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Salmonellas Antibiotic Sensibility and Resistance; The Sensitivity to Herb Extracts and New Synthesize Compounds

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1. Introduction

Although the use of antibiotics in the developed countries is done under certain principles, there is an opposite situation at the use of antibiotics in the underdeveloped countries which is irregular. The prevalence of antibiotic use in the fight against infectious diseases additionally raises the problem of increasing resistance of the micro-organisms against antibiotics. There is also an increase in the resistance of antibiotics which is used against *Salmonella*. The newly synthesized chemical compounds and extracts derived from plants which are alternative to existing antibiotics determining the sensitivity to *Salmonella*, plays an important role for increasing the options of alternative antibiotics.

1.1 Appearance and staining characteristics

Salmonella bacteria are asporogenic, capsule-free, motile via peritrichous cilium (*Salmonella gallinarium* or *Salmonella pulorum* are immotile), rod-shaped bacteria with an approximate length of 2,0-5,0 μm, width of 0,7-1,5 μm. They are stained well with bacteriologic stains and they are gram-negative (Picture 1). Most of them have type 1 (mannose sensitive (ms), hemagglutinating); S. Gallinarium and some origins have type 2 fimbriae. S. paratyphi As do not have fimbriae.

1.2 Reproduction and biochemical characteristics

Salmonella bacteria reproduce in many ordinary mediums. They are aerobe and facultative anaerobe. Their reproduction temperature limit is very wide even they reproduce at 37° C best. (20°C- 42°C). This is extremely important for reproduction of *Salmonellas* which cause food intoxication at room temperature. They like to produce at average pH of 7,2. They make homogenous turbidity in bouillon and similar liquid medium. They make round, slab sided, mostly tumescent colonies with a diameter of 2-3 mm, regular surface. In colonies of various *Salmonellas*, some differences may exist in terms of size, protuberance, surface and side. *Salmonella typhi* may also make gnome colonies which may reach to 0,2-0,3 mm diameter within the first 24 hours. Biochemical characteristics of bacteria which are obtained

from these colonies are same as normal colonies; and they are agglutinated with O serums only antigenically and they differ from bacteria in S colonies in terms of not reacting with anti H, anti Vi serums. If they are reproduced in mediums including sulfurous compounds, sulfates and tiosulfates which may be assimilated, normal colonies occur from bacteria that make gnome colonies.

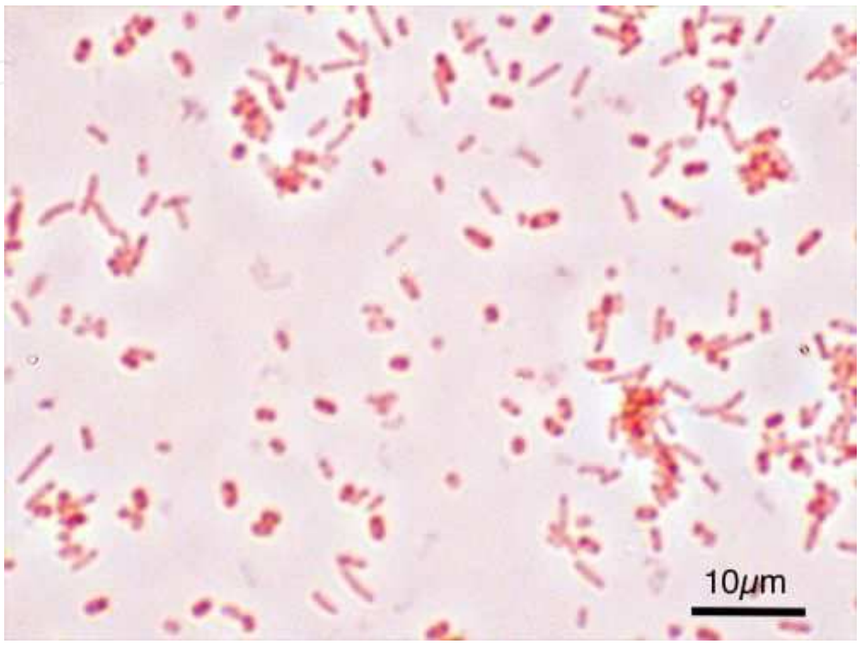

Fig. 1. Microscopic View of *Salmonella*

Some of *Salmonellas*, S. Schottemuelleri (s. paratyphi) in particular and some others form M colonies in appropriate mediums. It is detected that these bacteria have M antigens and agglutination is prevented by anti O and anti H serums. Furthermore, R colonies are formed by *Salmonella* which reproduce in inappropriate mediums (Picture 2).

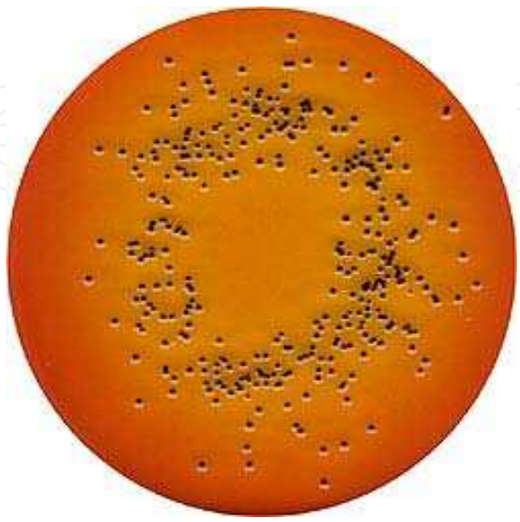

Fig. 2. *Salmonella* colonies

Salmonellas are not effective on lactose. This characteristics is important in first differentiation from Escherichias. As these bacteria which are planted in a separator plaque medium (endo, EMB) including lactose and an appropriate reagent are not effective on lactose, they make colorless colonies; however those effective on lactose make dark red, black, greenish bright colonies (Picture 3).

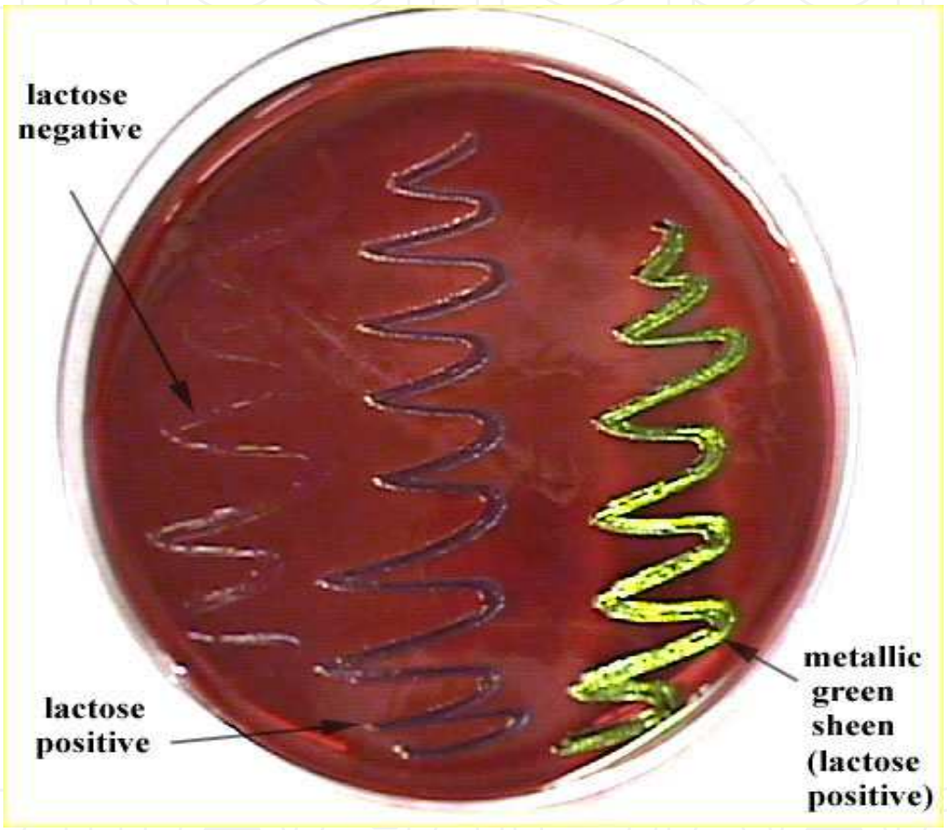

Fig. 3. View of *Salmonella* and Lactose Positive Colonies

Salmonellas do not effect on sucrose, adonitole and salicin in usual other than lactose. They digest glucose, mannite and maltose by producing acid and gas except *Salmonella typhi* and S. gallinarum; and *Salmonella typhi* and gallinarum digest them by producind acid only. They produce H2S in general (except S. paratyphi A); they are indole negative, methyl red positive, Vogesproskauter negative and they reproduce in citrated mediums (Simmon), they do not digest urea. They could not be produced in KCN (potassium cyanide 0,5%) mediums. ONPG (orthonitro phenyl galactopyranoside) assay is negative. (They do not have beta galactosidase enzymes that may digest lactose). This assay is positive in Arizona. Biochemical characteristics of *Salmonellas* were shown in Table 1.

Motility	+
Indole	-
H2S	+
Oxidase	-
Urease	-
Nitrate reduction	+
Citrate Utilization	+
MR	+
VP	-
Lysine decarboxilation	+
Ornithine decarboxilation	+
Phenylalanine deamination	-
Malonate Utilization	-
Lactose	-
Sucrose	-
Salicine	-
Inositol	-
Amygdalin	-
Gas Production from glucose	+
β-galaktosidase (ONPG Test)	-
Reproduction in KCN	-
	(-) Negative (+) Positive

Table 1. Biochemical characteristics of *Salmonellas*

2. Resistence in *Salmonellas*

Salmonella bacteria are resistless to heat. They die at 55°C in 20 minutes. They do not have resistance to dryness. But they may stay alive in humid environments away from daylight, sewages, well water and soil for a long time. They are very resistant to cold. Staying alive in cold food and drinks has an epidemiological importance. They may stay alive in liyophilized situations for years.

Antiseptics effect rapidly providing the direct contact. Chlorine within normal concentrations kill *Salmonellas* in the water. However, effect of these agents to *Salmonellas* in stool particles and other organic substances lower.

To differ them from other bacteria in terms of accompanying coliform and intestinal settlement habits, their status against various chemical agents and stains were examined and consequently special cases of *Salmonellas* appeared against some of them.

Malachite green, a stain kills E.coli or slower their reproduction within suitable densities, however it does not effect S. typhi. Similarly, while paratyphoid bacillus are very resistant and typhoid bacillus is quite resistant to Brillant green stain, dysentery bacillus and coliforms are very sensitive. Although lithium chloride inhibits E.coli similarly, it s inefficient to *Salmonella typhi*. Differently, sodium tetrathionate increases Slamonella reproduction although it is noneffective to coliforms.

To preserve *Salmonellas*, after making immersing culture to a vertical agar including 1% agar and 2% Na H PO_4 $12H_2O$ and reproduction is provided, they may stay alive in tightly closed agar in the dark for months. Furthermore, *Salmonellas* may be lyophilized.

Although sodium deoxycholate reduces reproduction of coliforms, they do not effect reproduction of *Salmonellas* and Shigellas.

Resistance against chemotherapeutics in *Salmonellas* has appeared quite lately. Although an important resistance has not been observed in *Salmonellas* against antimicrobic agents such as chloramphenicol, tetracyclines, kanamycin, ampicilin, streptomycin, sulfonamids until 1960, increasingly resistant strains against tetracyclins, ampicilin, streptomycin, sulphonamids have been detected from that day to this. Resistance to chloramphenicol develops slower than others. Resistance depends on plasmids.

3. Antibiotic resistance in microorganisms

Microorganisms may become resistant to antibiotics that they are in effect spectrum in time due to any reason. Antibiotic resistance in bacteria is called as inability to treat the infection disease against treatment doses of antibiotics that are in effect spectrum of microorganisms naturally and used routinely.

There are various predictions recently about where antibiotic resistance genes which are thought to be appeared after wide usage of antibiotics in medicine come from. According to an opinion, resistance genes has developed as a protection mechanism in bacteria species that produce antibiotic first. Resistance genes in these species are in the same group with antibiotic production genes. Another possibility is that antibiotic resistance genes has developed from normal genes present in the microorganism. However, possibility of development of antibiotic resistance due to spontaneous mutation is very low. (10^{-5}-10^{-10}).

3.1 Resistance problem against antibiotics

Antibiotics which has been started to be used within last 50-60 years provided the most important contribution in human life and enabled treatment of many infectious diseases successfully. Antibiotics which are one of the most important inventions of humanity lost their effects significantly because of resistance developed after inappropriate and unnecessary usage. Microorganisms gain a sustaining power, namely resistance eventually against antimicrobic agents which are used to destroy themselves. The resistance developed against antimicrobic agents is a very important problem which will threat all humanity today. Hospital infections which develop by resistant origins against many drugs mainly in hospitals increase hospitalization and death rates and cause more additional cost. Today, resistance not only in hospital sources, but also in sources acquired from the society increases significantly and this case augments the problem and carries it to serious levels.

Resistance may also develop to other antimicrobis which are close in terms of structure or effect form to a chemotherapeutic agent in a microorganism species which has become resistant to such antimicrobic agent; and this condition is called as cross resistance. The condition that a microorganism becomes resistant to many antimicrobic agents with different structure and effect is called as multiple-drug resistance.

3.2 Natural (intrinsic) resistance

It is a resistance type without hereditary characteristic. This means becoming resistant of a microorganism due to its structure. Absence of the target molecule that the antimicrobic agent is effective by binding is responsible from the natural resistance in general. Any origin of the resistant species to an antimicrobic agent is not effected from such antibiotic.

Many gram negative bacteria show resistance to vancomycine and methicillin and enterococcus show resistance to cephalosporin because of their cell wall structure. As passage of aminoglycosides into cell membrane is an oxygen dependent, energy requiring case, aminoglycosides are not effective on anaerobes since sufficient drug can not enter into the cell in obligate anaerobe bacteria that oxidative phosphorylation is not present.

3.3 Acquired (hereditary) resistance

It is the resistance type acquired. Here, the drug is effective when bacteria population contacts the antimicrobic agents first; however, resistance develops against the antimicrobic agent in the microorganism population during the contact period or repeated treatments. The resistance developed against antimicrobics occurs by this manner and resistant origins appear and diffuse by selection after genetic change. Genetic resistance is under control of chromosome, plasmide, transposon. Microorganisms become resistant against antimicrobics by using one or more resistance mechanisms.

4. Mutation

Changes which has been occurred in alignment or structure of nucleotide pairs forming gene structure in DNA and modified protein structure coded and function as well are called as mutation. As a result of mutations, mutants which show resistance to various drugs, disinfecting agents, chemotherapeutics, inhibitors, ultraviolet beams, phages and such agents may appear.

5. Genetic material transfer between bacteria

In bacteria, genetic material may be transferred into another bacteria partially and three basic mechanisms play role in genetic material transfer after these transfers.

5.1 Transformation

It is recombination of free DNA fragment which was left into the environment by the donor bacteria without any mediator (another bacteria or bacteriophage) in the environment with the receiver's own genetic elements. In another words; if a microorganism is produced in an environment including genetic material (DNa fragments) of another microorganism which is very close in terms of DNA composition and planting from this liquid medium into a solid medium after a certain period, it is seen that some colonies have different morphologies and genetic material of them are similar to original colonies of dead microorganisms which give the genetic material.

Some specific characters has been able to be transferred to receiver bacteria by transformation. These include lactose and galactose positive genes, resistance against

antibacterial substances, virulence etc. These factors are transferred into mRNA by transcription after combination with donor DNA and it is transloced from here and causes appearance of new characters in the cell. Although transformation is not successful among enterobacters, it is reported recently that transformation was detected in high calcium ion concentrations in Escherichia coli.

5.2 Transduction

Transfer of genetic material from a donor bacteria into a receiver bacteria via bacteriophages is called as transduction. Gen transfer via transduction is detected in Gram negative (*Salmonella*, E.coli, Shigella, Proteus, Vibrio, P. Aeruginosa etc.) and Gram positive (staphylococcus and bacillus) microorganisms.

5.3 Conjugation

Conjugation is a method of gene transfer that genetic material transfer realized as a result of physical relation of donor cell with the receiver cell. For realization of conjugation between two cells, cells should contact with each other. Generally, contact occurs via sex pilus which are synthesized by special genes in sex factors in the cell. These are longer and thicker tan other normal pilus (fimbria). They serve as a pipe or passage bridge as they are hollow. Genetic material passes from here and is transmitted into the receiver. Normal pilus do not have roles on gene transmission.

Another way in conjugation is direct contact. In this way, membranes of two bacteria combine and form a pore on the combination point. And DNA transmission occurs via this pore. Receiver population is very wide in conjugation and it may occur between different species and genus.

There are two types of conjugative structures consisting of chromosomal elements known as conjugative transposones and plasmides.

5.3.1 Conjugative transposons

Conjugative tronsposons are genetic elements of a bacteria which may replace from chromosome of a bacteria to its plasmid or to a chromosome or plasmids. They show similar characteristics with plasmids and bacteriophages. The difference from plasmids is inability to replicate by themselves. These structures are in Gram positive bacteria and Bacteroides species and cause diffusion of antibiotic resistance genes among various bacteria groups. Conjugative transposons form a circular intermediate form by binding covalently after leaving from bacterial DNA. This intermediate form may be transmitted to another region in the same cell or to another cell and be bounded to genomes or plasmids of the receiver cell.

5.3.2 Plasmids

Genes that make bacteria resistant against antimicrobial agents may be present on chromosomes of bacteria as well as they are carried on small DNA fragments called as plasmid. The term plasmid was first used by Lederberg to define all extrachromosomal hereditary elements. Today, this phrase was limited by extrachromosomal DNA which replicates independent from chromosome. Plasmids are circular extrachromosomal DNA

molecules with fibril pairs and they code various activities which are not required for aliveness of the bacteria in natural environments and conditions.

The most common antibiotic resistance in bacteria caused by plasmid. Genes that control antibiotic resistance of bacteria exist on R (resistance) plasmids and these plasmids cause spreading of the resistance by being transmitted to other bacteria. Existence of resistance genes on plasmids and their ability to be transmitted showed that plasmids are basic vectors for spresding of resistance genes among bacteria populations. Plasmids on bacteria of enteobacteriacea family are related with transmission of various genetic characteristics such as drug resistance, hemolysin, enterotoxin and co-lysin production, tolerance to heavy metals, resistance to ultraviolet beams, carbohydrate fermentation and H2S production.

Plasmids acquire their multiple drug genes via 2 paths.

1. To form plasmids that show multiple drug resistance via subsequent transposon insertion (This path is not used by many plasmids).
2. To acquire multiple drug resistance genes by receiving linear DNA fragments that may be inserted into DNA like transposons called as integrons.

Integrons are integrated only to a single point differently from transposons and they do not code transposase. Integrase enzymes are coded by plasmids that they are integrated. Many integrons carry promoter-free antibiotic resistance genes and are integrated to plasmids specific to direction. Many resistance gene may be present consecutively on a plasmid.

6. Resistance mechanisms of bacteria against antibiotics

Various resistance mechanisms has developed in bacteria which are coded by any of abovementioned paths against antibiotics which are compounds with low molecular weight and suppress bacteria reproduction or kill them.

6.1 Resistance dependent on external membrane in gram negative bacteria

Target regions of beta lactam antibiotics are on the outer surface of the cytoplasmic membrane and targets of many other antibiotics are in the cell. Therefore, all antibiotics should pass the external membrane barrier to reach to target regions in gram negative bacteria. Passage from the external membrane is via pores. Requirement of antibiotics diffusing from pores gives a minor resistance to all Gram negative bacteria (5 to 10 times) and mutations that may occur in pores may increase this resistance ratio. Some of external membrane pores are specific and some of them are non-specific pores. Mutations on non-specific pores may provide resistance to more than one antibiotic type.

6.2 Enzymatic inactivation of antibiotics

An important resistance mechanism especially in gram negative bacteria against beta-lactam antibiotics is beta-lactamase. The beta-lactamase enzyme which is present on some microorganisms (Staphylococcus aureus, E. coli, *Salmonella* spp., Shigella spp., etc.) and is coded by R-plasmids hydrolyzes C-N bound in beta-lactam bond in the structure of antibiotics such as penicillin, cephalosporin, ampicillin, cloxacilin etc. and inactivates antibiotics (Arda, 2000). Beta lactamases are released into periplasmic space in Gran

negative bacteria and into extracellular environment in Gram positive bacteria. Since external membrane pores limits antibiotic passage and beta lactamases are released into periplasmic space, resistance of gram negative bacteria can be obtained with a lower enzyme level than gram positive bacteria.

The most important mechanism of the resistance against aminoglycosides is enzymatic inactivation of the antibiotic. Aminoglycoside modifying enzymes inactivates antibiotic by addition of a group such as phosphoryl, adenyl or acetyl. These enzymes are on the outer side of the cytoplasmic membrane in gram negative bacteria. Therefore, a decrease occurs both in passage of antibiotics from cytoplasmic membrane and in their power to inhibit protein synthesis. Although an enzyme which inactivates tetracycline in aerobe conditions recently, it was understood that this enzyme is not effective clinically and in terms of total resistance of tetracycline.

6.3 Active ejaculation of antibiotics

Ejaculation type resistance is first observed in tetracycline. A cytoplasmic membrane protein catalyzes energy dependent ejaculation of bacteria of tetracycline. Genes that code this protein are both in Gram negative (tet A- tet G) and in Gram positive bacteria (tet K - Tet L). This type of resistance was detected in staphylococcus species for macrolide antibiotics. Another ejaculation system with a low efficiency was also found for fluoroquinolones.

6.4 Modification of target areas of antibiotics

The resistance mechanism which plays role in the resistance against beta lactam antibiotics is changing specific binding regions of penicillin binding proteins (PBP) which are the target region of the antibiotic. This type of resistance is common among Gram positive bacteria. The "mec" gene which gives methicillin resistance is the best defined gene among these resistance genes. Furthermore, this type of resistance was also detected against glycopeptides, tetracyclines, macrolides, lincosamide, quinolon, rifampicin, trimethoprim and sulphonamides.

7. Antibiotic resistance conditions of *Salmonella* origins

Resistance against chemotherapeutics in *Salmonellas* has appeared quite lately. While no resistance has been observed in *Salmonellas* against chloramphenicol, kanamycine, ampicillin, streptomycin and sulphonamides until 1960, an increasing resistance has been developed against tetracyclins in particular, ampicillin, streptomycin and sulphınamides since 1960s. However, the most effective chemotherapeutic agents on *Salmonella* species are chloramphenicol, tetracyclines, ampicillin and gentamicin.

S. typhi origins were sensitive to all antibiotics including chloramphenicol in particular until 1970s. After a wide epidemia created by chloramphenicol resistant S. typhi origins in 1972, these resistant sources were found in many countries mostly in India, Mexico, Thailand and Vietnam.

First trimethoprim resistance for S. typhimurium has been detected in 1973 and then it is reported in 7% of human sources. No trimethoprim resistance was detected in S. typhi until 1980. During past 20 years, antibiotic resistance and multiple drug resistance has increased

in *Salmonella* species. Resistance cases were reported in non-typhoidal *Salmonellas* in many countries in South America, Middle-east and South Asia. *Salmonella* sources that show aggressive multiple drug resistance is a big problem in many countries. Antibiotic resistance is commonly under control of plasmids in *Salmonella* sources. Plasmids are gained as a result of antibiotic pressure which is used common in feeds of livestock and in medicine and in veterinary. Resistance plasmids gains resistance genes from other plasmids in the same source or plasmids that is carried by other bacteria origins involved in chromosome or host organism. Resistance may also develop spontaneous mutation of a chromosomal gene as a response to selective antibiotic pressure.

8. Determination of antimicrobial characteristics of essential oils and methods used

Herbal extracts and essential oils have been used for long years for different purposes. However, their use for wider purposes by utilizing from their different features and studies related with them are continued rapidly. The most emphasized subject is antimicrobial characteristics. By using these characteristics, essential oils are started to be used in protection of raw and processed food, as additives in modern drugs and in natural treatments. There are many articles published associated with research of antimicrobial characteristics of essential oils and herbal extracts. In these studies, a kind of essential oil is studies by targeting against a pathogen microorganism.

This information is usually useful, however every study has procedural differences. Antimicrobial test methods used differ from each other. Furthermore, there are differences between selected oils or plants that they were extracted in terms of place where they are picked up and extraction methods. It is more likely that study results may differ due to these factors.

Until 1960s, many methods for drug, especially antibiotic sensitivity tests of microorganisms and many different modifications of these methods were reported. Superiority and area of use of every methods are limited. To interpret results with a highest level, all characteristics of the method should be understood well. There are two basic methods that are used to detect antibiotic sensitivity of bacteria. These methods are “Titration (Dilution) Methods” that antibiotics interact with microorganisms after serial dilution and "Diffusion Methods" which are performed by placing a test substance impregnated paper disc to medium surface after planting the culture to be tested.

Essential oils have some characteristics such as volatility, hydrophobicity and having special odors activating in respiratory system. Essential oils are heterogenous mixtures that organic substances are present as a mixture. These final characteristics reveal that especially odorous oils may be biologically active. In fact, essential oils have various pharmacological activities. The most reported characteristic is antimicrobial effect. Tests that reveal such characteristics does not depend on a certain standardization and they may be performed in any laboratory randomly. Techniques used are generally agar diffusion and dilution methods.

Dilution techniques were developed to detect sensitivity of a microorganism to antibiotics. However, they are also used to determine antimicrobial characteristics in plant extracts or essential oils. It depends on serial dilution of antimicrobial agent and inoculating bacteria culture. After incubation, the effect of antimicrobial agent tested with which concentration

against the microorganisms is determined according to presence and absence of reproduction. Presence and absence of reproduction is performed via turbidity detection and low final concentration value that no reproduction occurs is defined as Minimum Inhibiting Concentration(MIC) value. This technique is macro-both dilution technique which has been performed in standard assay tubes for a long time. Recently, a method that acts with the principle of this method but requires very less medium and test material was started to be used in testing synthetic and natural antimicrobial agents excepts antibiotics. The method which is more advantageous mostly than other diffusion techniques and reveal MIC value accurately is micro tube dilution or microbroth dilution methods. In this methods, plates including 80, 96 or more pits which were developed commercially are used. Material dilutions are prepared in these pit series and agent and the microorganism are activated by adding less culture. After the incubation, absence and presence of reproduction by turbidity determination. Turbidity detection procedure may be performed by simply observation or by using special turbidity readers. Although this method is mostly used for antibiotics, they are also used for plant extracts and essential oils. The most important advantage is to perform the assay with 10 to 25 µl. Because it is very hard to obtain essential oils plenty. Another advantage is to allow testing many agents at the same time.

Another method used in antimicrobial tests is agar diffusion method. This technique is commonly preferred due to easy testing of essential oils. Agar diffusion technique has been used to determine antimicrobial characteristics of various agents since early 1940s. Qualitative and quantitative information may be revealed by this method. In agar diffusion technique, an appropriate medium including test organism is used by a hole system including the substance to be tested. The essential oil which was dissolved homogenously is put on medium with certain volumes. Holes contact with the medium. By this method, sometimes essential oil impregnated paper discs are used instead of making a hole on the medium. Consequently, essential oil is diffused to previously microorganism vaccinated medium from holes or paper discs. Structural characteristics of the agent used may be effective on diffusing percentage or period and may effect test results. If the agent used is effective at the end of incubation period, significant, inhibition zones occur around holes indicating no reproduction. Oil quantity applied and diameter of the disc or hole used are important parameters in this method. Because, diameters of inhibition zones occurred at the end of incubation are under the control of these parameters. Thickness of the medium that the hole is opened effects the diameter of inhibition zone. A certain period should pass for formation of inhibition zone. This period is called as "critical period" (T erit). Before this period, inhibition zones may not become significant or when incubation is performed over this period, occurred zones start to disappear. Furthermore, density of inoculum used should be certain and fixed. Because, an agent which be effective normally will be seemed as ineffective due to microorganism concentration and will not form an inhibition zone or will not be within real sizes. Therefore, inoculum concentration should be hold in critical level. If density of microorganism is at the required level, duration of the incubation period is not very important. Diameters of inhibition zones formed is measured via a scale and recorded. Increasing or decreasing concentrations of the agent is put into the pits and increase or decrease in diameters of zones proportionally are expected. However, it is reported that there is a definite parallelism between zone diameter values obtained via agar diffusion method and corresponding agent concentrations and real MIC values, but zone diameters obtained are not compatible with MIC values.

Another method which has been started to be used frequently to determine microbiological activity of essential oils recently is bioautography. Bioautography method is very easy and correct results giving method to try plant extracts or pure substances against botanic and human pathogens. In this method, it is revealed that which of organic compounds forming the essential oil is responsible from the activity as well as antibacterial characteristics of the essential oil. This method bases on principles of agar diffusion technique. However, it may differ in terms of application of the substance to be tested and evaluation of results. The biggest difference is that thin layer chromatography (TLC) is used in the method and the essential oil is activated by test microorganisms after application to TLC plates. By the help of TLC technique, compounds in the essential oil are removed roughly and the compound which is responsible from the activity is revealed. Test substance is applied to both TLC plates in the method and one of plates are accepted as reference plate. The other is the plate that microorganisms are applied. Fractions are marked by making the reference plate colored with reagents or examining under 254 or 336 nm UV light. After incubation of the plate used in the assay, the inhibition zone presence is determined by such substance and Rf value of that substance is calculated. Rf value (Retention Factor) is found by calculation the ratio of the distance that such substance has moved on the plate to the distance that dissolving agent has moved. Referans olarak saklanan İTK plağındaki maddeler ile inhibisyon zonlarının oluşturduğu maddelerin R_f değerleri karşılaştırılarak zonu oluşturan madde işaretlenmekte ve bu aşamadan sonra zonu oluşturan madde çeşitli yöntemlerle referans plaktan izole edilerek tayin yoluna gidilmektedir. In fact, bioautography method is suitable to reveal compounds with high antimicrobial activity such as antibiotics. It reveals the most active components among plant extracts or similar organic compounds. Three bioautography was reported since today. These are;

a. Direct bioautography method that microorganism is directly placed on TLC plate,
b. Contact biouatography method that the substance moved on TLC plate is isolated and transferred into inoculated medium by the microorganism.
c. Immersion bioautography or "Agar-overlay biouautography" that is performed by pouring the medium which was inoculated by a certain microorganism on TLC plate.

The last method is formed by combining direct bioautography and contact bioautography. Direct biouatography is especially used for bacteria and spore producing fungus. This method is quite sensitive and very net inhibition zones may be observed at the end of the assay. However, disadvantage of this method is difficulty of development of microorganisms on TLC plate. This problem is not present in contact biouatography method, but isolation and transfer of the substance from TLC plate cause some problems. Bigger than required inhibition zones appear and this causes difficulty in discrimination between active components. It is reported that immersion bioautography which is the combination of both methods are generally used for yeasts and bacteria. By pouring certain medium on TLC plate, active substances are tested in place and no reproduction problem occurs because sufficient medium is used. The problem of this technique is different diffusing coefficients of active substances. As a solution, a softer medium is obtained by decreasing the agar amount on the medium which is poured on TLC plate and diffusions of components on the plate into inoculated agar is facilitated. Whether which of these three methods is used, tetrazolium salts are used in general to provide determination of inhibition zones that are expected to form after incubation or to visualize. These reactive substances provide microorganisms to be colored purple and formation of colorless inhibition zones are provided at backstage.

There are various methods for determination of antimicrobial effects of volatile substances, especially essential oils in steam phase. The most applicable method is micro-atmosphere method. It is reported that this method is more appropriate for filament fungus in general. Petri plates with a size of 120 mm including medium is used in the method. Fungal spores which are prepared in sterile distilled water are inoculated on the centre of the medium as final concentration to be 10^4 spore/spot. The oil to be tested is impregnated purely with different amounts to the filter paper which has the same size with the petri plate. Sole microorganism is inoculated to one of the plates to use as control, no test substance is applied. Separate petri plates are used to try different quantities of the essential oil. Prepared filter paper is placed into the cap of the petri and petri plate is closed and incubated in a reverse position for 2 to 12 days. Plates are controlled during this 12 days and development status of inoculated microorganisms are controlled. Usability of essential oils in elimination or disarming of fungal or bacterial load in the air has been searched. So, it has been stated that there would be chance to protect the atmosphere of library, museum, hospital, cinema etc places without damaging to people against to microbial flora through essential oil. Some of researchers observed the inhibition instead of emission the pure essential oil into filter paper to put directly into cover section of petri cup. To prevent lost of essential oil, around of petri cup is covered with parafilm and is let to incubation in converse type. At the end of 3 days incubation period, petri cup is opened and steam of essential oil is released and it is left 3 days more to incubation. This second time applied incubation provides to determine whether antimicrobial affect of essential oil is at bactericidal feature or not. If essential oil steam only inhibited development of microorganism and it has been informed that the microorganisms at the plate will be developed at the end of this period. Some of the researchers searched whether steam pressure has any affect or not through emission the essential oil in various diameter filter papers and applying different quantities. For this purpose, volatile pure materials have been tested and consequently it has been set forth that steam pressure doesn't support steam activity of essential oil.

9. The methods that are used to determine antimicrobial features

9.1 Resuscitation of microorganisms

The bacteria occurring in lyophilize cultures, are extracted from their tubes under aseptic terms and they are transmitted into Nutrient Broth tubes to resuscitate. At the end of incubation period at 37°C for 24 hours, the cultures are planted in sole colony into Mueller Hinton Agar (MHA) plates and then they are left into incubation again. At the end of this period the purity of microorganisms are checked and they are transmitted to 2 ml micro-reaction tubes (eppendorf) including 1,5 ml 15% sterile glycerol solution which was prepared previously. Those tubes are kept in -85°C as to be used later.

9.2 Agar diffusion method

For the Agar diffusion method, first of all 25 ml Mueller Hinton Agar (MHA) has been poured into sterile glass petris in 120 mm diameter and the petris have been left for frozen on a smooth surface. The bacteria that will be used are developed in 5 pursuant to McFarland cloudy gauge at Müller Hinton Broth (MHB). Then 1 ml is taken from bacteria

solution and poured into petri cups in 120 mm diameter that were prepared previously and the bacteria is provided to spread over medium through sterile drigalski spatula. Then those petris are left to dry in sterile cabinet with semi opened cover. Approximately six reservoirs at per petri is opened on drying surface medium through sterile corc-borer with 6 mm diameters. Essential oil are weighted in 1 mg and solved in 1 ml DMSO and double layer serial dilutions are prepared. Essential oil concentration from 1 mg/ml to 15.6 µl is obtained. Those dilutions are pipette in 50 µl into opened holes on mediums. After completing pipette process, the materials are stored in fridge for 30 minutes for diffusion of materials into medium and then they are left into incubation at 37°C for 24-48 hours.

9.3 Microbroth dilution method

The cultures are cultivated in petries which include MHA to regenerate them and incubated for a period of 24 hours after they are taken out from -85°C. After incubation, some colonies are developed in medium and these colonies are transferred to 10ml of tubes that contain Mueller Hinton Broth (MHB) and again incubated at 37°C for a period of 24 hours. After a period of 18-24 hours of incubation, the cultures developed in liquid medium are again transferred to double power of MHB tubes in definite amounts after they are tuned up in haze as per Mc Farland No: 0.5 (approximately 10^8 cfu/ml).

The essential oils to be tested are transferred to 4mg of sterilized flakons and these are dissolved by adding 2ml of sterilized Dimethyl-Sulfoxide(DMSO) in 25% ratio. The essential oils should be fully dissolved in DMSO and a homogenous mixture should be obtained. Beginning from stocked solutions obtained, essential oils in micro-reaction tubes (eppendorf) are diluted with sterile distilled water and a series of combinations (1, 1/2, 1/4, 1/8, ...) are prepared from 2mg/ml to 1.95 µg/ml.

Micro titration petries (Brand) having 96"U" type bores are used for experiment. 100 µl of diluted mixtures are transferred to each bore column series via micropipettes. In addition to the essential oils to be tested, to control the solvents the DMSO and Standard antibiotic chloramphenicol (Sigma) are tested as positive control. After all concentrations are transferred to the bores, the microorganisms are added to them. To do this, the microorganism cultures whose haze is tuned up as per McFarland No:05 before are transferred to the reservoirs that are produced proper to multichannel automatic pipetors and 100 µl of mixture are pipeted to each line of bores as one microorganism is in the same eline. After these procedures, the cover of micro titration petries are closed and they are incubated at 37°C for period of 24 hours. At the end of incubation period, to be able detect the regeneration some TTC salt is sprayed over petries. Later it is left to incubation and coloring process at 37°C for a period of 3 hours. At the end of incubation, the areas that are not colored are the ones that no regeneration is obtained.

9.4 Detection of antimicrobial compounds in essential oils by bio-autography method

9.4.1 Thin layer chromatography (TLC) system

Thin layer chromatography plaques (Aldrich) that are coated with silica gel 60 GF_{254} adsorbent and in 0.2mm of height are used in appropriate sizes over aluminum supports. These plaques are kept in their special protected boxes in room temperature, droughty, and dark places. Pure

essential oils are applied over plaques as 1 µl by using capillary tubes. 9:1 (v/v) proportion Hexane-Ethyl Acetate dissolvent as 20 ml is prepared and filled in a covered rectangular glass container. The TLC plaque where essential oil applied parts are marked is immersed vertically into dissolvent, but one should be careful as the dissolvent not to reach the oily parts. The TLC plaques are developed in this system as twins. While one of the plaques is taken out and kept aside for experiment, the other one is cleaned out from dissolvent by evaporating it and analyzed under 254/364nm wave lengths and UV active spots are marked. To be able to detect the compounds that do not absorb UV, anisaldehyde / H_2SO_4 color reactive is sprayed over the plaque and it is heated at 110°C for a period of 1-3 minutes.

9.4.2 Preparation of microorganisms

A cell suspension of approximately 1.5 x10^9 cfu/ml in MHB is prepared for bio-autography method which contains bacterial culture that is prepared the day before in MHB medium and tuned up in haze as per McFarland No:5.

9.4.3 Assessment of activity

Pre-prepared molten agar that is delivered in 20ml of Erlenmeyer is kept in water-bath and then 2ml of bacterial suspension culture is added into agar and a final concentration of 1.5x10^8 cfu/ml is obtained. TLC plaques which are pre-prepared and not processed with reactive are placed in a petri that is filled with Nutrient Agar. Then molten agar that is kept in hot water-bath and inoculated with microorganisms in a well stirred situation is added into the petri as a thin layer not much than 1mm. These plaques are incubated for a period of 24-48 hours at 37°C. At the end of incubation period, formed inhibition zones and their R_f values of corresponding reference plaque fractions are measured and registered.

9.4.4 Gas chromatography (GC)

The compounds in essential oil are isolated and evaluated under following conditions by taking into account their attachment periods in gas chromatography (R_t) and their relative rates.

GC Analysis Conditions	
System	Shimadzu GC-17A
Column	CPSil 5CB
Carrying Gas	Nitrogen (1ml/min.)
Splitting Rate	50:1
Detector	FID
Temperatures	
Injection	250°C
Column	60°C//5°C/min//260°C-20 min
Detector	250°C,

Table 2.

9.4.5 Gas chromatography / mass spectrometer(GC/MS)

After isolating compounds in essential oil within a gas chromatography column, spectrums of each compound is determined by using a mass spectrometer. All evaluations are made primarily by using "TBAM Essential Oil Compounds Library". Furthermore, Wiley and Adams-LIBR library scanning software" and ''The Wiley/NBS Registry of Mass Spectral Data'' systems are used.

GC/MS Analysis Conditions	
System	Shimadzu GCMS-QP5050A
Column	CPSil 5CB (25mx0.25mm i.d.)
Carrying Gas	Helium (1ml/min)
Temperatures	
Injection	250^{o}C
Column	60^{o}C//5^{o}C/min.//260^{o}C-20 min.
Splitting Rate	50:1
Power of Electrons	70 eV
Mass Spectrum	35-400 m/z

Table 3.

9.4.6 Dispense of preparative TLC plaque

Readymade TLC plaque coated in size of 20x20 is used for preparative purposes to isolate active compounds in essential oil. Neutral essential oil is applied over plaque as a thin layer and eliminated in hexane: ethylacetat (9:1 v/v) dissolvent system within a TLC vessel. At the end of this process, a narrow fraction of TLC plaque is cut out vertically and this sample is analyzed under 254-364nm UV light and R_f value of this to be isolated fraction is determined by the application of anisaldehyde/ H_2SO_4 reactant. Subsequent to this process, sample plaque fraction is laid alongside of TLC plaque that is not processed with reactant yet and by taking into account the R_f value, the area on the plaque where the target substance is present is determined and silica gel over the aluminum support is rubbed out. Silica gel and substance compound is transferred to a glass funnel where glass wool is present and acetone is poured over them as a solvent. The substance is dissolved with acetone and resolving silica gel in the funnel gathers in the Erlenmeyer downside. Later on by using a vacuumed rotavapor, the solvent is removed in 40^{o}C.

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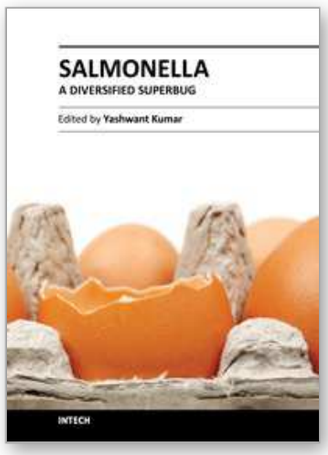

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Salmonella is an extremely diversified genus, infecting a range of hosts, and comprised of two species: enterica and bongori. This group is made up of 2579 serovars, making it versatile and fascinating for researchers drawing their attention towards different properties of this microorganism. Salmonella related diseases are a major problem in developed and developing countries resulting in economic losses, as well as problems of zoonoses and food borne illness. Moreover, the emergence of an ever increasing problem of antimicrobial resistance in salmonella makes it prudent to unveil different mechanisms involved. This book is the outcome of a collaboration between various researchers from all over the world. The recent advancements in the field of salmonella research are compiled and presented.

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