Interaction between some Plant Extracts with some Antibiotics against *Staphylococcus aureus* from Chickens

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**Abstract**

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 (geranium, turmeric, thyme, ginger and green tea) methanol extract prepared by ultrasonic-assisted (UAE) combined with antibiotics (amoxicillin, doxycycline, gentamicin and difloxacin) against 16 *Staphylococcus aureus* chickens 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 were evaluated. Methanol extracts had high total phenolic compounds which used as a source of natural antioxidants. The results revealed that synergistic effects appear thyme with amoxicillin and gentamicin and difloxacin. Turmeric had synergistic effect with amoxicillin, doxycycline, and difloxacin. Synergistic activity against Gram-positive 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.

**Keywords**

Decimal Assay for Additivity (DAA), Antiradical, Antimicrobial, Amoxicillin, Doxycycline, Gentamicin, Difloxacin

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**Introduction**

The incorrect and overuse of existing antimicrobials was becoming a formidable threat in the fight against disease due to the emergence of multi-drug resistant strains (Lambert, 2000; Van Vuuren et al., 2009). Drug synergism between known antimicrobial
agents and bioactive plant extracts is a novel concept and has been recently reported (Nascimento et al., 2000; Aqil et al., 2005; Betoni et al., 2006 and Mhanna and Adwan, 2008).

In the recent time, spread of multidrug resistance as a phenomenon among bacterial pathogens had been a major problem confronting the field of antibacterial chemotherapy (Stefanovic and Comic, 2012). To overcome this problem some medicinal plants as a source of multidrug resistance inhibitor were utilized in combination antimicrobial agents (Eze et al., 2013).

The Minimum Inhibitory Concentration (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 resistance to antimicrobial agents and done by broth dilution methods (Handa et al., 2008).

Nowadays to overcome environmental pollution caused plant residues, numerous studies focused on recovering, recycling of plant residues as it has potential biological effects (Cioffi et al., 2010 and Gavaric et al., 2015). About 99% of residues after extraction 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). This study was carried out to evaluate the interaction of some plant extracts with some antibiotics against *Staphylococcus aureus* from chickens and determine minimal inhibitions concentration (MIC) for each antibiotics and plant extracts by using decimal assay for additivity (DAA) method to detect effect of interaction between antibiotics and plant extracts.

Materials and Methods

Bacterial strains

Standard strain (ATCC)

The tested microorganisms were provided from the culture collections of the Microbiological Department National Research Center (NRC) Dokki, Giza, Egypt. These include *Staphylococcus aureus* ATCC.

Field strain

Sixteen isolates out of 150 samples of clinical diseased chickens which isolated from different farms in Dakhliagovernate, and Sharkiagovernate was shown in Table 1. Under aseptic condition, samples were transferred to bacteriological laboratory for bacteriological examination.

Plants

Plant materials

Five plant including green tea (*Camellia sinensis*) leaves, thyme (*Thymus vulgaris*) leaves, ginger (*Zingiber officinale*) rhizomes, turmeric (*Curcuma longa*) rhizomes and geranium leaves (*Pelargonium graveolens*) leaves.

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

Modern techniques as extraction by ultrasound to overcome (time-solvent) consuming and increase extraction efficiency Betancount (2008) was shown in Table 2.

Isolation and identification of the suspected bacteria

Samples were identified by Gram staining, colony morphology, motility test, coagulase test, catalase reaction, Vogues- Proskaur test,
and sugar fermentation, as described by Barrow and Gelthan (1993) and Harrigan (1998).

**Antimicrobial susceptibility testing**

**Disk Diffusion Method**

According to guidelines set by the Clinical Laboratory Standards Institute (2011), (CLSI-2011) the diameters of the zones of inhibition were measured in millimeter and classified as resistant, intermediate or sensitive and done to plant extract and, antibiotics alone and also to combination between them by disk diffusion method to detect the effect of ten standard antibiotic discs (Oxoid®) and five selected extracts against *Staphylococcus aureus*.

**Minimal inhibitory concentration (MIC)**

The isolated strain matches the 0.5 McFarland standards (1.5*10^8 CFU mL ^{-1}) and results of antibiotics and plant extracts showed no visible bacterial growth considered as MIC and interpreted with recommendations of the National Committee for Clinical Laboratory standards (2011).

**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 2,2-diphenyl-1-picrylhydrazyl (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 prevalence of *Staphylococcus aureus* and resistance patterns in *Staphylococcus aureus*. A total of 150 samples were aseptically in farms located in Sharkia and Dakahlia governorates with bacteriological examination. Positive samples revealed the presence of 16 Staphylococcosis out of 150 specimens and isolates with percentages of (10.6 %) respectively in Table 1.

For further identification of Gram positive biochemical tests such as catalase test and coagulase test were used under standard conditions as discussed in the methods in Table 3. *Staphylococcus aureus* showed positive results with catalase test, also positive result with coagulase.

Antimicrobial susceptibility testing showed the highest sensitivity rate of *Staphylococcus aureus* strains that recorded to amoxycillin (6%) of sensitive strains, the highest intermediate with doxycycline and gentamicin (6%) of intermediate strains and the highest resistant with colistin, streptomycin, and cefotaxeme (16%, 15% and 12% respectively).
of the resistant strains which shown in Table 4.

As for *Staphylococcus aureus*, the extract of turmeric with lowest concentration had an inhibition zone of 11mm, the green tea with lowest concentration had an inhibition zone of 14mm, the thyme with lowest concentration had an inhibition zone of 13mm, the geranium with lowest concentration had an inhibition zone of 10mm and ginger with lowest concentration had an inhibition zone 12mm against field isolated *Staphylococcus aureus* strains in Table 5.

On the other hand antibiotics (amoxicilin, doxycycline, gentamicin, and difloxacin) exhibited different inhibition zone from (14 to 15mm on field isolated *Staphylococcus aureus* strains) for amoxicilin, from (15 to 17 mm on field isolated *Staphylococcus aureus* strains), for doxycycline from (12 to 16 mm on field isolated *Staphylococcus aureus* strains), while (12 mm on field isolated *Staphylococcus aureus* strains) for gentamicin, finally (16 to 19 mm on field isolated *Staphylococcus aureus* strains) for difloxacin which shown in Table 5.

Five plant extracts were subjected to a broth macrodilution assay and after twenty four hours observation of *Staphylococcus aureus* growth to determine the MIC values.

As the MIC of geranium was (4 µg/mL on field isolated *Staphylococcus aureus* and 2 µg/mL on standard strain of *Staphylococcus aureus*), green tea was (16 µg/mL on field isolated *Staphylococcus aureus* and 4 µg/ML on standard strain of *Staphylococcus aureus*), ginger was (16 µg/ML and 4µg/mL on standard strain of *Staphylococcus aureus*), thyme was (4 µg/mL on field isolated *Staphylococcus aureus* and 1µg/ML on standard strain of *Staphylococcus aureus*) and turmeric was (16 µg/mL on field isolated *Staphylococcus aureus* and 2µg/mL on standard strain of *Staphylococcus aureus*) which shown in Table 6. In this study each four antibiotics was subjected to a broth macrodilution assay and after twenty four hours observation of bacterial growth to determine the minimal inhibition concentration (MIC) values on *Staphylococcus aureus*.

MIC values amoxicillin was (8 µg/ml for *Staphylococcus aureus* and 4µg/mL on standard strain of *Staphylococcus aureus*), doxycycline was (16µg/ml for on field isolated *Staphylococcus aureus* and 2µg/ mL on standard strain of *Staphylococcus aureus*), and gentamicin was (16 µg/ml on field isolated *Staphylococcus aureus* and 4µg/mL on standard strain of *Staphylococcus aureus*), and difloxacin was (0.5 µg/ml on field isolated *Staphylococcus aureus* and 0.125µg/mL on standard strain of *Staphylococcus aureus*)which shown in Table 7.

Antimicrobial activities of methanol different plant extracts in combination with antimicrobial agents on selected *Staphylococcus aureus* isolates as Interactions between these components lead to antagonistic, additive and synergistic effects as additive effect was observed when the combined effect was equal to the sum of the individual effects, antagonism was observed when the effect of one or both compounds was less when they were applied together than when individually applied and Synergism was observed when the effect of the combined substances was greater than the sum of the individual effects.

Results of synergy between antibiotics/plant extracts on *Staphylococcus aureus* were presented in Table 8.

Amoxicillin showed synergistic action in combination of with thyme by level (7:3) and by (5:5) while with turmeric by level (7:3),
according to doxycycline showed synergistic action with geranium by level (7:3), (6:4) and by (5:5) and with turmeric by level (7:3) and (6:4), also gentamicin recorded synergistic action in combination of with thyme by level (7:3), (6:4) and by (5:5). Finally difloxacin reported synergistic action in combination of with ginger by level (7:3), (6:4) and by (5:5) and with turmeric by level (7:3), (6:4) and by (5:5) and with thyme by level (5:5).

According to total phenolic compound, Table 9 illustrated that thyme had high phenolic compounds than genarium and finally green tea, on other hand ginger and turmeric had nearly the same results with respective values of 253.01, 219.38, 190.33, 43.96 and 41.92 mg GAE g⁻¹ extract TPC expressed as Gallic acid equivalent (GAE) was calculated using the following linear equation based on the calibration curve.

According to DPPH result, ginger and turmeric revealed higher extension than green tea than geranium, Also thyme extract has shown lower extent of DPPH neutralization (EC50 = 128.49 mg/mL) than oil obtained in our investigation as show in Figure 1.

Table.1 Number of *Staphylococcus aureus* isolates obtained from various specimens collected from different localities in Sharkia and Dakalia

| Locality                        | No. of cases | *Staphylococcus aureus* |
|---------------------------------|--------------|-------------------------|
| Dakahlia Farms                  |              |                         |
| Private farms (Mahtet Elsalam, Mahtet ElAml, Tawonya) | 30           | 5                       |
| Private farms (Gamsa, Sherbin)  | 20           | 0                       |
| Sharkia Farms                   |              |                         |
| Farms in Sharkiagovernate Elsalehia project | 40           | 5                       |
| Private farms in Sharkiagovernate | 60           |                         |
| Total                           | 150          | 16 (10.6%)              |

Table.2 List of the methanolic extract obtained 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) |
|--------------------------|------------|-------------|---------------------------|-----------------------|--------------------------|
| *Pelargonium graveolens* | Geraniaceae| Geranium    | 138.0542                  | 134.5216              | 3.53                     |
| *Camellia sinensis*      | Theaceae   | Green tea   | 172.3789                  | 169.9189              | 8.9                      |
| *Zingiberofficinale*     | Zingiberiaceae | Ginger      | 177.5154                  | 176.9858              | 1.02                     |
| *Thymus vulgaris*        | Thymeleaceae | Thyme      | 191.9960                  | 187.3328              | 4.66                     |
| *Curuma longa*           | Zingiberiaceae | Turmeric    | 193.6245                  | 192.6999              | 0.42                     |
Table.3 Culture and biochemical identification of Gram-positive isolates *Staphylococcus aureus*

| Nutrient agar       | smooth, had a low convex profile with an entire edge and pigmented yellowish colonies |
|---------------------|-------------------------------------------------------------------------------------|
| Haemolytic activity | B-hemolytic                                                                         |
| Mannitol salt agar  | yellow halo zone surrounding their growth                                           |
| Baired Parker agar  | black, shiny, convex and surrounded by a clear zone                                 |
| E-Pigmentation      | golden yellow pigment on milk agar                                                  |
| Catalase test       | +                                                                                    |
| Coagulate test      | +                                                                                    |
| Total NO.           | 16                                                                                   |
| % of isolate        | 10.6%                                                                                |

Table.4 Antimicrobial susceptibility on *Staphylococcus aureus* by agar disc diffusion method

| Antimicrobial agents | Antibiotic disc/conc.(µg) | S  | I  | R  |
|---------------------|---------------------------|----|----|----|
| Amoxicillin         | AML-25 µg                 | 6  | 4  | 6  |
| Colistin            | CT-10 µg                  | 0  | 0  | 16 |
| Difloxacin          | INN-5 µg                  | 5  | 5  | 6  |
| Doxycycline         | DO-30 µg                  | 4  | 6  | 6  |
| Gentamicin          | CN-10 µg                  | 3  | 6  | 7  |
| Erythromycin        | E-15 µg                   | 5  | 5  | 5  |
| Flurophenicol       | F-30 µg                   | 2  | 4  | 10 |
| Cefotaxime          | CTX-30 µg                 | 1  | 3  | 12 |
| Streptomycin        | S-10 µg                   | 0  | 1  | 15 |

R (resistance), S (susceptibility), I (intermediate)

Table.5 Zone of inhibition (mm) of antibiotics and plant extracts alone and in combination on field and standard strains of *Staphylococcus aureus*

| Isolates | Inhibition zone (mm) | Combination |
|----------|----------------------|-------------|
| Plant    | Plant alone          | Antibiotics alone | AML/18 (mm) | Do/ 18 (mm) | CN/15 (mm) | INN/22 (mm) | AML | DO | CN | INN |
|----------|----------------------|---------------|-------------|-------------|-------------|-------------|-----|----|----|-----|
| geranium | 10                   | 16            | 14          | 12          | 17          | 16          | 19  | 13 | 20 |
| green tea| 13                   | 15            | 13          | 12          | 16          | 13          | 12  | 10 | 14 |
| ginger   | 12                   | 15            | 12          | 12          | 16          | 16          | 17  | 13 | 22 |
| thyme    | 14                   | 17            | 16          | 12          | 17          | 18          | 16  | 17 | 22 |
| turmeric | 11                   | 15            | 13          | 12          | 19          | 19          | 18  | 13 | 22 |

AML: amoxicillin. DO: doxycyclin.
CN: gentam INN: difloxacin
Table 6. Minimum inhibitory concentration (MIC) of plant extracts on *Staphylococcus aureus*

| Plants     | Staphylococcus aureus | MIC (µg / ml) |
|------------|-----------------------|---------------|
| Geranium   | Field Strain          | 4             |
|            | Standard strain       | 2             |
| green tea  | Field Strain          | 16            |
|            | Standard strain       | 4             |
| Ginger     | Field Strain          | 16            |
|            | Standard strain       | 8             |
| Thyme      | Field Strain          | 4             |
|            | Standard strain       | 1             |
| Turmeric   | Field Strain          | 16            |
|            | Standard strain       | 2             |

Table 7 Minimum inhibitory concentration (MIC) of antibiotics on *Staphylococcus aureus* (field and standard strains)

| Antibiotic | Staphylococcus aureus | MIC (µg / ml) |
|------------|-----------------------|---------------|
| AML(20%)   | Field strain          | 8             |
|            | Standard strain       | 4             |
| DO(20%)    | Field strain          | 16            |
|            | Standard strain       | 2             |
| CN(10%)    | Field strain          | 16            |
|            | Standard strain       | 4             |
| INN(10%)   | Field strain          | 0.5           |
|            | Standard strain       | 0.125         |

AML: amoxicillin. DO: doxycyclin. CN: gentamicin. INN: difloxacin.
**Table 8** Combination activity of antibiotics with plant extracts using Decimal Assay for Additivity (DAA)

| Plant extracts | Antibiotics   | DAA | MIC | effect     |
|----------------|---------------|-----|-----|------------|
|                | AB | E | DAA | AB alone |
| Geranium       | a) | doxycycline | 7  | 3  | 8  | 16 | Synergy (S) |
|                | 6  | 4  | 8   | 16     |
|                | 5  | 5  | 8   | 16     |
| Thyme          | a) | amoxycillin | 7 | 3  | 4  | 8  | Synergy (S) |
|                | 5  | 5  |     |       |
|                | b) | gentamicin | 7 | 3  | 4  | 16 | Synergy (S) |
|                | 6  | 4  |     |       |
|                | 5  | 4  |     |       |
|                | C) | difloxacin | 5  | 5  | 0.25 | 2 |
| Turmeric       | a) | amoxycillin | 7 | 3  | 0.25 | 0.5 | Synergy (S) |
|                | 6  | 4  |     |       |
|                | b) | doxycycline | 7 | 3  | 2   | 4  | Synergy (S) |
| Ginger         | a) | difloxacin | 7 | 3  | 0.5 | 2  | Synergy (S) |
|                | 6  | 4  | 1   |       |
|                | 5  | 5  | 0.5 |       |

**Table 9** Yield of extracts (g/100g) for different plants

| Plants          | extract yield | % Extract | TPC mg GAE/g extract |
|-----------------|---------------|-----------|----------------------|
| Green tea       | 8             | 40        | 190.33               |
| Thyme           | 4.66          | 23.3      | 253.01               |
| Ginger          | 1.02          | 5.1       | 43.96                |
| Turmeric        | 0.42          | 2.1       | 41.92                |
| Geranium        | 3.53          | 17.65     | 219.38               |

GAE: gallic acid equivalents.
TPC: Total phenolic compound
Figure.1  Antioxidant DPPH radical-scavenging activity

![INHIBITION Of DPPH](image)

GAE: gallic acid equivalents
TBHQ: Tert-butyl hydroquinone

*Staphylococcus aureus* was recorded from chickens in Dakahlia and Sharkia governorates in Egypt in percentage 10.6% (16 out of 150) which was nearly coordinated with Lee *et al.*, (2003) who recorded *Staphylococcus aureus* from feces, feed, joint and trachea of chickens, and isolated *Staphylococcus aureus* from each sample in the percentage of 13%, 5.7%, 11.1%, 27.7%, 13.5%, respectively.

Biochemical tests as catalase test and coagulase test showed positive results with tested strains this results agreed with Normannoa *et al.*, (2004).

As for *Staphylococcus aureus* the extract of turmeric with the lowest concentration had an inhibition zone of 11 mm, the green tea with the lowest concentration had an inhibition zone of 14 mm, the thyme with the lowest concentration had an inhibition zone of 13 mm, geranium with the lowest concentration had an inhibition zone of 10 mm and the ginger with the lowest concentration had a inhibition zone of 12 mm showed in. So this result agreed with Hamed *et al.*, (2013) in turmeric, Archana and Abraham (2011) in green tea, Fayed *et al.*, (2013) in thyme, while in geranium Oulkheir (2017) disagreed with us, finally in ginger was not parallel to Kredi (2016).

Our results suggested that thyme extract could be combined with antibiotics like amoxicillin, gentamicin and difloxacin as it has been successful in combating *Staphylococcus aureus*.

As extracts from turmeric shows synergistic effects against *Staphylococcus aureus* compared to doxycycline, difloxacin antibiotics and this agreed with Teow *et al.*, (2015).

On the other hand in this study demonstrated synergistic effects of ginger amoxicillin and difloxacin against *Staphylococcus aureus*, these results were similar to Shekhan *et al.*, (2012).
There were antagonist effects results of green tea with difloxacin similar to Tuysuz et al., (2017).

The study showed that geranium and doxycycline combinations were synergistic against *Staphylococcus aureus*. Rosato et al., (2007) illustrated that there were synergistic effects of geranium and gentamicin combination against *Staphylococcus aureus*, these results disagreed with our study results. Activity of extract compared to unstable oil is probably due to presence of nonvolatile phenol compounds. In addition, some of the compounds with a different polarity, which are present in very small amounts in the extract, are also able to contribute to better an oxidative activity of extract. Some compounds can originate in extract during hydrolysis or other processes of decomposition. Some chemical reactions initiated by heating can also drive up to activities changes of complex extract, composed of a number of compounds with different chemical and physical properties (Singh et al., 2005).

The free radical activity of the plant extracts was performed according to the DPPH free radical method, described by Brand-Williams et al., (1995).

It can be noticed that the degree of DPPH neutralization depended on incubation time, for all investigated concentrations of oil. The highest degree of DPPH radicals’ neutralization is for 60 minutes incubation.

In conclusion, the demonstration of synergistic activity by the antibiotic and extracts against Gram-positive bacteria is an indication that plants can be a source of bioactive substances that could possess broad spectrum of activity most especially when it is combined with antibiotic. Thus, there is increasing need for researchers to investigate the synergistic capacity of plants or other natural products, independent of the antimicrobial activity. These findings also suggested that the need for understanding of synergism mechanism is fundamental to development of pharmacological agents to treat diseases by various bacteria using medicinal plants in combination with antibiotics.

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