Synergistic antimicrobial combination with cumin extract and some antibiotics on *Staphylococcus aureus* from chickens

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A B S T R A C T

In the present study, antimicrobial activity of methanol Cuminum cyminum extract (50mg/ml) in combination with some antibiotics were investigated against *Staphylococcus aureus* isolates. The antimicrobial sensitivity pattern of these isolates in vitro was done and the intermediate isolates were checked for combination with methanol Cuminum cyminum extract after detecting their antimicrobial activities against these isolates by both disc diffusion test and MIC and the results showed that the antimicrobial activities of rifampicin, enrofloxacin, doxycycline, gentamicin, and erythromycin were enhanced in combination with methanol Cuminum cyminum extract the obtained data showed that enhancement by both disc diffusion test and MIC for methanol Cuminum cyminum extract and each of mentioned antibiotics in decimal assay of additivity ratio (0.5 for rifampicin and erythromycin and 0.5 for extract ,0.7 for enrofloxacin, and 0.3 for extract,0.6 for doxycycline, gentamicin and 0.4 for extract) against *S.aureus* isolates .

Keywords: *Antimicrobial combination, Staphylococcus aureus, cumin extract, antibiotics, synergism.*

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1. INTRODUCTION

The emerging and sustained resistance to antibiotics and the poor pipeline of new antibacterial is creating a major health issue worldwide. Bacterial pathogens are increasingly becoming resistant even to the most recently approved antibiotics. Few antibiotics are being approved by regulatory organizations, which reflect both the difficulty of developing such agents and the fact that antibiotic discovery programs have been terminated at several major pharmaceutical companies in the past decade (Zapun et al., 2008). As a result, the output of the drug pipelines is simply not well positioned to control the growing army of resistant pathogens, although academic institutions and smaller companies are trying to fill that gap. An emerging option to fight such pathogens is combination therapy. Combinations of two antibiotics are emerging as a promising therapeutic approach (Cottarel and Wierzbowski, 2007). New antibiotics were produced by pharmacological industries in the last three decades (White et al., 1993). Thus, it is extremely important to find new antimicrobial agents or new ways that are effective for the treatment of infectious diseases caused by drug-resistant bacteria (Taylor et al., 2002). Few studies have found that the efficacy of antimicrobial agents can be improved by combining them with plant extracts against different multidrug-resistant pathogens (Horiuchi et al., 2007; Ibezim et al., 2006). There is a crucial and urgent need to develop new classes of antibiotics or to revitalize existing antibiotics (Tan et al., 2000). Reasons that justify the use of antimicrobial combinations are broad-spectrum coverage for the initial therapy of severely infected patients, polymicrobial infections, and prevention of selection of resistant microorganisms when a high mutation rate of the causal organism exists to the antibiotic indicated, reduction of dose-related toxicity and antimicrobial synergistic activity (Acar, 2000).

Biological effects of these plants on prokaryotic and eukaryotic organisms have been discussed by few studies (Ababutain, 2011). Because of their low toxicity, there is a long tradition of using dietary plants in the treatment of infectious disease

in Cameroonian folk medicine (Djeussi et al., 2013). In recent years, staphylococcosis has become one of the most important bacterial diseases of poultry. Chickens have been highly selected for their ability to achieve rapid growth. Such selection has added more stress by creating various leg problems that predispose poultry to the development of staphylococcosis. Historically, staphylococcosis has been a significant problem because of the ubiquitous nature of the bacterium in the poultry farm environment. When the door is left open, *staphylococcus aureus* has been able to localise the disease (Norton et al., 1994). In this respect, Awan et al. (2013) studied Chloroformic and isoamyl alcohol extracts of *Cinnnamomum zylanicum*, *Cuminum cyminum*, *Curcuma long Linn*, Trachy spermum ammi and selected standard antibiotics were investigated for their in vitro antibacterial activity against six human bacterial pathogens. The antibacterial activity was evaluated and based on the zone of inhibition using agar disc diffusion method. The tested bacterial strains were *Streptococcus pyogenes, Staphylococcus epidermidis, Klebsiella pneumonia, Staphylococcus aurues, Serratia marcesnces, and Pseudomonas aeruginosa*. Ciprofloxacin showed highly significant action against K. pneumonia and S. epidermidis while Ampicillin and Amoxicillin indicated lowest antibacterial activity against tested pathogens. Among the plants chloroform and isoamyl alcohol extracts of C. cyminum, S. aromaticum and C. long Linn had significant effect against P. aeruginosa, S. marcesnces and S. pyogenes. Comparison of antibacterial activity of medicinal herbs and standard antibiotics was also recorded via activity index. Used medicinal plants have various phytochemicals which reasonably justify their use as antibacterial agent.

So, the aim of this study is synergistic antimicrobial combination with Cumin extract with some antibiotics on *Staph. aureus* isolated from chicken.

2. MATERIALS AND METHODS

2.1. Samples collection:

A total 150 samples were aseptically collected from visceral organs (liver, gall bladder, spleen, kidney, cecum, heart and lung) of clinically diseased and dead chickens of different ages reared in farms located in Sharkia and Dakahlia governorates in period between 2014 till 2015.

2.2. Bacteriological examination:

2.2.1. Isolation of Gram positive bacteria (Staph. aureus)

In laboratory, the collected samples were transferred to sterile tryptone soya broth and incubated at 37 ºC for 24 hrs. A loopfull from each incubated broth was streaked onto the surface of mannitol salt agar, nutrient agar, blood agar, and baired Parker agar. The inoculated plates were incubated at 37°C for 24-48 hours and examined for bacteriological growth which appeared as yellow halo zone surrounding their growth on mannitol salt agar, smooth colonies had a low convex profile with an entire edge and pigmented yellowish on nutrient agar, zones of beta hemolysis, which were clearly visible after incubation on blood agar and black, shiny, convex colonies and surrounded by a clear zone of about 2-5mm in diameter on baired parker agar . One single colony which was showed typical colonial appearance and morphological characters was picked up and streaked onto semisolid agar media and was incubated at 37°C for 24 hours for further identification (Koneman et al., 1992).

2.2.2. Microscopic examination of Staph. aureus:

Modified Gram's stain used as described by Cruickshank et al. (1975).

2.2.3. Biochemical identification of Staph.aureus

The methods of biochemical tests used for identification were carried out according to the schemes described by Koneman et al. (1992).

2.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility test for all isolates was done according to description of Smith et al. (1997).

2.4. Preparation of methanol plant extract:

The collected plant was cleaned from other contaminated plants &the fresh plant was collected and air dried away from sun light then the dried plants were crushed to powder using grinder. The powdered plant stored in tightly closed sterile containers until use (Sooad et al., 2012).

2.4.1. Preparation of plant extract.

Ten grams of powdered samples was dissolved in 100 ml of methanol in conical flask, plugged with cotton wool and then kept in rotatory shaker at 190-220 rpm for 24 hr. The extract was then filtered using conical flask with side arm, a filter funnel size (size 2) and a 90-mm diameter filter paper. Filtered extract was then poured in weighed 500 ml round bottom flask Solvent was evaporated with rotatory evaporator. Temperature of the water bath in the rotatory evaporator was set at 40 c. This temperature was used because the evaporation

under reduced pressure makes it possible to evaporate at much lower temperature. Finally, the extracts were preserved in sterilized dishes at refrigerator and to prevent from the light effect, they were wrapped with aluminum covers (Sooad et al., 2012).

2.4.2. Preparation and dilution of stock solution

200 mg of the dry plant residue were provided and solved in 5% dimethyl sulfoxide (DMSO) in 1ml sterile distilled water (stock solution). Extraction with concentration of 100, 50, 25, 12.5, 6.25, 3.15, 1.56 and 0.78 mg/ml distilled water were prepared (Sooad et al., 2012).

2.4.3. Preparation of discs susceptibility testing containing Plant extract.

This was carried out using the modified method of Bauer et al. (1966). Plain discs were sterilized by autoclaving at 121°C for 15 min. Cool, flamed forceps were used to firmly apply discs, within 15 min of plate inoculation, to avoid decreased zone diameters. Different concentration (100, 50, 25, 12.5 and 6.25 mg/ml) of plant extract were kept on Plain discs using micro titer pipette with sterile tips.

2.4.4. Antimicrobial susceptibility testing of plant extraction.

2.4.5. Detection of MIC of each drug and plant extraction.

Minimum inhibitory concentration for each selected antibiotic and plant extract was done according to description of Smith et al. (1997).

2.5. Decimal Assay for additivity of drugs permits delineation of synergy and antagonism (Chirstine et al., 1993).

Decimal assay for additivity Disk diffusion assays were performed initially with each antibiotic alone over a range of drug masses in order to derive a standard dose-response curve by linear regression analysis. For each antibiotic, there is a linear relationship between the log1o of the drug mass (in micrograms) on the disk and the diameter of the zone of inhibition (in millimeters) produced. Once a standard curve for each drug alone had been derived, a target zone for each combination to be tested was selected. This target was selected to represent a zone in the midrange of the standard curve for each drug so that increases or decreases in the zone size resulting from drug interactions could be reliably detected. Once a target zone size was selected for a particular combination, the mass of each drug alone (in micrograms) required in order to produce this zone was calculated from the formula for the standard dose response curve. This mass was defined as the biologic equivalence factor (BEF), since it represented the mass of each of the two drugs that would produce the same zone size. Once the BEF had been calculated, a series of 11 decimal mixtures of the two drugs to be examined in combination was prepared. This series represented all possible mixtures of the two drugs that, when combined in 10 parts, added up to one BEF. Thus, if no positive or negative interaction between the drugs occurred, each mixture should have generated the target zone size. Since there are inherent errors in any standard curve and in preparation of the decimal mixtures, controls for these potential errors consisted of each drug added to itself in a similar series of decimal mixtures. The results generated by these 11 single-drug decimal mixtures represented the true additive response. Therefore, results obtained with combination decimal mixtures had to lie outside the range of those obtained with the single-drug decimal mixtures in order to be considered indicative of a positive or negative interaction. Data were also analyzed statistically (Stat View II, Abacus Concepts, Berkeley, Calif.). The mean zone size (k drug) was calculated from the data obtained with the 11 single-drug decimal mixtures, and 95% confidence intervals (t distribution) were determined. For the combination, decimal mixtures, the mean zone size (x comb) was calculated by using data obtained with mixtures 1 through 9 only, since mixtures 0 and 10 represented each drug alone. Ninety-five percent confidence intervals (t distribution) were calculated for this mean as well. Results obtained with the combination were considered indicative of synergism if X comb was larger than -drug A and X drug B and the 95% confidence intervals for X comb did not overlap those for X drug A or X drug B. Results were considered indicative of antagonism if x comb was smaller than-drug A and X drug B and the 95% confidence intervals did not overlap. All other results were considered additive.

3. RESULTS

A total of 150 samples were aseptically collected from visceral organs (liver, gall bladder, spleen, kidney, cecum, heart and lung) of clinically diseased and dead chickens

Table (1): Antimicrobial activities of methanol cumin (*Cuminum cyminum*) extract in combination with antimicrobial drugs on selected *S.aureus* isolates.

M.O *S.aureus*	Zone of inhibition (mm)			MIC µg / ml			D.D.A		REACTION
	EXTRACT Cumin (50mg/ml)	ANTIBIOTIC	COMBINATION	EXTRACT Cumin (50mg/ml)	ANTIBIOTIC	COMBINATION	AB	Extract	
ST.4	9	AML 22	22	64	AML 16	16	-	-	NO
ST.14	9	RA 21	22	64	RA 8	4	0.5	0.5	ENHANCEMENT
ST.2	10	ENR 16	21	128	ENR 8	4	0.7	0.3	ENHANCEMENT
ST.6	9	DO 13	17	64	DO 32	8	0.6	0.4	ENHANCEMENT
ST.7	9	GN 13	19	128	GN 16	4	0.6	0.4	ENHANCEMENT
ST.13	10	E 14	20	64	E 16	4	0.5	0.5	ENHANCEMENT
ST.6	9	F 13	13	128	F 16	16	-	-	NO
ST.16	9	CTX 22	22	64	CTX 64	64	-	-	NO
ST.19	10	S 12	12	64	S 16	16	-	-	NO

AML = Amoxicillin RA= Rifampicin ENR= Enrofloxacine DO= Doxycycline
CN = Gentamicin E= Erythromycin F= Flurofenicol CTX = Cefotaxime
S= Streptomycin D.D.A= Decimal assay for additivity ratio

of different ages reared in farms located in Sharkia and Dakahlia governorates in period between 2014 till 2015. The bacteriological and biochemical examination of these samples revealed the presence of 16 Gram positive isolates out of 150 specimens (*Staph. aureus*) with percentages 10.6%. The in-vitro antimicrobial sensitivity pattern of these isolates was done and the intermediate isolates were checked for combination with methanol cumin extract after detecting their antimicrobial activities against these isolates by both disc diffusion test and MIC and the obtained data that: Antimicrobial activities of rifampicin, enrofloxacin, doxycycline, gentamicin, and erythromycin were enhanced in combination with methanol cumin extract the obtained data showed that enhancement by both disc diffusion test and MIC for methanol cumin extract and each of mentioned antibiotics in decimal assay of additivity ratio (0.5 for rifampicin and erythromycin and 0.5 for extract ,0.7 for enrofloxacin**,** and 0.3 for extract,0.6 for doxycycline, gentamicin and 0.4 for extract) against *Staph. aureus* isolates as shown in (table 1).

4. DISCUSSION

The antimicrobials resistance is nowadays considered as a great obstacle for combating infectious diseases, so the aim of this study is to threw spots up on some antimicrobials interactions with some plant extracts against some bacterial isolates to give an idea for practitioners & specialists of infectious diseases to lay strategic control of the epidemics by using those antimicrobials to save animal wealth. In this study a total of 150 samples were aseptically collected from visceral organs (liver, gall bladder, spleen, kidney, cecum, heart and lung) of clinically diseased and dead chickens of different ages reared in farms located in Sharkia and Dakahlia governorates in period between 2014 till 2015. *Staph. auerus* was isolated from chickens in Dakahlia and Sharkia Governorates in Egypt with a percentage 10.6% (16 out of 150) the results in this study nearly coordinated with Lee (2003) who isolated S. aureus from feces, feed, joint and trachea of chickens, and isolated S. aureus from each sample in the percentage of 9/69 (13%), 2/35 (5.7%), 4/36 (11.1%), 33/119 (27.7%) and 5/37 (13.5%), respectively. In this study, antimicrobial activity for methanol cumin extract was detected by both disc diffusion test and MIC against *S. aureus* isolates and this attitude was supported Hamdy et al. (2013) who studied the antimicrobial activity and synergistic/antagonistic effect of interactions between antibiotics and some spice essential oils against pathogenic and food spoiler microorganism. Antimicrobial activities of rifampicin, enrofloxacin, doxycycline, gentamicin, and erythromycin were enhanced in combination with methanol cumin extract the obtained data showed that enhancement by both disc diffusion test and MIC for methanol cumin extract and each of mentioned antibiotics against *S. aureus* isolates, this attitude was supported by Hamdy et al. (2013) who studied the antimicrobial activity and synergistic/antagonistic effect of interactions between antibiotics and some spice essential oils against pathogenic and food spoiler microorganism but no enhancement was observed to amoxicillin and cefotaxime antibiotics against *S. aureus* isolates in combination with methanol cumin extract.

5. CONCLUSION

From the results of this study, one can conclude that**:** Antimicrobial activities of rifampicin, enrofloxacin, doxycycline, gentamicin, and erythromycin were enhanced in combination with methanol cumin extract the obtained data showed that enhancement by both disc diffusion test and MIC for methanol cumin extract and each of mentioned antibiotics against *S. aureus* isolates.

6. REFERENCES

Ababutain, I.M., 2011. Antimicrobial Activity of Ethanolic Extracts From Some Medicinal Plant. Australian Journal of Basic and Applied Sciences 5, 678-683.

Acar, J.F., 2000. Antibiotic synergy and antagonism. Med Clin North Am 84, 1391-1406.

Awan, U.A., Andleeb, S., Kiyani, A., Zafar, A., Shafique, I., Riaz, N., Azhar, M.T., Uddin, H., 2013. Antibacterial screening of traditional herbal plants and standard antibiotics against some human bacterial pathogens. Pakistan journal of pharmaceutical sciences 26, 1109-1116.

Bauer, A.W., Kirby, W.M.M., Sherris, J.C., Turck, M., 1966. Antibiotic Susceptibility Testing by a Standardized Single Disk Method. Am J Clin Pathol 45, 493-&.

Chirstine, C., Sanders, W., Eugene, S.J.R., Ellen, S.M., 1993 Decimal Assay for Additivity of Drugs Permits Delineation of Synergy and Antagonism Antimicrobial agents and chemotherapy 260-264.

Cottarel, G., Wierzbowski, J., 2007. Combination drugs, an emerging option for antibacterial therapy. Trends in biotechnology 25, 547-555.

Cruickshank, R., Duguid, J.P., Marmion, B.P., Swain, R.H.A., 1975. Medical Microbiology, 12th ed. Churchill living stone, Edenburg, London and New York.

Djeussi, D.E., Noumedem, J.A., Seukep, J.A., Fankam, A.G., Voukeng, I.K., Tankeo, S.B., Nkuete, A.H., Kuete, V., 2013. Antibacterial activities of selected edible plants extracts against multidrug-resistant Gram-negative bacteria. BMC complementary and alternative medicine 13, 164.

Hamdy, A.S., Mohamed, B.M.A., Lamyaa, E.E., May, M., A. , 2013. Study on the antimicrobial activity and synergistic /antagonistic effect of interactions between antibiotics and some spice essential oils against pathogenic and food-spoiler microorganisms. Journal of Applied Sciences Research 9, 5076-5085.

Horiuchi, K., Shiota, S., Kuroda, T., Hatano, T., Yoshida, T., Tsuchiya, T., 2007. Potentiation of antimicrobial activity of aminoglycosides by carnosol from Salvia officinalis. Biol Pharm Bull 30, 287-290.

Ibezim, E.C., Esimone, C.O., Nnamani, P.O., Onyishi, I.V., Brown, S.A., Obodo, C.E., 2006. In vitro study of the interaction between some fluoroquinolones and extracts of kola nitida seed. African Journal of Biotechnology 5, 1781-1784.

Koneman, E.W., Allen, S.D., Janda, W.M., Schreckenberger, P.C., Winn, W.C., 1992. Color atlas and textbook of diagnostic microbiology, 4th ed. Lippincott, Philadelphia.

Lee, J.H., 2003. Methicillin (oxacillin)-resistant Staphylococcus aureus strains isolated from major food animals and their potential transmission to humans. Applied and Environmental Microbiology 69, 6489-6494.

Norton, R.A., Bayyari, G.R., Skeeles, J.K., Huff, W.E., Beasley, J.N., 1994. A survey of two commercial turkey farms experiencing high levels of liver foci. Avian Dis 38, 887-894.

Smith, C.E., Foleno, B.E., Barrett, J.F., Frosco, M.B., 1997. Assessment of the synergistic interactions of levofloxacin and ampicillin against Enterococcus faecium by the checkerboard agar dilution and time-kill methods. Diagn Microbiol Infect Dis 27, 85-92.

Sooad, A., Manar, A., Nora, A., Rawan, A., Amal, B., Seema, Z., Ramesa, S.B., 2012. Antibacterial activity and phytochemical screeninigof some medicinal plants commonly used in Saudi Arabia against selected pathogenic microoganisms. Jouranl of King Saud university 25, 115-120.

Tan, Y.T., Tillett, D.J., McKay, I.A., 2000. Molecular strategies for overcoming antibiotic resistance in bacteria. Mol Med Today 6, 309-314.

Taylor, P.W., Stapleton, P.D., Paul Luzio, J., 2002. New ways to treat bacterial infections. Drug Discov Today 7, 1086-1091.

White, R.L., Kays, M.B., Friedrirch, L.V., Del-Ben, V.E., 1993. Impact of different statistical methologies on the evaluation of the in-vitro MICs for bacteroides fragilis of selected cephalosporins and cephamycins. J. Antimcrob. Chemother 31, 57-64.

Zapun, A., Martel, C.C., Vernet, T., 2008. Penicillin-binding proteins and b-lactam resistance. FEMS Microbiology Reviews Volume 32, 361–385.