while the antimicrobial activities were assessed by using disc diffusion method.Total phenolic content (TPC) of plant extracts was determined by the Folin–Ciocalteu method.Also the antioxidant activity of the extracts was determined by the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay. Results revealed synergistic effects appear in rosemary with amoxicillin by ratio (7:3),(6:4) and gentamicin by ratio (7:3),(6:4) and difloxacin with ratio (7:3),(6:4),(5:5) while Dill and amoxicillin by ratio (7:3) and gentamicin by ratio (7:3),(6:4),(5:5) and Peppermint with doxycycline only with (7:3),(6:4),(5:5).

Keywords: *E.coli; antibiotics; plant extracts; TPC; antioxidant; DPPH; DAA.*

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1. INTRODUCTION

Antimicrobial resistance is one of the most common serious threats facing poultry industry (Enayat et al., 2013) as it can transfer to other pathogenic bacteria, causing compromise in treatment of severe infections (Stefanovic & 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 an In-vitro test for antimicrobial agents used in combination with antibiotics that is DAA which is based on disc diffusion assay, designed to define the interactions if greater or less than additivity, which respectively, defined as synergism or antagonism.

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 by aromatic plant extract residues, numerous studies focuses in recovering, recycling of residues as it has potential biologicals (Cioffi et al., 2009; Gavaric et al., 2015) as 99% of residues after extraction are rich with secondary metabolites and bioactive compounds e.g. natural antioxidants and phenolic compounds (Zhao and Gao, 2014) which play an important role in protection against infection, preventing oxidation and degenerative diseases (Singleton et al., 1965 and Valko et al., 2006). Finally, this study was carried out to evaluate the Interaction of some plant extracts with some antibiotics against *E.coli* from chickens and Determination of MIC, MBC for each antibiotics and plant extracts by using DAA method to detect effect of interaction between antibiotics and plant extracts and detection of antioxidant and total phenolic compound.

2. Materials and methods

2.1. Bacterial strains:

a) Standerd strain: the tested *E.coli* (ATCC 10536) were provided from the culture collections of the Microbiological Dept., National Research Center (NRC) Dokki, Giza, Egypt.

b) Field strain: 79 isolates out of 120 diseased poultry samples which isolated from different poultry farms in Dakahlia governorates (Mahtet Elsalam, Mahtet ElAml, Tawonya) and in Sharkia governorates (Gamsa, Sherbin, Elsalehia project).

2.2. Plants:

a) Plant materials: Five plant samples: rosemary leaves (*Rosmarinus oficinalis*), marjoram leaves (*Origanum majorana*), mint leaves (*Mentha spicata*), dill seeds (*Anethum graveolens*), neem leaves (Azadirachta Meliaceae) were obtained from Faculty of Agriculture., Zagazig University.

b) 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).

2.3. Isolation and Identification of the suspected bacteria: in Animal Health Research Institute Zagazig lab, 79 poultry samples were subjected to biochemical identification described by Harley and Prescott (2002).

2.4. Antimicrobial susceptibility testing:

2.4.1. Disk Diffusion Method: 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 (Bauer *et al*., 1966) and five selected extracts (Oxoid®) against *E.coli*.

2.4.2. Minimal inhibitory concentration (MIC): the isolated strain 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; Dorman & Deans, 2000).

2.5. Evaluation of combined activity of antibiotics and extracts using Decimal Assay for Additivity (DAA):

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.

2.6. Determination of total phenolic compounds (TPC):

Measured by UV spectrophotometer Škerget et al.,(2005) using Folin-Ciocalteu reagent AOAS, (1990) 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) for different plants.

2.7. Antioxidant DPPH˙ radical-scavenging activity:

The ability of the extracts for electron donation was measured by bleaching of the purple colored solution of DPPH (2, 2-diphenyl-1-picrylhydrazyl) to the yellow colored Gulcin *et al.,* (2004). 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).

3. RESULTS

This study focused on the incidence of *E.coli* in a total of 120 samples that were aseptically collected from visceral organs. Samples revealed 79 *E.coli* out of 120 specimens with percentages of (65.8%).

Identification of Gram negative isolates, biochemical tests as IMVIC were used under standard conditions which discussed in Table (4), as *E.coli* show negative results with Indole and Citrate and positive result with Indole and M.R.

Antimicrobial susceptibility testing showed highest sensitivity rate of *E.coli* strains to colistin, doxycycline and flurofinecol (38 , 31 and 27 %) respectively, intermediate sensitivty recorded to difloxacin, gentamycin and amoxicillin (54, 42 and 32%) respectively; while the highest resistant rates were recorded to erythromycin, cefotaxime and streptomycin with (58, 53 and 42 %) respectively in (Table 5).

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, Majoram , Peppermint , Dill and Neem) with the lowest concentration had a ZI 8 ,15.2 ,15, 0 and 10 mm, respectively.on the other hand antibiotics as (amoxicillin, doxycycline, gentamycin, difloxacin) exhibited different I.Z like 10-15 mm amoxycilin, 0-16 mm doxycycline,13 mm around gentamycin and 18-19 mm difloxacin respectively against field isolate of *E.coli* in (Table 3).

The result of MIC of plant extract on field strain is compared with their results on standard strain as Rosemary was (1µg on field and 0.25µg on standard), Peppermint was (64µg on field and 16µg on standard), Majoram was (4µg on field and 1µg on standard), Dill was (8µg on field and 2µg on standard), Neem was (32µg on field and 8µg on standard).

The result of MIC of antibiotics on field strain is compared with their results on standard strain as AML was (8µg on field and 4µg on standard), INN was (1µg on field and 4µg on standard), DO was (0.5µg on field and 0.125µg on standard), GN was (16 µg on field and 4µg on standard).

Determination of synergistic effect between plant extracts and antibiotics was evaluated by comparing the size of inhibition zone of plant alone and antibiotics alone on *E.coli* with size of inhibition zone of plant extracts and antibiotics together as results revealed that synergistic effects appear in rosemary and amoxicillin with ratio (7:3),(6:4), rosemary and gentamicin with ratio (7:3), (6:4),rosemary and difloxacin with ratio (7:3),(6:4),(5:5) while Dill and amoxicillin with ratio (7:3), Dill and gentamicin with ratio (7:3),(6:4),(5:5) and Peppermint with doxycycline only with ratio (7:3),(6:4),(5:5).

In this study each 4 antibiotics was subjected to a broth microdilution assay and after twenty four h observation of bacterial growth to determine the MIC values on *E.coli* which shown in (Table 6).

each 5 plant extracts were subjected to a broth microdilution assay and after twenty four h observation of *E.coli* growth to determine the MIC values ,As the MIC of Rosemary is 1 µg/ ml, Majoram is 4 µg/ml, Peppermint is 64 µg/ ml, Dill is 8 µg/ml and Neem is 32 µg/ml.

Antimicrobial activities of methanol plant extracts in combination with antimicrobial agents on selected *E.coli* 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 ,as in (Table 8) as amoxycillin shows synergistic action with Rosemary by level (7:3), (6:4) and with dill by level (7:3). While doxycyclin shows synergistic action in combination of with Peppermint by level (7:3), (6:4) and by (5:5). Also gentamicin shows synergistic action in combination of with Rosemary by level (7:3), (6:4) and with dill by (7:3), (6:4) and (5:5) and difloxacin shows synergistic action in combination of with Rosemary by level (7:3), (6:4) and (5:5).

Extraction with organic solvents is the best way to extract antioxidant according to the polarity as solvents seemed to be preferable to either nonpolar or polar solvents Pokorný *et al.,* (2000) So the extraction was methanol 80% with ultrasonic-assisted was better solvents for extracting phenolic compounds owing to their higher polarity and good solubility Wieland *et al.,* (2006) and also the ultrasound waves cause tissue or cell wall disruption in a short period of time, increase in solvent transfer into the biological matrix, and release of cellular content, consequently leading to higher surface mass transfer Vilkhu *et al.* (2008), the result showed that Neem, marjoram and rosemary had high yield with respective weights 24.8, 16.2 and 13.4 g extract/100 g plant also rosemary and mint had high phenolic compounds with respective values of 186.25 and 143.45 mg GAE g^{-1} extract. Variation in the extraction yields is referred to the polarity of compounds found in plants Jayaprakasha et al., (2003) as during the extraction of U.M.E. The Folin-Ciocalteu method estimates the reduction of the reagent by phenolic compounds for formation a blue complex that can be measured at 763 nm against gallic acid equivalent (GAE) as a standard phenolic compound Imeh and Khokhar, (2002) (Table 9).

Inhibition of DPPH radicals: Antioxidants react with $DPPH^{\bullet}$ means reducing the number of $DPPH^{\bullet}$ free radicals to the number of their available hydroxyl groups. Therefore, the absorption at 515 nm is relative to the amount of residual DPPH Juan et al., (2005). It is visually noticeable as a discoloration from purple to yellow. Figure (1) represented that extracts contained high amount of total phenolic compounds showed high antioxidant activity as strong as that of TBHQ and gallic acid. As Heim et al., (2002) proved that antioxidant activity of plant extracts is mainly due to the concentration of phenolic compounds in the plant. The results recorded rosemary extract has the best scavenging activity of DPPH-free radical with activity 80.4% against synthetic antioxidant (TBHQ) with 94.62% and gallic acid 94.43%.Then followed by mint, neem, marjoram and dill extracts with respective data 56.58%, 41.88%, 39, 96% and 16.91% (Figure 1).

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Table 1: No. of E.coli isolates obtained from various specimens collected from different chicken localities in Sharkia and Dakahlia governorates.

Locality		No. of cases	*E.coli*
Dakahlia farms	Private farms (Mahtet Elsalam,Mahtet ElAml,Tawonya)	19	25
	Private farms (Gamsa,Sherbin)	18	30
Sharkia farms	Farms in Sharkia governate	29	44
	Elsalehia project		
	Private farms in Sharkia governate	13	21
Total		120	79 (65.8%)

Table 2: List of the concentration of the methanol extract obtained from leaves from different plant parts g/ml spices in Stock solution.

Scientific name	Family	Local name	Wt.of extract in tube (g)	Wt.of empty tube (g)	Total Wt.of extract (g)
Rosmarinus officinalis	Lamiaceae	Rosemary	149.0201	146.3335	2.6
Origanum Majorana	Lamiaceae	Majoram	135.1119	131.8644	3.2
Mentha piperita	Lamiaceae	Peppermint	180.0534	177.5157	2.5
Anethum graveolens	Umbelliferae	Dill	142.3050	140.2561	2.1
Azadirachta Meliaceae	Meliaceae	Neem	138.0542	134.5216	3.5

Table 3: Diameter of zone of inhibition (mm) of antibiotics and Plant extracts only and combination on E.coli isolates.

Isolates	Inhibition zone (mm)								
	Plant	Antibiotics alone				Combination			
E.coli	alone	Amoxy	Doxy	Genta	Diflo	Amoxy	Doxy	Genta	Diflo
Rosemary	$8_{\pm0.82}$	$15_{\pm0.82}$	---	$13_{\pm0.82}$	$19_{\pm0.95}$	$19_{\pm0.41}$	---	$18_{\pm1.25}$	$22_{\pm1.25}$
Majoram	$15.2_{\pm1.02}$	$15_{\pm0.41}$	---	$13_{\pm 1.63}$	$18_{\pm1.10}$	$15_{\pm 0.41}$	---	$13_{\pm2.05}$	$18_{\pm2.05}$
Peppermint	$15_{\pm0.82}$	$10_{\pm0.62}$	---	$13_{\pm 0.47}$	$19_{\pm1.14}$	$10_{\pm0.74}$	---	$13_{\pm0.54}$	$18_{\pm1.03}$
Dill	---	$15_{\pm0.74}$	$16_{\pm0.82}$	$13_{\pm 0.41}$	$19_{\pm1.47}$	$18_{\pm0.82}$	$16_{\pm0.47}$	$15_{\pm1.31}$	$19_{\pm0.80}$
Neem	$10_{\pm1.25}$	$15_{\pm0.85}$	---	$13_{\pm 1.10}$	$18_{\pm0.77}$	$15_{\pm0.62}$	---	$13_{\pm0.59}$	$18_{\pm0.68}$

Table 4: Summary of the Biochemical characteristics of isolated bacteria by the IMViC results of some important species.

Species	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
Citrobacter freundii	Negative	Positive	Negative	Positive

Table 5: Antimicrobial susceptibility of E.coli spp. (n = 79) by agar disc diffusion method.

Antimicrobial agents	Trade name	S	I	R
Amoxicillin	AML	25	29	25
Colistin	CT	38	30	11
Difloxacin	DIF	8	54	17
Doxycyclin	DO	31	24	24
Gentamycin	GN	27	34	13
Erythromycin	E	--	21	58
Flurophenicol	F	24	42	13
Cefotaxime	CTX	16	10	53
Streptomycin	S	26	10	42

Table 6: Determination of Antibacterial Activity of plants extract by Minimal Inhibitory Concentration (MIC) on E.coli spp.

Plants	*E.coli* (E.coli isolates)		MIC µg / Ml
Rosemary	Field	E.29	1 ±0.41
	Standard strain		0.25 ±0.04
Pepermint	Field	E.29	64 ±3.27
	Standard strain		16 ±1.63
Majoram	Field	E.29	4 ±0.82
	Standard strain		1 ±0.41
Dill	Field	E.29	8 ±1.25
	Standard strain		2 ±0.82
Neem	Field	E.29	32 ±2.49
	Standard strain		8 ±0.82

Table 7: Determination of Antibacterial Activity of antibotics by Minimal Inhibitory Concentration (MIC) on E.coli spp.

Antibiotic	*E.coli* (*E.coli* isolates)		MIC µg / ml
AML	Field	E .3	8 ± 0.81
		E. 29	4 ±0.81
	Standard strain		1 ±0.41
INN	Field	E.21	4 ±0.81
		E. 29	0.5 ±0.20
	Standard strain		0.125 ±0.02
DO	Field	E.33	16 ±1.63
		E. 29	4 ±0.81
	Standard strain		2 ±0.81
GN	Field	E.40	4 ±0.81
		E. 29	2 ±0.81
	Standard strain		0.05 ±0.03

Table 8: Combination activity of antibiotics with extracts using Decimal Assay for Additivity (DAA).

Plant extracts	antibiotics	E.coli				effect
		DAA			MIC	
		AB	E	DAA	AB alone	
Rosemary	a) Amoxycillin	7	3	0.5	4	Synergy (S)
		6	4	0.5	4	
	b) Gentamicin	7	3	0.5	2	Synergy (S)
		6	4	0.5	2	
	c) Difloxacin	7	3	0.06	0.5	Synergy (S)
		6	4	0.125	0.5	
		5	5	0.25	0.5	
Peppermint	a) Doxycycline	7	3	2	4	Synergy (S)
		6	4	2	4	
		5	5	2	4	
Dill	a) Amoxycillin	7	3	1	4	Synergy (S)
	b) Gentamicin	7	3	1	2	Synergy (S)
		6	4	1	2	
		4	6	1	2	

Table 9: Total phenolic compounds (mg gallic acid /g extract) in U.A.E and M.A.W extracts.

plants	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

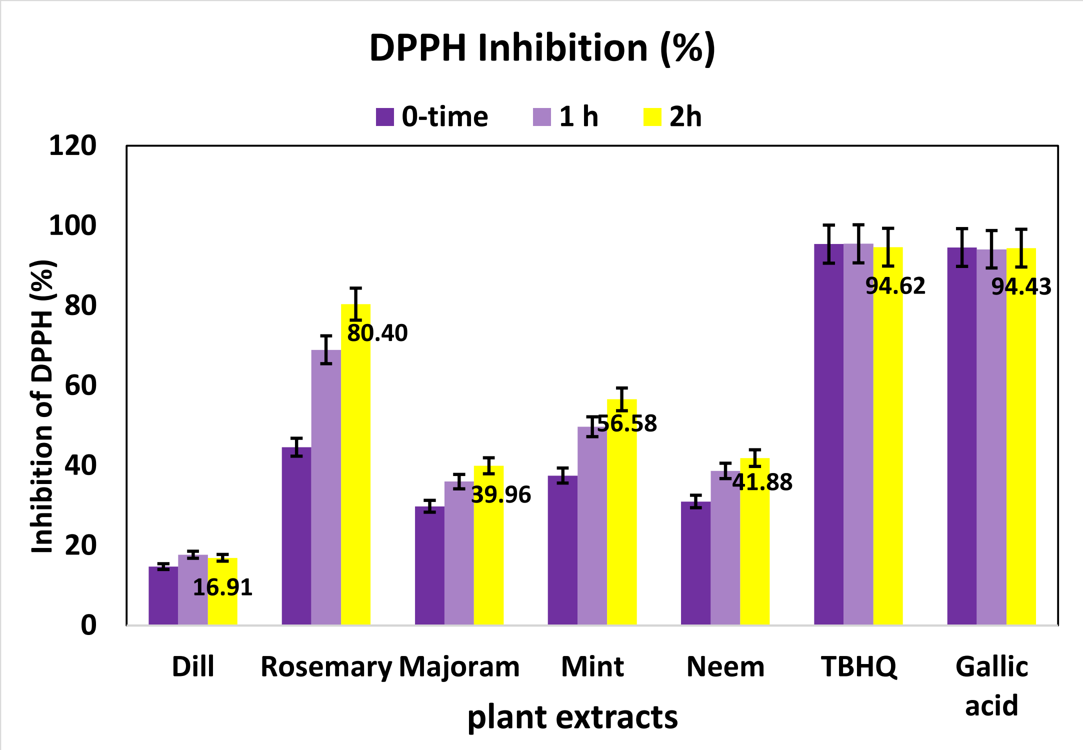

Fig.1. Antioxidant DPPH˙ radical-scavenging activity.

4. DISCUSSION

E.coli infection is the major bacterial disease in poultry industry worldwide and our data are parallel to those of (Kabir, 2010) and Markov et al., (2009) who talked about 95% of *E.coli* are ingested through food.

Biochemical tests as IMViC with recoded results agreed with (Hendriksen, 2011).

Antimicrobial susceptibility testing on clinical veterinary *E.coli* strains showed results similar to other pattern of Threlfall et al., (1996) which recorded highest sensitivity rate to colistin, doxycycline and intermediate action to gentamicin and amoxicillin while the highest resistant to streptomycin.

The zone of inhibition was obtained for *E.coli* to show antibacterial activity of different plant extracts and antibiotics on it and the results revealed that the diameter of inhibition zone of different plant extract varied from 8 to 15 mm, this results are disagree with Marzouk *et al.*, (2006) with Z.I of Rosemary ranged of (14-26) mm in diameter, matched with (Shaza and Frdoos 2014) with Z.I of Marjoram (16) mm in diameter, also agree with reports of Hawrelal *et al.*, (2009) with Z.I of Pepermint (17) mm in diameter, not accordance with previous published data with Z.I of Dill (10) mm in diameter. On the other hand, Neem results totally disagree with the finding of Hoque *et al*., (2007) who reported that extract of neem did not show antimicrobial activity against any of the Gram-negative bacteria. The synergistic interaction was determined between rosemary with amoxicillin, difloxacin and gentamicin against Escherichia coli which agree with Nazzaro *et al.*, (2003) therefore rosemary increased the permeability of cell to antibiotics that lead to reduced the MIC when combined with gentamicin, In another study Ghusoon A, (2017) demonstrated the combination of gentamicin and ciprofloxacin with rosemary EO were increased the antimicrobial activity of these antibiotics (synergetic effect) against *E. coli*.

Our results suggested that Dill extract could be combined with antibiotics like amoxicillin and gentamicin as it has been successful in combating *E.coli* infection, similar results have been obtained by other researchers. Markov et al., (2009) as extracts from dill seeds shows higher antimicrobial activity on *E. coli* compared to amoxicillin antibiotics and this contrast with Ljiljana P. *et al*., (2016) which reported that dill effect was observed the highest effect on Gram-positive m.o. On the other hand in this study, doxycycline antibiotic and Mint combinations may be recommended for *E.coli* infections, Similarly, Tuysuz *et al*., (2017) examined the antimicrobial activities of 31 different herbal teas for examples (Mint) both alone and in combination with antibiotics while Lee *et al*., (2005), also showed that Mint and ciprofloxacin combinations were synergistic against *E. coli.*

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

Higher antioxidant activity has been positively correlated with the concentration of phenolic compounds in extracts, as Rosemary and Mint had high phenolic compounds with respective values of 186.25 and 143.45 mg GAEg/l extract.On the other hand rosemary and Mint extracts have the best scavenging activity of DPPH-free radical with activity 80.4% and 56.58% against synthetic antioxidant (TBHQ) with 94.62% and gallic acid, our obtained results were very close to data with (Bryngelsson *et al.*, 2002; Sun et al., 2007).

5. Conclusion

Synergistic activity by antibiotic and plant against Gram negative bacteria demonstrated that plant can be a source of bioactive substances with broad spectrum of antibacterial activity especially when combined with antibiotic, in addition, the methanol plant extract have higher total antioxidant and phenolic compounds which used in pharmaceutical products as a source of natural antioxidants plus that more research is required to investigate the synergistic capacity of plants with antimicrobial activity.

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