The Effectiveness of Methanolic Extracts of Five Plants on Different Salmonella Isolates

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ABSTRACT
Salmonellosis is known to be one of important issues that affect poultry industry as well as it can affect human health. Recently, multiple challenges are facing the use of natural antibacterial compounds, such as herbal extracts to overcome the massive increase in bacterial antibiotic resistance. Different Salmonella serotypes were recovered throughout examination of diarrheic poultry. These strains showed multidrug resistance by disc diffusion methods also, the resistance genes qnrS and aac (6')-Ib-cr were detected in S. Enteritidis and S.Typhimurium which isolated from broiler's organs and muscles. The methanolic extracts of five plants (Alhagi maurorum, Conyza dioscoridis, Coriander sativum, Caracuma longa and Cuminum cyminum) were tested for their antibacterial activity against different isolated Salmonella serotypes using minimum inhibitory concentrations (MIC). Conyza dioscoridis was the most effective extract retarding microbial growth of Salmonella Enteritidis, while other plant extracts showed variable antimicrobial activity. These results are promising in the way of replacing the antibiotic therapy with natural substances to overcome the multidrug resistance.

Key words: Salmonella Enteritidis; Salmonella Typhimurium; Broiler; Methanolic plant extracts; Resistance genes.

INTRODUCTION
Expanding of antibiotic resistance and the scarceness of new antimicrobial agents have been long acknowledged as a major challenge in global health (Walsh and Toleman, 2011). There has been an urgent need for effective and reasonable medications to overcome bacterial infections, particularly in the developing nations. Salmonellosis is a typical infectious zoonotic disease that occurs frequently in poultry with many incriminated Salmonella serotypes. Salmonella species are the most common causes of food borne illness worldwide and the two most commonly involved foods are poultry meat and eggs (Dar et al., 2017). The prevalence of Salmonella enterica in poultry is different across the world which marked much more in developing countries, while in the first class countries, Salmonella species positivity was reported in 1% of poultry flocks (Rychlik et al., 2014; Shaimaa et al., 2018). This pathogen can persist in poultry for a prolonged period of time, turning human to reservoir particularly enterica subtype (Rychlik et al., 2014; Soliman et al., 2020). Multidrug resistant Salmonella have been showing high prevalence in foods across many different parts in the world. Unfortunately, there were many reports related to treatment failures particularly with ciprofloxacin in S. Typhi cases in different continents (Nkemungu et al., 2005).

Yamane et al., (2007) showed that there are two molecular groups involved in the plasmid-mediated fluoroquinolones resistance mechanisms. Qnr peptides, QnrA, QnrB and QnrS were detected in different Salmonella species, and it has been thought that these peptides affect DNA gyrase by resembling DNA. Moreover, aac (6')-Ib-cr gene was assumed to have an N-acetylation activity toward the piperazinyl substituent of ciprofloxacin and norfloxacin, and it has been reported to be globally spread.

Medical uses of plants were recognized by former medical practitioners because they used the medicines derived from plants from their local environment (Emeka et al., 2012). To recover the active component of the plant, extraction is the first vital step. Numerous extraction procedures and solvents were used to purify extracts that have antimicrobial effects from different plants. The most common methodologies used for extraction are maceration, Soxhlet extraction, solvent extraction, microwave-assisted extraction and sonication (Bicchi et al., 2000).

Antimicrobial activity of Coriander sativum, Cumin seeds (Cuminum cyminum) and Conyza dioscoridis plant extract had been reported to be effective against many strains of Gram positive and negative bacteria as well as some fungi involved in food poisoning (Zain et al., 2012).

Different extracted samples of Alhagi maurorum, Conyza dioscoridis, Coriander sativum, Caracuma longa, Cuminum cyminum) have been investigated against S. Typhimurium and S. Entriditis along with testing their use as a proper, safe and easy degradable antimicrobial alternative.

MATERIALS AND METHODS

Sample collection, bacterial isolation and identification

Fifty samples (17 liver -13 gallbladder and 20 muscles) were collected from fifty broiler chickens which were obtained from El-Giza broilers farms. Each sample was collected aseptically in a separate plastic page and transported in ice boxes packed with ice to the Microbiological lab as soon as possible. For isolation of Salmonella strains, organs were inoculated into Pre-enrichment broth medium (buffer peptone water) and incubated in 37°C for 16-18 hr. For selective isolation of Salmonella according to ISO 6579-1: 2002 Rappaport-Vassiliadis medium (RV) was used as enrichment broth and incubated at 41.5°C for 24hr. For selective isolation of Salmonella, XLD agar was used. Translucent pink colonies with black centers on XLD agar were suspected as Salmonella spp. Further confirmation was done depending on culture characters, microscopical identification using Gram staining, and biochemical tests (urease test, triple sugar iron test)

Serological identification of Salmonella species according to ISO 6579-3: 2014 reading with Kauffman – White scheme (Grimon and Weill, 2007) to determine Somatic (O) and flagellar (H) antigens using Salmonella antiserum (Sifin Co., Japan).

Antibiotic susceptibility testing of Salmonella isolates

The antibiotic sensitivity testing of the isolates was done according to the modified Kirby-Bauer disk diffusion method according to Manual for Identification and Antimicrobial Susceptibility Testing (CDC, 2003) and the interpretation was following CLSI (Clinical and Laboratory Standards Institute) (CLSI, 2017). The antibiotic disks (Oxoid) used were Gentamicin (GEN), Neomycin (N) Streptomycin (S), Amoxicillin-clavulanic acid (AMC), Ampicillin(AMP), Enrofloxacin (ENR) Ciprofloxacin (CIP), Ceftriaxone (CRO) Cefazidime (CAZ) Doxycycline (DO) Tetracyclin (TE) Chloromphenicol (C) Sulphatrim (Trimethoprin+sulfamethoxazole) (SXT).

Molecular identification of antibiotic resistance genes

Isolated Salmonella strains were investigated for the presence of antibiotic resistance genes (qnrS and aac(6’)-Ib-cr) which are associated with Plasmid mediated quinolone resistance (PMQR). For the identification of these genes, DNA was extracted from pure Salmonella isolates using QIAamp DNA Mini Kit, (Qiagen, Germany, GmBH) Catalogue no.51304 (according to kit instructions). Genomic DNA quality was checked using gel electrophoresis and absorbance was taken at A260/A280 and A260/A230 ratios using the spectrophotometer. The primers used for amplification as shown in (Table 1) (Lunn et al., 2010, Robicsek et al., 2006). The reactions were performed in thermal cycler (Applied Biosystem). The amplicon was separated by electrophoresis on 1.5% agarose gel according to (Sambrook et al., 1989) in 1x TBE buffer at room temperature. For examination of gel, 20 µl of the products was loaded in each gel well. To determine the size of product, 100 bp DNA Ladder (Qiagen, Germany) was used and the gel was photographed by transilluminator.

Methanolic extraction of the Natural medicinal plants: (Sulaiman, 2013).

These plants were collected from different sources at Plant Research Institute and Drug during period from December (2017) to January (2018). The families, genera and plant species were identified scientifically according to the flowering plants catalogue (Cappers and Bekker, 2013) as shown in Table (2). One kilogram of the proper parts of each plant was collected. These parts were cleaned using water before drying in the shade for seven to ten days. Then they were crushed in mortar. The extraction process was held in soxhlet cold extractor using methanol 80% as solvent for three successive days. The extracts were filtered using Whitman filter paper then dried in rotary evaporator under reduced pressure at 45°C. The extraction and evaporation processes were processed three times. The yield of the extracts was weighted by grams and stored in dark containers in refrigerator at 4°C.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of plant extracts

Fresh pure culture of S. Enteritidis and S. Typhimurium were suspended in buffer peptone water and their concentration were adjusted to 7.5x10⁵ CFU/ml (Elisha et al.,2017). Stock solution of the plant extracts were made up in DMSO (1.5%) to ensure complete solubilization. The concentration of plant extract was adjusted to be 100 mg/ml and prepare serial dilution till 0.19 mg/ml. In a sterile microtiter plate (96 well plates), wells of each row were filled with100 µl of serially diluted plant extract then 100 µl of the prepared bacterial culture were added to each dilution. Positive control (bacterial culture only) and negative control (for each tested plant extracted) were used.

The MICS were reported at the lowest concentrations showing no growth by visual inspection. To establish MBC, aliquots from each well were removed and each dilution was plated on MacConkey's agar. After 24 h of incubation at 37°C, the colony-forming units per millilitre (CFU /mL) were then determined. The MBC values were demarcated at the lowest concentration of the extract resulting in no growth. These assays were performed in triplicate. (Forbes et al., 2007).
Table 1: Oligonucleotide primers sequences and amplify specific product

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence (5’-3’)</th>
<th>Annealing</th>
<th>Amplified product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>aac(6′)-Ib-cr</td>
<td>F: CCCGCTTTCTCGTAGCA</td>
<td>52˚C/30 sec</td>
<td>113 bp</td>
<td>Lunn et al. (2010)</td>
</tr>
<tr>
<td>qnrS</td>
<td>F: ACGACATTGCACACTGCAA</td>
<td>55˚C/ 40 sec</td>
<td>417 bp</td>
<td>Robicsek et al. (2006)</td>
</tr>
</tbody>
</table>

Table 2: Scientific name, family, local name and used part for extraction of the active principles:

<table>
<thead>
<tr>
<th>Scientific name</th>
<th>Family</th>
<th>Used part for extraction of the active principle</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Alhagi maurorum</td>
<td>Leguminosae</td>
<td>Leaves and flowers</td>
</tr>
<tr>
<td>2-Conyza dioscoridis</td>
<td>Asteraceae</td>
<td>leaves and flowers</td>
</tr>
<tr>
<td>3-Coriander sativum</td>
<td>Umbelliferae (Apiaceae)</td>
<td>Seeds</td>
</tr>
<tr>
<td>4-Caracuma longa</td>
<td>Zingiberaceae</td>
<td>Rhizomes</td>
</tr>
<tr>
<td>5-Cuminum cyminum</td>
<td>Apiaceae</td>
<td>Seeds</td>
</tr>
</tbody>
</table>

Fig. 1: Agarose gel electrophoresis showing amplification of 113bp for the qnrS gene of S. Enteritidis and S. Typhimurium.

Fig. 2: Agarose gel electrophoresis showing amplification of 417bp for the aac (6′)-Ib-cr gene of S. Enteritidis and S. Typhimurium.

Fig. 3: MIC of the five plant extracts against S. Typhimurium and S. Enteritidis.

RESULTS

A total of 6 Salmonella strains were recovered from 50 samples of different organs of broiler chickens suffered from diarrhea in percentage of 12% (6/50). Out of which, the predominant were from Liver 23.5% (4/17) followed by gallbladder 7.7% (1/13) and muscles 5% (1/20). Depending on O and H antigen Salmonella spp were serotyped into three isolates of S. Enteritidis (1,9,12; g,m), and three isolates of S. Typhimurium (1,4, (5),12; i;1,2).

The antibiogram profile of S. Enteritidis and S. Typhimurium isolates showed the highest resistance (83.33%) to Ciprofloxacin, Tetracycline, Neomycin and Amoxicillin clavulanic acid, while 50% resistance was observed against Ceftriaxone, Doxycycline, Gentamicin and Ampicillin. There was no significant association of the prevalence of antibiotic resistant strains with the type of Salmonella strains (P>0.05).

The qnrS and the aac(6′)-Ib genes were detected in Salmonella strains; S. Typhimurium and S. Enteritidis. As illustrated in Fig (1) and Fig (2). The use of 1000 g dried plant extract with methanol yielded plant extract residues ranged from 31.2 g to 93.8 g. The highest yield of plant extract was obtained from Alhagi maurorum (93.8 g) followed by Conyza dioscoridis (62.2 g), Coriander sativum (56.2 g) and Caracuma longa (41.5 g) while Cuminum cyminum give the lowest extract yield (31.2 g).

The five plant species were investigated for their antibacterial activity against S. Typhimurium and S. Enteritidis by estimation of MIC and MBC. Results of antibacterial activity were recorded in Fig (3). These results showed that all plant extracts were potentially effective in inhibition of microbial growth of Salmonella isolates with variant potency. Conyza dioscoridis was the most effective extract impeding microbial growth of S. Enteritidis at a concentration of 1.56 mg/ml and 3.125 mg/ml to S. Typhimurium. Moreover, the MIC of Caracuma longa and Cuminum cyminum were 3.125 mg/ml for both S. Enteritidis and S. Typhimurium while other plant extracts showed variable antimicrobial activity against salmonellae. The MBC was confirmed by the absence of bacterial growth of the tested strains obtained from inhibition well corresponding to their lowest MIC.
DISCUSSION

The haphazard use of antimicrobial agents against enteric bacteria in poultry is the main source of increasing the antimicrobial resistance and transmission to human and animals. The present study tried to investigate the role of plant extracts as a natural source of antimicrobial agents considered as safe and easy degradable.

Six isolates were recovered from internal organs or muscle of 50 birds with a prevalence of salmonellosis about 12% which was near to Ammar et al., (2016) who reported 17% rate of salmonella in Egypt. Higher prevalence rates (21.9% and 52.2%) were found in studies of (Rahman et al., 2004 and Yang et al., 2011) in Bangladesh and China respectively. While, lower percentage of salmonella were reported by (Rehan 2004, Zoo El-faker and Rabie 2009 and Medeiros et al., 2011) from poultry 7.5%, 9.8% and 2.7%, respectively.

Inne et al.,(2009) reported that the result of serotyping showed that most isolates were belonged to S. Enteritidis and S. Typhimurium. S. Enteritidis infections has been reported as main cause of food-borne salmonellosis globally. S. Typhimurium has also been implicated in non-typhoidal salmonellosis and has a significance on public health.

In the present study the two strains of Salmonella ( S. Enteritidis and S. Typhimurium) were resistant to Ciprofloxacin, Neomycin, Tetracycline and Amoxicillin clavulanic acid. These results are concordant with Pribul et al., (2016) who reported that high prevalence of resistance to quinolone ciprofloxacin was identified through the disc diffusion among Salmonella strains isolated from poultry. On the other hand, Purturu et al., (2013) reported that S. Enteritidis was 100% sensitive for ciprofloxacin followed by chloramphenicol, gentamycin amoxicillin and streptomycin (96%, 90%, 82%, 80% receptively).

Chen et al., (2004) stated that there are many factors involved in bacterial resistance to antimicrobial agents such as alteration permeability of the microbial cell wall or enzymatic effects that result in the breaking down of drugs. Nabi (2017) stated that antibiotic resistance in Salmonella species may result from the misuse between humans and animals and the presence of at least one of the qnr genes may be a cause for the antibiotic resistance to quinolone.

Robicsek et al., (2006) said that several bacterial drug-resistance genes had been identified via molecular methods especially PCR; the first was plasmid mediated quinolone resistance gene “qnrA” reported in 1998. In the current work, qnrS gene has been detected as quinolone resistance gene synthesized by Salmonella. The used primer of this gene multiplies a region of 417bp in all identified strains. Similarly, aac (6’)-Ib-cr gene has also been detected; it’s widely known as ciprofloxacin resistance gene. The used primer of this gene multiplies a region of 113bp in all identified strains (S.Typhimurium and S. Enteritidis).

The massive increase in multidrug-resistant bacteria alarm for the urgent need for development of innovative and protective antimicrobial choices (Jorgensen and Ferraro, 2009). Different methods have been used to assess antimicrobial effects of natural and synthetic compounds; the most widely used techniques are the disk diffusion test and the minimal inhibitory concentration test. MIC is the most preferred method due to the generation of a quantitative result (Jorgensen and Ferraro, 2009).

Many research articles studied the role of plant extracts as alternative antimicrobial compounds especially in control of food borne and spoilage bacteria. Some scientists stated that terpenoid, alkaloid and phenolic compounds of the plant extracts react with enzymes and proteins of bacterial cell membrane lead to dispersion of protons outside the cell so, enhance cell death and inactivate the enzymes required for vital biosynthesis (Gill and Holley, 2006). The antimicrobial activity of Alhagi maurorum plants was related to their high flavonoid contents. The possible mechanism of its antimicrobial action may be related to inhibition of cell reproduction and DNA replication. Moreover, these phenolics not only hinder DNA replication but also the formation of free radicals via chelating metals and inactivation of enzymes involved in the initiation reaction (Russo et al., 2002). In this study, Alhagi maurorum showed antibacterial activity against S. Typhimurium and S. Enteritidis with MIC 6.25 and 12.5 mg/ml respectively. Nearly to this result, Zain et al. (2012) said that Alhagi maurorum showed inhibitory concentration against S. Typhimurium with MIC 4 mg /ml. Sulaiman (2013) stated that the ethanolic extract of the A. maurorum showed significant antimicrobial activity against Gram negative bacteria as Salmonella.

In this study Conyza dioscoridis has antibacterial activity against S. Typhimurium and S. Enteritidis with MIC (as a quantitative result) 3.125 and 1.56 mg/ml respectively. Zalabani et al. (2013) stated that the antimicrobial activity of Conyza dioscoridis due to the presence of phenolic compounds with MIC ranging from 50-200 µg/ ml.

In our study Coriandrum sativum has in vitro antibacterial activity against S. Typhimurium and S. Enteritidis with MIC 25 mg/ml and 12.5 mg/ml respectively. These results were concordant with Silva and Domingues (2017) who stated that Coriander (Coriandrum sativum L.) is exhibiting potent antimicrobial action against bacteria and some yeasts and fungi, Mahdi (2011) found that essential oil of Coriandrum sativum was screened for antimicrobial activity against Salmonella Typhi. Also Kubo et al. (2004) showed that Corriander sativum had antibacterial activity against S. choleraeaeus. Similarly Silva and Domingues (2017) demonstrated in vitro effect of coriander oil against many foodborne pathogens such as S. aureus, C. jejuni, Shiga and non-Shiga toxin producing E.coli, L. monocytogenes, Y. enterocolitica and S. Typhimurium.

It was founded that Caracuma longa extract has antimicrobial activity against S. Typhimurium and S. Enteritidis with MIC 3.125 and 3.125 mg/ml, respectively. Hosny et al. (2011) reported that aqueous Curcumin extract (0.3%) achieved a reduction of bacterial counts about one log of S. Typhimurium, two log of P. aurogenosa and E. coli 0157:H7, respectively. Infante et al. (2014) showed that Curcumin inhibited Salmonella growth. Gul and Bakht, (2015) found that extracts of...
turmeric exhibited different ranges of activity against *Calibicans*, *S. Typhi*, *S. aureus* and *E. coli*. Álvarez et al., (2016) denied the presence of antimicrobial effect as well of *Curcuma longa* against salmonella which may be due to the presence of curcuminoid, a phenolic compound. Oppositely Franco et al. (2007) evaluated the antimicrobial activity of *Curcuma longa* essential oil on *S. aureus* ATCC 6538, *E. coli* O:158 and *S. choleraesuis* ATCC 10708 growth using agar diffusion and verified that only *S. aureus* ATCC 6538 growth was inhibited.

Our study revealed that *Cuminum cyminum* extract has antibacterial activity against *S. Typhimurium* and *S. Enteritidis* with MIC 3.125 and 3.125 mg/ml respectively. Dua et al. (2013) also reported that cumin had a potent effect with MIC extended from 6.25 to 12.5 mg/ml. On the other hand, cumin extract with concentration of 60 mg/ml may be required to be effective against bacteria causing food spoilage and these results were parallel with that previously reported by Sheikh et al. (2010). Oppositely Shan et al. (2007) found that Cumin had no active against Salmonellae.

Conclusion

Detection of multidrug resistance genes is a necessary step in establishing of effective strategies for controlling this antibiotic resistance phenomenon within pathogens, such as Salmonella, which can cause serious and potentially life-threatening disease in humans. Natural plant extract is an acceptable alternative safe way that replaces antibiotics without hazard of antimicrobial resistance transmission to human or animals. Conyza dioscoridis was the most effective extract retarding microbial growth of *S. Typhimurium* and *S. Enteritidis* then *Caracuma longa* and *Cuminum cyminum*.

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