Anti-Microbial Effects of Olive Oil and Vinegar against *Salmonella* and *Escherichia coli*.

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ABSTRACT

The objectives of the present study were to evaluate and compare the antimicrobial activities of olive oil and vinegar against *Salmonella* and *Escherichia coli.* The spread plate method was used to determine the growth of *Salmonella* and *Escherichia coli*. The concentration of preservatives used for inhibition of *Salmonella* and *Escherichia coli* was determined by using minimum inhibitory concentration (MIC) method.

The results of the present research indicate that acetic acid in vinegar and phenolic compounds in olive oil had strong antimicrobial activity against *Salmonella* and *Escherichia coli*. However, vinegar showed stronger antimicrobial activity as compared to virgin olive oil. The presence of acetic acid in vinegar may make it more strong antimicrobial compound as compared to olive oil that contains phenols and poly phenols. The findings of the present study clearly met with the hypothesis that if olive oil and vinegar are used as preservatives in food, they inhibit the growth of *Salmonella* and *Escherichia coli*.

The findings of the present study show, vinegar and olive oil exert a protective effect against microorganisms and could be used as food preservatives for the inhibition of *Salmonella and Escherichia coli*.

(Keywords: antimicrobial activity, vinegar, olive oil, *Salmonella, E. Coli*, *Escherichia coli*)

INTRODUCTION

The development of food production methods and sensitive techniques has comparatively minimized microbial contamination in commercial products. However, outbreaks of food-borne illness are still an increasingly important public health problem. Wagner et al. (2008) reported that above ninety-percent of the food poisoning cases occur each year which are caused by *Staphylococcus aureus, Salmonella, Clostridium perfringens, Campylobacter, Listeria monocytogenes, Vibrio parahaemolyticus, Bacillus cereus,* and entero-pathogenic *Escherichia coli*. Similarly the CDC estimates that each year roughly 48 million people in United States get sick; 128,000 are hospitalized; and 3,000 die of food-borne diseases (Elaine et al., 2011).

Most of the food-borne diseases are periodic and often not reported. These disease outbreaks may take on massive proportions. An outbreak of salmonellosis due to contaminated ice cream occurred in the USA in 1994, affecting an estimated 224,000 persons. In 1988, an outbreak of hepatitis A, resulting from the consumption of contaminated clams, affected some 300,000 individuals in China (Food Safety and Foodborne Illness, 2007). Similarly *E. coli* O157 and *Listeriosis* are important food-borne diseases which have emerged over the last decades. Although their incidence is relatively low, their severe and sometimes fatal health consequences, particularly among infants, children and the elderly, make them among the most serious food borne infections, (Food Safety and Foodborne Illness, 2007). However, no

outbreaks of food-borne illness linked to olive oil have been reported in the literature, Similarly literature describes that food-borne pathogens are not likely to occur in extra virgin or virgin olive oils. (Palumbo et al., 2011).

Many natural products including plants, herbs, and certain foods containing antimicrobial substances have been studied for their antimicrobial activity. Medina et al. (2007) evaluated the survival of *Salmonella Typhimurium, Shigella sonnei,* and *Escherichia coli* in beverages like beer, cola, milk, and wine. The antibacterial effect was found higher in the wine.

Abo-El Seoud, et al. (2005) studied the antimicrobial activity of some essential oils against some plant pathogenic microorganisms. Medina et al., in 2007, reported about a natural antimicrobial system in the milk; the enzyme lecto-peroxidase has been used to preserve the raw milk quality.

The high quantity of acetic acid in vinegar made it very effective in preventing bacterial food poisoning (Medina et al., 2007). Olive oil has a vast use in homemade and commercial food products such as tuna, mayonnaise, tomato, toast and fresh salads, etc.

Most people are oriented towards the use of foods possessing natural biopreservatives. Ponce, et al. (2011), observed the natural preservatives in combination with other factors are an alternative to control the pathogen growth minimizing undesirable changes in organoleptic characteristics. The use of olive oil and vinegar products have increased dramatically with the arrival of new flavored oils and infused oils. The mixtures of oil and vinegar result in delicious dressings, toppings, sauces, vinaigrettes, and marinades.

Olive oil is known to have high levels of antioxidants, and is recognized for its natural composition of monounsaturated fatty acids, which unlike animal fats, is good for health. Poly phenols available in olive natural products such as olive fruits, leaves and oil possess activity against broad spectrum of microorganisms (Medina et al., 2007). However, literature review showed a relatively little amount of work done on antimicrobial activity of olive oil and vinegar against food borne pathogens. The results of the study showed that vinegar had strongest bactericidal effect, followed by the aqueous extract of virgin olive oil. Vinegar reduced the count of *Salmonella Enteritidis* and *E.coli* (Medina et al., 2007). The present research has been planned to study the growth of microorganisms such as *Salmonella* and *Escherichia coli* in the food having olive oil and vinegar, and to evaluate the antimicrobial activity of the oil and vinegar.

Media: Trypto Soya Agar (TSA), Trypto Soya Broth (TSB), MacConkey (MAC), and Nutrient Broth were used as media. TSA is general media that is good for spread plate method (Zimbro et al., 2003). TSB is general broth. It is nutrient rich media for general use. Testing of all types of pathogens can be performed by the use of this media, especially for diagnostic research. Nutrient broth is a pre-enrichment medium. This medium is used when food and dairy products are being tested, especially for *Salmonella*.

MacConkey agar (MAC) is used to isolate and differentiate member of Enterobacteriaceae, based on the ability to ferment lactose. MAC is used to confirm the organism *E. Coli*, (Zimbro et al., 2003).

Methods: The “minimum inhibitory concentration (MIC)” and “spread plate/dilution methods” were administered for the evaluation of growth of the microorganisms. These methods are the most appropriate in order to find out the countable microorganism with the help of spread plate method. Similarly, MIC method was applied to find out the minimum concentration of oil and vinegar, used to inhibit the growth of microorganism. Different biochemical tests, Citrate, Urease, SIM, Methyl Red (MR), and Voges–Proskauer (VP), were performed to confirm about the microorganisms.

Minimum Inhibitory Concentration Method is a standard against which other methods are assessed. MIC Method uses the lowest concentration of antimicrobial agent that inhibits the visible growth (Collins et al., 1999).

In Spread Plate/Dilution Technique, the mixed culture of microorganisms is diluted in a series of tubes to reach the countable range. Dilution systematically reduces cell density and provides a mathematical frame work by which to find out unknown original cell density with known number

of colonies on the plate (Leboffe et al., 2005). A drop of so diluted liquid from each tube is placed on the center of an agar plate and spread evenly over the surface by means of a sterilized bent-glass-rod.

The medium is now incubated. When the colonies develop on the agar medium plates, it is found that there are some plates in which well-isolated colonies grow. This happens as a result of separation of individual microorganisms by spreading over the drop of diluted liquid on the medium of the plate (Collins et al., 1999).

MATERIALS AND METHODS

Organisms and Chemicals

Salmonella enteriditis 13076, *E. coli* 25922. Distilled water, crystal violet, de-colorizer (alcohol, or acetone), Safranin, peptone water, saline, and sterile water.

Samples and Media

Virgin olive oil (President Choice made in Italy), pure white vinegar (no name, product of Canada). SIM medium 445**,** Citrate – medium, Urease – 541, broth or agar slant, M-43 (MR), VP, Nutrient broth, TSA, T.S.B broth.

1. Sulfur Indole and Motility (SIM) Preparation

Suspended 30 gram of the powder (Pancreatic digest of casein 20.0 g, Peptic digest of animal tissue 6.1 g, Ferrous ammonium sulfate 0.2 g, Sodium thiosulfate 0.2 g, Agar 3.5 g) in one liter of purified water. Mixed thoroughly, heated with frequent agitation and boiled for one minute to completely dissolve the powder. Then dispensed and autoclaved the mixture at 121 C^0 for 15 minutes (Zimbro and Power, 2003).

2. Tryptic Soya Broth (TSB) Preparation

Suspended 30 gram (Bacto tryptone 17.0 g, Bactone (pancreatic digest of soyabean meal) 3.0 g, Dextrose 2.5 g, Sodium chloride 5.0 g, Di Potassium phosphate 2.5 g) of the powder in one liter of purified water. Mixed thoroughly, then sterilized for 15 minutes at 121 C^0 (Zimbro and Power, 2003).

3. Nutrient Agar Preparation

Dissolved 8 grams (Beef extract 3.0 g, Peptone 5.0 g) of the powder in one liter of purified water. Mixed it thoroughly and autoclaved at 121 C^0.

4. Tryptic Soya Agar (TSA) Preparation

Suspended 40 g (Casein15.0 g, Enzymatic digest of soya bean meal 5.0 g, Sodium chloride 5.0 g, Agar 15 g) of the powder in one liter of purified water. Mixed thoroughly, heated with frequent agitation and boiled for one minute. Finally autoclaved for 15 minutes at 121 C^0 (Zimbro and Power, 2003).

METHODS

1. Method for Gram Staining

Heated the loop and took a colony of microorganism, mixed the colony with one drop of water, and let it to dry. Added crystal violet as primary stain, washed with water for 5 seconds, and added gram iodine for one minute, washed and drained. Flooded with de-colorizer for 10 seconds; washed with water, and did counter staining with safranin for 30 seconds. Gram positive cells were stained violet and gram negative stained pink / red. (Leboffe, 2005).

2. Bio-Chemical Tests

A). Method for Sulfur Indole and Motility (SIM) Test: Organism inoculated in SIM by using inoculated needle. Incubated at 35 C^0 for 24 – 48 hours, Presence of black precipitate showed positive result.

B). Method for Citrate Test: Medium contains sodium citrate as the only carbon source along with ammonium as nitrogen source. Inoculated the organism with the help of loop. Citrate tube was inoculated by using loop, streak on surface, and incubated for 24 – 48 hours. Appearance of change in color from green to blue indicated a positive result (Leboffe et al, 2005).

C). Method for Urease Test: Urease tube inoculated by using loop, streaked on surface, and incubated for 24 – 48 hour, Appearance of change as a pink color indicated positive, while

orange or yellow was found negative respectively (Leboffe et al, 2005).

D). Method for Methyl Red Test: The tube of medium was inoculated with culture, and incubated at 35 C^0 for 24 – 48 hours. Then added 5 – 10 drops of methyl red, a red color formation indicated the positive result (Leboffe, et al, 2005).

E). Method for Voges–Proskauer (VP) Test: Medium was inoculated with culture, and incubated at 35 C^0 for 24 – 48 hours. Then added 6 drops of alpha – Naphthol and 2 drops of 40% potassium hydroxide (KOH). After 30 minutes pink color formation indicated positive result (Leboffe, et al., 2005).

3. Spread Plate Method

Microorganism was cultured in nutrient broth. One hundred micro-liters of diluted inoculums (in saline) from overnight broth cultured was added to 4 ml of olive oil and 4 ml vinegar respectively, and left for five minutes at room temperature. After 5 minutes these mixtures were plated on agar media, both spreading 0.1 ml on the surface and 0.1% peptone water. Each experiment was replicated twice and duplicates were also included. By counting, the survivors were determined (Jay et al. 2005).

4. Minimum Inhibitory Concentration Method (MIC)

Prepared a 48 hours culture of microorganism. Used 10 ml of TSB media and inoculated isolated colony of microorganism. Prepared TSB broth, made a serial dilution of oil using 10 sterile tubes, with 5 ml of sterile water, and performed 1/10 dilution. Added 5 ml of TSB broth to each tube, and divided each dilution in three tubes. Two tubes were used to test microorganism by duplicate. The third tube was taken as negative control. Added inoculum to each tube (0.1 ml aliquot to each tube). Made a positive control (broth + distilled water + inocula) and a negative control (broth + oil). Incubated test and controls tubes for 48 hours at 35 C^0. After incubation, examined the tubes for growth (MIC). Streaked the tubes without growth in TSA media (MBC), incubated the plates for 24 hours at 35 C^0, read the highest dilution with no growth along the streak line (Lennette et al., 1974). The same procedure as mentioned above was followed while performing with vinegar.

RESULTS AND DISCUSSION

Results of all methods performed during the research study including "Gram staining, biochemical tests, spread plate method and minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC)" methods are discussed below:

Results of Gram Staining

E. coli and *Salmonella* both are gram negative, and rod shaped.

Results of Bio Chemical Tests

Table 1: Showing the Results of Biochemical Tests for *E. coli* and *Salmonella.*

Test	*E. coli*			*Salmonella*		
SIM	H_2S	Indole	Motility	H_2S	Indole	Motility
	-	+	+	+	-	+
Citrate	-			+		
Urease	-			-		
Methyl red	+			+		
VP	-			-		

Indole and motility results of *E. coli* in SIM test were found positive and Sulfur was not reduced to H_2S so found negative. Similarly *E. coli* showed positive results with methyl red test, however, the results of *E. coli* with citrate, urease, and VP tests were found negative. *Salmonella* showed positive results with Sulfur and motility but Indole was found negative in SIM test. Similarly *Salmonella* showed positive results with citrate and methyl red. However, the results of this organism in urease and VP biochemical tests were found negative.

Results of Spread Plate Method

Table 2: Showing the Results of *E. coli* and Vinegar in Spread Plate Method.

E. coli + vinegar	Growth	No Growth
Plate 1	-	No Growth observed
Plate 2	-	No Growth observed

The growth of *E.coli* was not observed in both plates in presence of vinegar. The same results were observed in the duplicate test.

Table 3: Showing the Results of *E. coli* and Olive Oil in Spread Plate Method.

E. coli + olive oil	Growth	No Growth
Plate 1	Growth observed	-
Plate 2	Growth observed	-

Growth of *E. coli* was observed in both plates in presence of olive oil. The same results were observed in the duplicate test.

Table 4: Showing the results of *Salmonella* and Vinegar in Spread Plate Method.

Salmonella + vinegar	Growth	No Growth
Plate 1	-	No Growth observed
Plate 2	-	No Growth observed

The growth of *Salmonella* was not observed in both plates in presence of vinegar. The same results were observed in the duplicate test.

Table 5: Showing the Results of *Salmonella* and Olive Oil in Spread Plate Method.

Salmonella + vinegar	Growth	No Growth
Plate 1	Growth observed	-
Plate 2	Growth observed	-

Growth of *Salmonella* was observed in both plates in presence of olive oil. The same results were observed in the duplicate test.

Results of Minimum Inhibitory Concentration (MIC) Method

Table 6: Showing results of *E. coli* and Vinegar in Minimum Inhibitory Concentration (MIC) Method.

Dilution	Tube 1 T1	Tube 2 T2	Control
1/ 40	-	-	-
1/ 80	-	-	-
1/ 160	-	-	-
1/ 320	+	+	-
1/ 640	+	+	-
1/ 1280	+	+	-
1/ 2560	+	+	-
1/ 5120	+	+	-
1/ 10240	+	+	-
1/ 20480	+	+	-

The minimum concentration of vinegar observed to inhibit the growth of *E.coli* was 1/160. The same results were found in the duplicate test.

Table 7: Showing results of *E. coli* and Olive Oil in Minimum Inhibitory Concentration (MIC) Method.

Dilution	Tube 1 T1	Tube 2 T2	Control
1/ 40	+	+	-
1/ 80	+	+	-
1/ 160	+	+	-
1/ 320	+	+	-
1/ 640	+	+	-
1/ 1280	+	+	-
1/ 2560	+	+	-
1/ 5120	+	+	-
1/ 10240	+	+	-
1/ 20480	+	+	-

The growth of *E.coli* was observed positive on all dilutions as shown in the table above. So MIC > 1/40. The same results were found in the duplicate test.

Table 8: Showing results of *Salmonella* and Vinegar in Minimum Inhibitory Concentration (MIC) Method.

Dilution	Tube 1 T1	Tube 2 T2	Control
1/ 40	-	-	-
1/ 80	-	-	-
1/ 160	-	-	-
1/ 320	+	+	-
1/ 640	+	+	-
1/ 1280	+	+	-
1/ 2560	+	+	-
1/ 5120	+	+	-
1/ 10240	+	+	-
1/ 20480	+	+	-

The minimum concentration of vinegar observed to inhibit the growth of *Salmonella* was 1/160. The same results were observed in the duplicate test.

Table 9: Showing results of *Salmonella* and Olive Oil in Minimum Inhibitory Concentration (MIC) Method.

Dilution	Tube 1 T1	Tube 2 T2	Control
1/ 40	+	+	-
1/ 80	+	+	-
1/ 160	+	+	-
1/ 320	+	+	-
1/ 640	+	+	-
1/ 1280	+	+	-
1/ 2560	+	+	-
1/ 5120	+	+	-
1/ 10240	+	+	-
1/ 20480	+	+	-

The growth of *Salmonella* was observed positive on all dilutions as shown in the table above. So MIC > 1/40. The same results were found in the duplicate test.

Results of Minimum Bactericidal Concentration (MBC) Test for *E. coli* and *Salmonella*

Table 10: Showing the results of MBC Test for Tubes without growth for *E. coli* and *Salmonella* on TSA.

Dilution	*E. coli* + vinegar	*Salmonella* + vinegar
1/ 40	No growth observed	No growth observed
1/ 80	No growth observed	No growth observed
1/ 160	No growth observed	No growth observed

The results of minimum bactericidal concentration (MBC) test for tubes without growth both for *E.coli* and *Salmonella* with vinegar are shown above. No growth was observed on TSA.

DISCUSSION

Streaking on TSA and gram staining (Leboffe et al, 2005) were the methods performed for the identification of *E. coli* and *Salmonella*. The results of both tests mentioned above showed that *E. coli* was a gram negative, and rod shaped bacteria. *Salmonella* was also observed a gram negative, and rod shaped bacteria. Findings of this research study about tested organisms are supported by an earlier research work done by Joklik et al. (1992).

Biochemical tests performed for confirmation of *E. coli* and *Salmonella* were sulfur indole motility (SIM), Citrate, Urease, Methyl red, and Voges Proskauer (Leboffe et al., 2005). The results of biochemical tests as mentioned in (Table 1) indicate that sulfur test for *E. coli* was found negative as there was no production of Hydrogen sulfide (H_2S), Indole result was positive due to the cherry red color appearance, and motility was observed positive in SIM test. Similarly *E. coli* showed positive result with Methyl red. However, the results of *E. coli* were found negative with citrate, urease, and VP biochemical tests, respectively.

The microorganism *Salmonella* in the present study showed positive results with sulfur along with the production of H_2S, Indole was found negative, but it had positive result for motility in SIM test (see Table 1). Similarly *Salmonella* indicated positive results with citrate and Methyl red however, urease and VP biochemical tests of this organism were observed negative. The results of biochemical tests performed confirm that given organisms were *E. coli* and *Salmonella enteriditis*. The results of present study are supported by an earlier study conducted by Holt et al. (1994).

The spread plate method (Jay et al., 2005) was applied to find out the growth of microorganisms *E. coli and salmonella* in presence of vinegar and olive oil respectively. The results of spread plate method for *E. coli* and vinegar are shown in Table 2. It was observed that *E. coli* did not show growth in the presence of vinegar. These findings indicate the strong antimicrobial activity of

vinegar against the growth of *E. coli*. This strong antibacterial activity exhibited may be due to presence of acetic acid in the vinegar. Therefore acetic acid in the vinegar acts as a good inhibitor against the growth of *E. coli* and vinegar could be used an antimicrobial and protective agent in the food industry. Our findings have been supported by the same kind of research study already conducted by (Medina et al., 2007). However, growth of *E. coli* was observed in the presence of olive oil see Table 3. It shows the poly phenol and phenol compounds present in the olive oil exhibit comparatively weak antimicrobial activity then acetic acid in vinegar. The findings of present study are favored by same kind of study done by Medina et al. (2007). Similarly Karaosmanoglu et al. (2010) evaluated Turkish extra virgin olive oils and refined oils for antimicrobial activity against single strains of *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella enteritidis* and observed that populations of the pathogens with the extra virgin olive oils, was decreased from 5 log CFU/ml to below the limit of detection within one hour of exposure to two different extra virgin olive oils.

However, Medina et al. (2009) compared the bactericidal effects of several olive phenolic compounds with other food phenolic compounds and with synthetic disinfectants again*st* pathogens including *E. coli*. It was found that olive compounds with a dialdehydic structure exhibit strong bactericidal activity, and in the presence of organic material, stronger bactericidal activity than the synthetic disinfectants.

The results of *Salmonella* in presence of vinegar and olive oil are same as observed in case of *E. coli*. *Salmonella* did not show growth in the presence of vinegar. The same results were observed in the duplicate test as well (see Table 4). However, *Salmonella* showed its growth in the presence of olive oil (see Table 5). The same results were observed in the duplicate test. It indicates that olive oil has less antimicrobial activity as compared to vinegar.

The results of present study are in the favor that vinegar could be a more effective antimicrobial agent as compared to olive oil. Similar results were reported by a study conducted by Medina et al. (2007), describing that virgin olive oil in milk- or egg-based mayonnaises in combination with lemon juice reduce the populations of inoculated *Salmonella* enteritidis and *L. monocytogenes* by approximately 3 log CFU/g in 30 minutes.

Another study conducted by Jirawan et al. (2009) favored the findings of the current research that growth of *Salmonella enteritidis* was controlled by the use of vaporized fermented vinegar. Similarly the results of a study conducted by Teresa et al. (2008) suggest that natural extracts of olive and grape showed more antimicrobial activity in food products then shown by selected antioxidants alone against *E. coli, Salmonella, Bacillus cereus, Saccharomyces cervisiae,* and *Candidaalbicans.*

The second method applied in this research study was minimum inhibitory concentration (MIC) method (Lennette et al., 1974). In MIC different concentrations of olive oil and vinegar were used. The results presented in Table 6 show that I/160 were the minimum concentration of vinegar which inhibited the growth of *E. coli*. However, the growth of *E. coli* in the presence of olive oil was found positive at all dilutions/ concentrations ranging from 1/40 to 1/20480. The results have been shown in table 7. It indicates that MIC > 1/40. The same results were observed in the duplicate test.

The results of *Salmonella* in presence of vinegar given in Table 8 show the minimum inhibitory concentration of vinegar observed to inhibit the growth of *Salmonella* is 1/160. The results of *Salmonella* in the presence of olive oil have been shown in table 9. All the results are positive, which indicates that minimum inhibitory concentration is greater than 1/40. The MIC method performed on the organisms is a tool that decides a specific concentration of vinegar / olive oil that may cause the death of microorganism and maintain the antimicrobial effect.

The minimum bactericidal concentration (MBC) method was applied for the tubes. The results of MBC test did not show any kind of growth (see table 10). The results indicate that vinegar containing acetic acid has strong bactericidal effect.

The results of spread plate method and minimum inhibitory concentration methods, both supported the hypothesis that vinegar is a strong antimicrobial compound. These results confirm the strong antimicrobial activity of vinegar as compared to virgin olive oil.

CONCLUSION

The sample vinegar showed stronger antimicrobial activity against *E. coli* and *Salmonella* as compared to virgin olive oil. These results confirm the strong antimicrobial activity of vinegar due to the presence of acetic acid. The presence of acetic acid compound in vinegar proved it as a strong preservative as compared to the poly phenols and phenol compounds present in the olive oil. However, both compounds have antimicrobial effect and can be used as preservative in food products.

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