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EHLA and MAA conceived and designed the study; EHLA performed and analysed microbiology part; MAA done chemical part. EHLA and MAA wrote, revised and gave final approval for publication of the paper.

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Efficacy of Volatile Oils against *Salmonella* Isolated from Gizzard in Poultry

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Abstract:
Salmonella infection in poultry is one of the most important bacterial diseases which cause heavy economic loss through reduced production and mortality. Transmission of Salmonella is by direct contact with contaminated food of animal origins such as chicken meat and eggs. This study was conducted from April 2018 to May 2018 at store Ali Mohsen in Sana'a city, Yemen. A total of 60 samples of poultry gizzard were collected using a sterile sharp from store Ali Mohsen it was of Amran (20) samples, Thamar (10) samples, Hiziaz (20) samples, Al-hudaidah (10) samples and put it in sterile bottles. Salmonella were isolated from 40 (67%) gizzard samples while 20 (33%) gizzard samples were Negative. Positive samples were 14 (35%) from Amran, Hiziaz 12 (30%), Thamar 10 (25%) and 4 (10%) samples were positive from Alhudaidah. Volatile oils of *Origanum majorana*, *Nepeta delflersina,* and *Mentha x piperita* had antibacterial activities against *Salmonella* sp while isolated strains of *Salmonella* sp were resistant against volatile oils such as *Coriandrum sativum, Elettaria cardamomum, Trachys permum* and *Artemi judaica sia.* The volatile oil obtained from *N. deflersiana* contains sixteen identified and three unidentified constituents. The major constituents were 4aα, 7α, 7aα-Nepetalactone (77.7%). The main chemical constituent in the volatile oil obtained from *O. majorana* was Terpinen-4-ol (35.2%). The main constituent of *Mentha x piperita* volatile oil was Limonene (7.9%). *N. deflersiana*, *O.majorana*, and *M. x piperita* volatile oils showed an inhibition zone against the growth of *Salmonella* isolates at 30µl.

INTRODUCTION

Salmonella are one of the major causes of contaminated food in developing countries (Abdelkader *et al.,* 2019; Yaqub *et al.,* 2017). *Salmonella* species cause a zoonotic disease called Salmonellosis. They are bacilli Gram-negative bacteria that include the species, *S. bongori* and *S. enterica*, of which only the major one is of clinical significance to animals, especially humans. Transmission of *Salmonella* species is by direct contact with contaminated food from animal origin such as chicken meat and eggs (Mogollón *et al.,* 2016).

S. enteritidis is the major cause of human infection by Salmonellosis, and poultry products constitute the essential source of the bacterial infection. Salmonellosis is one of the major important bacterial infections in poultry causing heavy economic loss through reduced production and mortality (Haider *et al.,* 2004). Salmonellosis of avian may occur in poultry either chronic or acute form by one or more species of the genus *Salmonella* (Lutful Kabir, 2010).

Since poultry is the main food source of food for humans, its contamination by coliforms, mainly *Salmonella species* may result in the complications of human illness (Ashraf *et al.,* 2019; Iqbal and Iqbal, 2020; Shawish *et al.,* 2020). *Salmonella* is the main threat to public health of human, it has caused many foodborne outbreaks in USA and worldwide. Habitat of Salmonella in poultry is their stomach and intestine and excretes the bacteria through the stool contaminating the external environment. In addition, there are many sources of the *Salmonella* at the environment. Humans contract Salmonellosis disease by eating contaminated poultry meat and its eggs (Nair and Johny, 2019). *Salmonella* infection can cause both intestinal and extra-intestinal diseases.

Plants produce many substances, some of which have medicinal and pesticidal properties (Ashraf *et al.,* 2020; Iqbal and Ashraf, 2018; Ullah *et al.,* 2018). Although plants lack an immune system, they produce defensive chemicals to attack invading organisms. It has be documented that the defensive substances belong to different chemical classes such as flavonoids, terpenoids, alkaloids, phenolic acids, essential oils, and polyphenols (Al- Mahweety, 2016a,b; Cowan, 1999; Mubarak *et al.,* 2021). There has been an increased interest in the use of natural substances including plant volatile oils as alternative medicines against multidrug-resistant (MDR) microorganisms (Naveed *et al.,* 2013; Shahzad *et al.,* 2017).

The present investigation was initiated to isolate *Salmonella* spp. from gizzards of poultries and to study the effect of volatile oils on some isolates of *Salmonella*.

MATERIALS AND METHODS

Collection of Gizzard samples

Gizzard samples were collected from poultry using a sterile sharp at store Ali Mohsen that include (20) specimens from Amran, Thamar (10) specimens, Hiziaz (20) specimens, and (10) samples from Al-hudaidah. The specimens were placed in sterile bottles and transported to the microbiology laboratory for further examination.

Culturing of Gizzard samples

Each specimen was inoculated on *Salmonella Shigella* agar (S.S. agar), petri dishes, incubated aerobically at 37 °C for 24 hours (Cheesbrough, 1984).

Identification of the isolated bacteria

Bacterial colonies were identified by colony morphology; Gram stain and biochemical characters (Al-Khawlany *et al.,* 2021; Brenner *et al.,* 2005; Iqbal *et al.,* 2016; Yunus *et al.,* 2016).

Collection of plant samples

The samples of aerial parts of *Nepeta deflersiana, Origanum majorana, Mentha x piperita, Coriandrum sativum, Elettaria cardamomum, Trachys permum* and *Artemi judaica sia* were collected in Sana'a-Republic of Yemen. They were identified by botanists at the

Department of Biology, Faculty of Sciences, Sana'a University.

Extraction of volatile oils

One hundred gram of each plant leaves were subjected to hydro distillation for approximately three hours using a Clevenger type apparatus (Sharififar *et al.,* 2008).

Determination of the constituents of volatile oils using Gas Chromatography-Mass Spectrometry (GC-MS)

This part of the study was carried out in Department of Chemistry, The University of Alabama in Huntsville, USA. The composition of some volatile oils is described as follows:

Chemical analysis

Volatile oils were analyzed by Gas chromatographic-mass spectral (GC-MS) method using Agilent 6890 GC with Agilent 5973 mass selective detector [MSD, operated in the EI mode (electron energy = 70 eV), scan range = 45-400 amu, and scan rate = 3.99 scans/sec], and Agilent ChemStation data system. The GC column was HP-5ms fused silica capillary with (5% phenyl)-polymethyl siloxane stationary phase, film thickness of 0.25 µm, length of 30 m, and an internal diameter of 0.25 mm. The carrier gas was helium with column head pressure of 48.7 kPa and flow rate of 1.0 mL/min. Inlet temperature was 200°C and interface temperature was 280°C. The GC oven temperature program was used as follows: 40°C initial temperature, hold for 10 min; increased at 3°C/min to 200°C; increased 2°/min to 220°C. A 1%, w/v, solution of the sample in $CH_2 Cl_2$ was prepared and 1µL was injected using a splitless injection technique. Identification of the oil components was based on their retention indices determined by reference to a homologous series of n-alkanes, and by comparison of their mass spectral fragmentation patterns with those reported in the literature (Adams, 2007) and stored on the MS library [NIST database (G1036A, revision D.01.00) /ChemStation data system (G1701CA, version C.00.01.080]. The percentage of each component is reported as raw percentage based on total ion current without standardization.

Antibacterial activity of some of volatile oils

Screening of volatile oils for antibacterial activities were done by the disk diffusion method (Iqbal *et al.,* 2016; Mohamed *et al.,* 2020), which is normally used as a preliminary check and to select between efficient volatile oils. It was performed using 18 h cultivation at 37°C in 10 ml of Mueller Hinton Agar. The cultures were adjusted to approximately 10^6 CFU/ml with sterile saline solution. One hundred microliters of the suspensions were spread over the plates containing Mueller-Hinton agar using a sterile cotton swab in order to get a uniform microbial growth on both control and test plates. Paper disks (with diameter of 5mm) were then made in the agar. Volatile oil (30 µl) was introduced into each of the disks in appropriately labeled petri dish using a sterile micropipette.

Gentamicin (10µg/ mg) was used as positive control (El-Malti *et al.,* 2007). The zone of inhibition was taken to be the diameter of the zone visibly showing the absence of growth including the 5 mm disk. If there was no inhibition the value of 0 mm was assigned to the test sample (Iqbal *et al.,* 2015; Michael *et al.*, 2006; Saleem *et al.,* 2020).

The percentage of inhibition was calculated as follows:

$$\text{Inhibition (\%)} = \frac{\text{Growth diameterof the sample} - \text{Control growth diameter}}{\text{Control growth diameter}} \times 100$$

The zone of inhibition of the tested microorganisms by the volatile oil extracts were measured using HiAntibiotic Zone Scale - ^{TM}C reader model PW297 (India) (Shittu *et al.,* 2007).

RESULTS

Our results showed that *Salmonella* isolates were found in 40 (67%) poultry gizzard samples while 20 (33%) samples were negative (Figure 1).

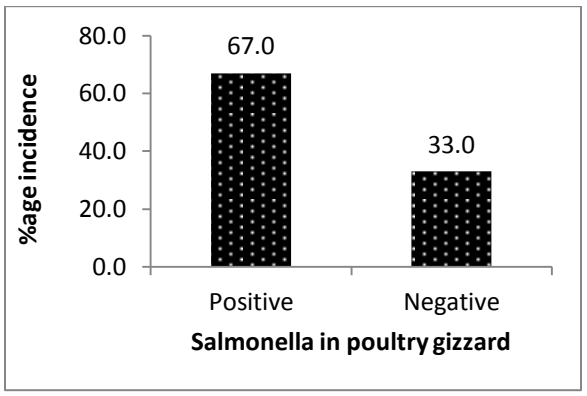


Fig. 1. Distribution of Salmonella in pus swab samples of poultry gizzard according to culture results.

Our results showed distribution of *Salmonella* isolates among four cities (Table 1)*, Salmonella* isolates in poultry gizzard samples from Amran were 14 (70%), while 6 (30%) samples showed negative culture. *Salmonella* isolates in poultry gizzard samples from Hiziaz were 12 (60%), while 8 (40%) samples showed negative culture. *Salmonella* isolates in poultry gizzard samples from Thamar were 10 (100%). *Salmonella* isolates in poultry gizzard samples from Alhudaidah were 4 (40%), while 6 (60%) samples showed negative culture. *Salmonella* isolated in Thamar 10 (100%) and *Salmonella* isolated in Alhudaidah was 4 (40%) while 6 (60%) showed negative culture.

Table 1. Distribution of samples of poultry gizzard among four cities.

Location of specimens	Positive culture		Negative culture		Total	
	No	%	No	%	No	%
Amran	14	70	6	30	20	100
Hiziaz	12	60	8	40	20	100
Thamar	10	100	0	0	10	100
Alhudaidah	4	40	6	60	10	100
Total	40	67	20	33	60	100

Our results showed that most of *Salmonella* were isolated from Amran 14 (35%) and least number of *Salmonella* isolates was from Alhudaidah 4 (10%) (Table 2).

Table 2. Percentage of Salmonella isolated according to city.

Name of city	Number of gizzard	Salmonella isolates	
		No	%
Amran	20	14	35
Hiziaz	20	12	30
Thamar	10	10	25
Alhudaidah	10	4	10

Our study showed that volatile oils of *Origanum majorana*, *Nepeta deflersiana* and *Mentha x piperita* had antibacterial activities against *Salmonella* spp. as 15mm, 11mm and 9 mm inhibition zone, respectively (Plate 1,2 and 3). It was found that *Salmonella* isolated were resistant against volatile oils such as *C. Sativum, E. Cardamomum, T. Permum* and *A. Judaica Sia*.

The main chemical constituents of volatile oils

Nepeta deflersiana

The results showed that the volatile oil obtained from *N. deflersiana* contains sixteen identified and three unidentified constituents (Table 3).

***Origanum majorana*.**

The results presented four identified compounds in the essential oil obtained from *O. majorana* (Table 4).

Mentha x piperita

The results presented two constituents, Limonene and 1,8-Cineole, in the volatile oil from *M. x piperita* (Table 5).

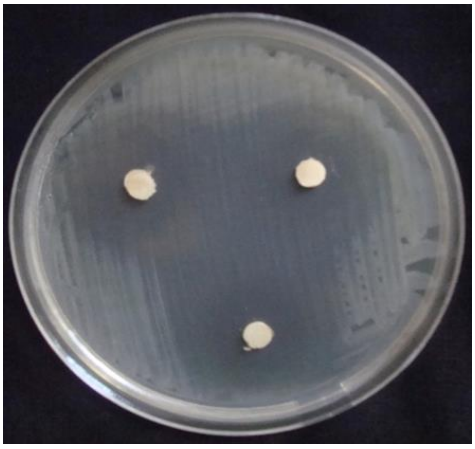


Plate 1. The antibacterial activity of *O. majorana* volatile oil against growth *Salmonella* at 30µl oil.

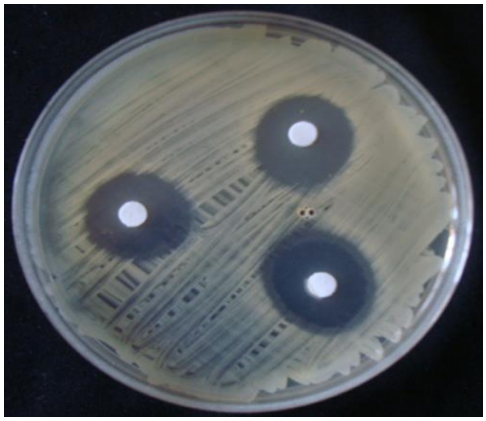


Plate 2. The antibacterial activity of *N. deflersiana* volatile oil against growth of *Salmonella* at 30µl oil.

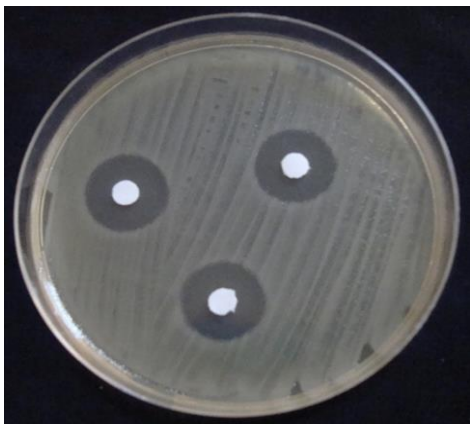


Plate 3. The antibacterial activity of *M. x piperita* volatile oil against growth of *Salmonella* at 30µl oil.

Table 3. The main chemical constituents of *Nepeta deflersiana* volatile oil.

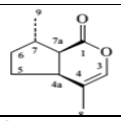
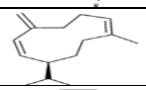
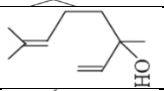
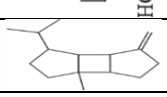
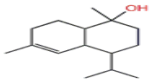
Constituents	%	Chemical structure
4aα,7α,7aα-Nepetalactone	77.7	
Germacrene D	6.0	
Linalool	2.4	
β-Bourbonene	1.3	
α-Cadinol	1.3	

Table 4. The major chemical compounds of *Origanum majorana* essential oil.

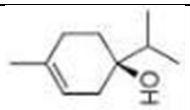
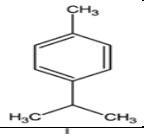
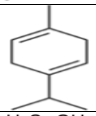
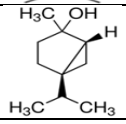
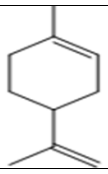
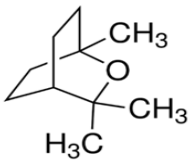
Constituents	%	Chemical structure
Terpinen-4-ol	35.2	
p-Cymene	9.8	
γ-Terpinene	7.7	
trans-Sabinene hydrate	6.8	

Table 5. The main chemical constituents of *Mentha x piperita* volatile oil.

Constituents	%	Chemical structure
Limonene	7.9	
1,8-Cineole	4.8	

DISCUSSION

Our results documented *Salmonella* isolates among 40 (67%) poultry gizzard samples while negative samples were 20 (33%). This data is similar to (Abdellah *et al.,* 2008).

Salmonella isolates were 40 maybe because of the poor health care in Quality Control and Standards, lack of cleanness in poultry farms, no care of poultry feeding, no continue test for poultry, and not removing birds with infected gizzard. The highest rate of infection was in Amran city maybe because unsuitable environmental conditions and poor health care. The lowest rate of infection was in Alhudaidah maybe because of suitable environmental conditions, availability of health care.

The incidence of Salmonella spp. In poultry has been reported in many studies (Abdelkader *et al.,* 2019; El-Aziz, 2013; Moawad *et al.,* 2017; Raji *et al.,* 2021). Tibaijuka et al. (2003) reported that the contamination level of Salmonella was higher in chicken giblets as compared to chicken meat.

The data in this investigation showed that the major constituents of *N. deflersiana* were 4aα, 7α, 7aα-Nepetalactone (77.7%). The major constituents of *O. majorana* volatile oils were terpinen-4-ol (35.2%). Our results differ to (Badee *et al.,* 2013) who showed that major constituents *O. majorana* were γ-terpinene (23.20%), α-terpinene (19.71%). Our results disagree with Cetin et al. (2011) who found that the major components of *Origanum* were carvacrol (47.5%) followed by p-cymene (22.2%).

The major constituent of *M. x piperita* oil was limonene (7.9%). Contrary it's different to the constituents of volatile oil of *M. piperita* leaves studied in Morocco by Derwich et al. (2011) where they found the main constituents in the leaves were: Menthone (29.01%) followed by menthol (5.58%). Our results disagree with the study by Dzamic et al. (2010), who found that major constituents in volatile oil of *Mentha longifolia* were trans- and cis- dihydrocarvone (23.64%) followed, by piperitone (17.33%).

In this investigation *O. majorana*, *N. delflersiana* and *Mentha x piperita* volatile oils had antimicrobial activities against growth of *Salmonella spp.* due to the presence of 4aα,7α,7aα-Nepetalactone (77.7%), Terpinen-4-ol (35.2%) and Limonene (8.9%) respectively. This result is in agreement with Tawfeeq and El-Moez (2014) and Moo-Young and Tae-Jin (2008) who found that *Salmonella* strains were the major sensitive isolates tested to the antibacterial activities of *O. marjoram* volatile oil. Similar results were observed by Lee et al. (2018) who found antibacterial activity of *N. delflersiana* volatile oil against the growth of *Salmonella spp.*

CONCLUSION

Results in this study showed that Salmonella cause contamination of chicken gizzard which needs obligate hygienic control to prevent transmission of Salmonella to human.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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