Pak. J. Agri. Sci., Vol. 57(3), 735-742 ; 2020
ISSN (Print) 0552-9034, ISSN (Online) 2076-0906
DOI: 10.21162/PAKJAS/20.8313
http://www.pakjas.com.pk

ANTIOXIDANT, ANTIBACTERIAL AND FUNCTIONAL-FOOD-PACKAGING POTENTIAL OF LEAF EXTRACT FROM PAKISTANI OLIVE CULTIVARS

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Olive leaves of eight varieties (Gemlik, Manzanilla, Sevillano, BARI-Zaitoon 1, BARI-Zaitoon 2, Earlik, Azerbaijan and Hamdi) procured from Barani Research Institute were processed to obtain olive leaf extract. The extract was analyzed for antioxidant capacity by measuring the concentration of oleuropein and rutin through HPLC and of total antioxidants through ABTS, FRAP and DPPH assay. Antibacterial potential of the extract was calculated against *Staphylococcus aureus*, *Escherichia coli* and *Salmonella typhimurium* through the methods of disc diffusion, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The extract from the variety Gemlik, due to its highest antioxidant and antimicrobial potential among the varieties, was used to prepare functional food packaging. The efficiency of Gemlik coated packaging was measured by its thickness, water vapor transmission rate, oxygen transmission rate and antibacterial potential through disc diffusion method. Olive leaf extract of Gemlik possessed the highest levels of oleuropein and rutin contents (511.67±1.45mg/g and 6.98±0.01 mg/g of extract, respectively). Gemlik had the highest mean contents of FRAP, ABTS and DPPH, always followed by Sevillano. Gemlik extract showed the highest zone of inhibition against *E. coli* (16.33±0.33 mm) and against *Salmonella typhimurium* (16.00±0.00 mm), while Manzanilla had the highest value against *Staphylococcus aureus* (15.00±0.00 mm). The mean MIC was significantly lowest for Manzanilla (1.30±0.13) against *E. coli*, for both Gemlik and Manzanilla (0.78±0.00) against *Salmonella typhimurium*, for Hamdi(0.78±0.00) against *Staphylococcus aureus* (0.78±0.00). The mean MBC was significantly lowest for both Manzanilla and Earlik (4.68±0.00) against *E. coli*, for Gemlik (1.56±0.00) against *Salmonella typhimurium*, for Sevillano (1.95±0.00) against *Staphylococcus aureus*. The studies of thickness, water vapor transmission rate, oxygen transmission rate and antibacterial potential of the packaging sheets proved olive leaf extract an effective active functional food packaging. We recommend cultivation of Gemlik in Pakistan and animal studies to explore health-promoting effects of locally grown olive cultivars.

Keywords: Functional food, olive leaf extract, antioxidant, antibacterial, food packaging

INTRODUCTION

In recent years, usage of plant extracts as antibacterial and antioxidant agents has gained popularity because many plant extracts have acquired GRAS (generally recognized as safe) status (Lee and Lee, 2010). Such plant extracts are used in medicine, cosmetics, food processing and food packing industries. Antibacterial and antioxidant packaging from GRAS plant extracts extends shelf life, enhances safety and quality of food items by reducing the growth rate of pathogenic microorganisms (Scorzoni *et al.*, 2007; Ahmed *et al.*, 2014).

Deterioration of vital chemicals, flavor, color and lipids are one of the most detrimental problems in food and cosmetic industries (Cao and Prior, 2001; Erel, 2004; Kiritsakis *et al.*, 2010). To reduce such problems on industrial scale, synthetic additives like Tertiary Butyl Hydroquinone, Butylated Hydroxytoluene and Butylated Hydroxyanisole have been used (Silva *et al.*,2006; Pazos *et al.*,2008). Risk of carcinogenesis and certain toxicological effects of these synthetic food additives have led to increasing use of natural sources as food additives (Moure *et al.*, 2001; Kiritsakis *et al.*, 2010; Cheng *et al.*, 2016). Food packaging from functional foods also acts as an antibacterial agent for food safety (Quintavalla and Vicini, 2002). Functional food additives as antibacterials can be mixed with food packaging materials or applied as a sheet on the packing material (Cha and Chinnan, 2004; Dogan *et al.*,2016). Due to the antibacterial and antioxidant activity of such natural packaging materials, the packaging sheet inhibits microbes by decreasing microbial growth rate (Quintavalla and Vicini, 2002).

Olive leaves are one of the main natural sources to be used as natural food additive and can improve oxidative stability, antioxidant and antibacterial capacity of food and edible oils along with health-promoting benefits (Salta *et al.*, 2007). Due to the presence of bioactive compounds governing

antioxidant, antitoxin, anticarcinogenic, antibacterial, antidiabetic, anti-hypertensive and cardiotonic properties; usage of olive leaf extract (OLE) and whole leaf as functional food materials and food additives has increased in pharmaceutical, cosmetic and food industries (Delgado *et al.,* 2000).

Olive leaves also contain antibacterial properties against fungi, bacteria including mycoplasma, viruses and against some microbial toxins (Benavente *et al.,* 2000; Furneri *et al.,* 2002). These properties of olive leaves are due to their polyphenols such as oleuropein, tyrosol, vanillic acid, caffeic acid, hydroxytyrosol, elenolic acid, tocopherol and *p*-coumaric acid. Moreover, the leaves have flavonoids, like diosmetin-7-glucoside luteolin, luteolin-7-glucoside, rutin, apigenin-7-glucoside and diosmetin (Delgado *et al.,* 2000).

Pakistan has 3.17 million hectares with the potential of olive production (Khanum *et al.,*2019). Presence of wild olive (Kahu) all around in Pothohar area of Pakistan indicates the possibility of successful cultivation and domestication of olive in this area (Khanum *et al.,*2019). Converting wild olive to commercial olive varieties in natural habitats and establishment of new olive plantations will impact socio-economic values. Keeping in view the huge potential of olive cultivation in Pakistan, there is an industrial demand to determine the quality of olive by-products after oil extraction in the country. Therefore, the current study was designed to determine the antioxidant, antibacterial potential and use as functional food packaging of most commonly cultivated olive varieties in Pakistan to recommend the best variety/varieties for cultivation in the country.

MATERIALS AND METHODS

The study was completed at the University of Agriculture Faisalabad, Pakistan (National Institute of Food Science and Technology and Department of Biochemistry) and Washington State University, Pullman, United States (Department of Food Science and Human Nutrition, and Department of Biological System Engineering). The olive leaves were procured from Barani Agriculture Research Institute, Chakwal, Pakistan. The leaves were from eight locally grown olive varieties: Gemlik, Manzanilla, Sevillano, BARI-Zaitoon 1 (BARI-1), BARI-Zaitoon 2 (BARI-2), Earlik, Azerbaijan and Hamdi. For extraction of OLE from the leaves, binary solution of 75% ethanol in water was used as described earlier (Khanum *et al.,*2019)**.** Briefly, leaves were washed to remove dust, dried in an air oven at 38°C for three days and ground to powder. Ten grams of the powder was extracted for two hours with 200 ml of aqueous solutions of 75% ethanol. Then the samples were centrifuged at 5000 rpm for 15 minutes and the supernatant was carried to a rotary evaporator (38°C, 120 rpm) to remove any solvent. The remaining aqueous solution was lyophilized at -50°C and 0.028 mbar. This crude extract was refrigerated in glass bottles until further analysis.

HPLC**:** Oleuropein and rutin in OLE were determined through HPLC (Varian pro star 230 HPLC, photodiode array detector model 330). Absorbance was measured at 280 nm at a flow rate of 1ml/min. Acetonitrile was used as mobile phase while varianmicrosorb-MV 100-5 C18 250 X 4.6mm was the stationary phase. The concentration of oleuropein and rutin in OLE was calculated by comparing its retention time curve to the curve for the coumarin which was used as standard for both oleuropein and rutin determination.

Determination of antioxidants**:**

FRAP**:** The ferric-reducing antioxidant power (FRAP) of OLE was determined following *Cao et al*. (2013) with minor modification. Briefly, the FRAP reagent was prepared by mixing acetate buffer (5.1 g sodium acetate and 20 mL acetic acid per 0.25 L, pH 3.6), 12.5 mM Ferrozine and 4 mM $FeCl_3 \cdot H_2O$, in 40 mM HCl at 10:1:1 (v/v/v). A 0.6 mL volume of this FRAP reagent was mixed with 10 µL of each diluted OLE sample, and incubated in 37°C for 10 min. Then, the absorbance of the reaction mixture was measured at 562 nm using a spectrophotometer. $FeSO_4 \cdot 7H_2O$ (0.2 mM) was used as a standard. The results were expressed as Fe^{2+} equivalent antioxidant capacity (µmol Fe_2+/g).

ABTS**:** The antioxidant capacity using ABTS method was determined following Cao *et al*. (2013). One ml of ABTS solution was mixed with 10 µL of OLE. The mixture was kept for 6 minutes at 30°C and then its absorbance was measured at 734 nm using spectrophotometer. Trolox in 80% ethanol (0 mM-2.5 mM) was used to prepare the standard curve. The results were expressed as mmol trolox equivalents /g dry olive leaves (mmol TE/g DOL).

DPPH assay**:** The electron donation ability of OLE was measured by bleaching of 1, 1-diphenyl- 2-picrylhydrazyl radical (DPPH) according to the method of Lee and Lee (2010). 1 ml of OLE sample was added to 0.25 ml of 0.20 mM DPPH methanol solution. After 30 min incubation at room temperature, the absorbance was determined against a blank at 517 nm using spectrophotometer. Percentage inhibition (PI %) of free radical DPPH was calculated as follow:

$$PI\% = [(A_{blank} - A_{sample})/A_{blank}] \times 100$$

Where A_{blank} is the absorbance of the control and A_{sample} is the absorbance for OLE. OLE concentration providing 50% inhibition was calculated from the log-dose inhibition curve regression equation prepared by using OLE concentration and the inhibition percentage. Butylated hydroxytoluene was used as a positive control.

Antibacterial activity of olive leaves**:** Antibacterial potential of olive leaf extract against three foodborne pathogens (*Escherichia coli, Staphylococcus aureus, Salmonella typhimurium*) was assessed through disc diffusion method, minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC).

***Disc diffusion method*:** The test was performed in petri plates containing 20ml Muller Hinton medium. Filter paper discs of 6 mm were soaked in 15µl OLE and placed 24mm apart from each other in the plates. 500 µL having 10^6-10^8cfu/mL of each bacterium was inoculated on the plates by spread plate method. The agar plates were incubated at 37ºC for16 to 18 hours. The diameters of the zones of complete inhibition were measured; including the diameter of the disc. The commercial antibiotic discs were used as control (Jorgensen, 1993).

***Minimum inhibitory concentration*:** Minimum inhibitory concentration of OLE was determined by the method of Pereira *et al*. (2007) with some modification. All the tests were performed in 96 wells plate using nutrient broth. 100 µL of the broth was added to each well of the plate. 20 µL of broth culture of each bacterium adjusted to 10^6-10^8cfu/mL, was added to respective wells. 100 µL of OLE was added to each treatment well using two-fold serial dilutions. Amoxicillin was used as control. The plates were incubated at 37°C for 24 h and then 10 µL of resazurin indicator solution was added to each well. The absorbance of each well was measured by microplate reader at 600 nm. Any color change from purple to pink or colorless was recorded as positive. The lowest concentration of two-fold serial dilution at which color changed was recorded as MIC value.

***Minimum bactericidal concentration*:** The MBC test was performed following Sudjana *et al*. (2009). Sub-culturing of 1 µl of the lowest concentration of OLE that had no visible growth in the MIC test was performed on an antibiotic-free agar. After incubation at 37°C for 24 h, colonies were counted. The growth of four or fewer colonies indicated a 99.8% or greater fall in the viable count (Sudjana *et al.*, 2009).

***Preparation of functional packaging and coating the films*:** Based on HPLC analysis, antibacterial potential and antioxidant activity, OLE of Gemlik was selected for the preparation of active packaging. For coating preparation, Gemlik OLE was added to methylcellulose (0.875 g) and hydroxypropyl methylcellulose (0.375 g). The mixture was homogenized at 7,000 rpm for 2 min by a homogenizer (Polytron PT 2500E). Then, ethanol (25 ml) and polyethylene glycol (0.75 ml) were added to the mixture and homogenized at 7,000 rpm for another 2 min. Then this film coating solution was degassed for approximately 5 min at room temperature. Total biopolymer concentration in the final solution was 70/30%: MC/HPMC on a dry weight basis (Neetoo *et al.*,2007). A control was prepared without olive extract. Threecommercial plastic films were coated by this coating solution: Sheet A= mLLDPE/LLDPE/LLDPE/Nylon/LLDPE/mLLDPE/mLLDPE, Sheet B= PET/EVOH-Polyethylene, and Sheet C= PET/Barrier PET/ Polypropylene. The films were taped to a smooth laboratory table surface, and the coating solutions with or without olive extract were cast onto the films with a wet film applicator rod (Mayer rods# 12). The thickness of the coating was fixed to 30.5 microns by the rod. The coated films were air-dried overnight at 37°C for 24 h (Neetoo *et al.*, 2007).

***Evaluation of the coated film*:** The thickness of the produced coating layer was determined by the difference in thickness between the non-coated and the coated film measured with a micrometer. Antibacterial activity of films was determined by the agar diffusion method (Aliabadi *et al.*, 2012). Briefly, OLE coated films were placed on MHA plates, which had been previously seeded with 0.1 mL of inoculum of the above-mentioned pathogens. The plates were incubated at 37°C for 24 h. The inhibitory zone, surrounding the film disc was measured with a scale. Films without olive extract served as control. The WVP test was conducted by using water vapor permeation analyzer. The OTR test was conducted by using oxygen transmission rate analyzer.

RESULTS

***Oleuropein and rutin concentration*:** Mean oleuropein contents of OLE from the varieties (Table 1) ranged from 305.33±3.28to 511.67±1.45 mg/g of extract. The highest oleuropein concentration (511.67±1.45mg/g) was found in Gemlik followed by Manzanilla, Hamdi, Sevillano, Earlik, Azerbaijan and BARI-1, respectively. Among all the varieties, Bari-2 exhibited the lowest (305.33±3.28) oleuropein contents. The mean oleuropein contents differed significantly among varieties except between Earlik and Azerbaijan, and between BARI-1 and BARI-2. Mean rutin contents of OLE from different olive varieties (Table 1) ranged from 1.60±0.0 to 6.98±0.01 mg/g of extract. The highest rutin contents of 6.98±0.01 mg/g of extract were found in Gemlik, followed in order by mean rutin contents for Hamdi, Manzanilla, Sevillano, Earlik, Azerbaijan and BARI-2. BARI-1 showed the lowest rutin contents of 1.55±0.02

Table 1. Mean ± SEM oleuropein contents (mg/g of extract) and rutin concentration (mg/g of extract) of olive leaf extract.

Method of extraction	Olive leaf varieties							
	Gemlik	**Manzanilla**	**Hamdi**	**Sevillano**	**Earlik**	**Azerbaijan**	**BARI-1**	**BARI-2**
Oleuropein concentration (mg/g of extract)	511.67±1.45a	463.33±2.02b	432.33±3.28c	400.33±6.81d	351.67±1.45e	345.67±1.45e	313.67±1.45f	305.33±3.28f
Rutin concentration (mg/g of extract)	6.98±0.01a	4.41±0.01b	4.85±0.05c	3.99±0.02d	3.11±0.0e	2.33±0.0f	1.55±0.02g	1.60±0.0g

Means with different small letters within the same row indicate significant difference (P<0.05).

Table 2. Mean ± SEM ferric reducing antioxidant power (µmol Fe^{2+}/g), ABTS scavenging activity (mmol TE/g of dry olive leaves) and DPPH scavenging activity (percent) of olive leaf extract.

Antioxidant potential	Olive leaf Varieties							
	Gemlik	Sevillano	Azerbaijan	Earlik	Manzanilla	Hamdi	BARI- 2	BARI-1
FRAP values (µmol Fe^{2+}/g of dry olive leaves)	423.1±1.78a	401±0.92ab	380.3±4.66ab	374.7±3.50bc	397.4±1.15ab	341.0±2.30cd	317.6±1.32d	305.4±4.78d
ABTS (mmol TE/g of dry olive leaves	1.56±0.017a	1.23±0.008b	0.90±0.008c	0.84±0.037d	0.73±0.014e	0.74±0.039e	0.41±0.006f	0.43±0.003f
DPPH scavenging activity (percent) of OLE	62.33±0.88a	57.33±1.45b	45±1.52c	41.3±1.45cd	38.66±0.88d	32.33±0.88e	33±1.15e	37.66±0.88d

Means with different small letters within the same row indicate significant differences (P<0.05).

Table 3. Mean ± SEM antibacterial activity of olive leaf extract against *Escherichia coli, Salmonella typhimurium and Staphylococcus aureus* using disc diffusion method.

Pathogens	Olive leaf varieties							
	Gemlik	Manzanilla	Sevillano	Azerbaijan	Hamdi	Earlik	BARI-2	BARI-1
E. coli	16.33±0.33a	14.66±0.33b	13.00±0.00cd	10.33±0.33gh	12.0±0.00def	9.33±0.33h	11.00±0.57fg	8.33±0.33h
Salmonella typhimurium	16.00±0.00a	15.00±0.00b	12.33±0.33c	12.00±0.00c	10.66±0.33d	11.66±0.33c	7.66±0.33e	7.66±0.33e
Staphylococcus aureus	14.00±0.00a	15.00±0.00b	10.66±0.33c	9.00±0.00d	10.33±0.33c	9.00±0.00d	8.66±0.33d	8.00±0.00e

Means with different small letters within the same row indicate significant difference (P<0.05).

mg/g of extract. The mean rutin contents differed significantly among varieties except between BARI-1 and BARI-2.

***Antioxidant potential of olive leaf extract*:** The mean FRAP values of OLE (Table 2) from all the varieties varied from 305.39±4.78 to 423.13±1.78 µmol Fe^{2+}/g of DOL. The highest FRAP value (423.13±1.78 µmol Fe^{2+}/g) was observed in Gemlik, which did not differ significantly from the mean for Sevillano, Azerbaijan, Manzanilla and Earlik. BARI-1 showed significantly lowest (305.39±4.78 µmol Fe^{2+}/g of DOL) FRAP value which was not significantly different from the mean for Hamdi and BARI- 2. The highest to the lowest mean FRAP values of OLE from all the varieties were in the order: Gemlik, Sevillano, Azerbaijan, Manzanilla, Earlik, Hamdi and BARI- 2, and BARI-1. The mean ABTS values for OLE from all the varieties (Table 2) varied from 0.41±0.006 to 1.56±0.017 mmol TE/g dry olive leaves (DOL). The mean ABTS+ scavenging activity was ranked as: Gemlik, Sevillano, Azerbaijan, Earlik, Manzanilla, Hamdi, BARI-1 and BARI-2. The mean for Gemlik (1.56±0.017 mmol TE/g DOL) was significantly highest than all other varieties. The mean ABTS contents differed significantly among all the varieties except between Manzanilla and Hamdi, and between BARI-1 and BARI-2. The mean DPPH values for the varieties (Table 2) ranged from 32.33±0.88%to 62.33±0.88%. The mean DPPH concentration was the highest in Gemlik (62.33±0.88%) followed in order by Sevillano, Azerbaijan, Earlik, Manzanilla, BARI-1, BARI-2 and Hamdi (32.33±0.88%). The mean DPPH differed significantly among varieties except between Azerbaijan and Earlik; among Earlik, Manzanilla and BARI-1; and between BARI-2 and Hamdi. For all three of FRAP, ABTS and DPPH, Gemlik has the highest mean value always followed by Sevillano. While, BARI-1 and BARI-2 showed the least antioxidant capacity in terms of FRAP, ABTS and DPPH.

***Antibacterial Potential of Olive Leaf Extract*:**

***Disc diffusion method*:** The mean values for the zone of inhibition using disc diffusion method (Table 3) against *E. coli* was significantly highest for Gemlik (16.33±0.33 mm) than all other varieties. The zone of inhibition decreases in order from Manzanilla to Sevillano, Azerbaijan, Hamdi, BARI-2, Earlik and BARI-1. BARI-1exhibited the lowest mean value (8.33±0.33 mm) which was not significantly different from Earlik. Moreover, no significant differences in the zone of inhibition were observed between Azerbaijan and Hamdi, however, differences among all other varieties were significant. Against *Salmonella typhimurium* (Table 3), Gemlik had significantly highest zone of inhibition (16.00±0.00 mm) than all the other verities. The mean was followed in order by Manzanilla, Sevillano, Azerbaijan, Earlik and Hamdi. Both BARI-2 and BARI-1, had significantly lowest mean zone of inhibition (7.66±0.33). The mean different significantly among all varieties except among Sevillano, Azerbaijan and Earlik; and between BARI-2 and BARI-1. Against *Staphylococcus aureus* (Table 3), Manzanilla exhibited significantly highest zone of inhibition (15.00±0.00 mm) than any other variety. Whereas, no significant difference was observed between Hamdi and Sevillano; and among Azerbaijan, Earlik, and BARI-2. Overall, the zone of inhibition against *S. aureus* was ranked as Manzanilla, Gemlik, Sevillano, Hamdi, both Azerbaijan and Earlik, and BARI-2. BARI-1 had significantly lowest mean than all other varieties. Gemlik had significantly highest zone of inhibition against gram-negative *E. coli* and *S. typhimurium* followed by Manzanilla. While, Manzanilla had significantly highest zone of inhibition against gram-positive *S. aureus* followed by Gemlik.

***Minimum inhibitory concentration*:** Against *E. coli*, mean MIC of OLE (Table 4) from Manzanilla was significantly

Table 4. Mean ± SEM minimum inhibitory concentration (mg/ml) of olive leaf extract against *Escherichia coli, Salmonella typhimurium and Staphylococcus aureus*.

Pathogens	Olive leaf varieties							
	BARI-1	**Azerbaijan**	**BARI-2**	**Sevillano**	**Earlik**	**Gemlik**	**Manzanilla**	**Hamdi**
Escherichia coli	3.63±0.26a	6.25±0.00b	4.42±0.26c	3.12±0.00d	4.42±0.26c	2.60±0.26e	1.30±0.13f	1.82±0.13g
Salmonella typhimurium	4.68±0.00a	1.82±0.13b	2.21±0.13c	3.38±0.25d	3.12±0.00e	0.78±0.00f	0.78±0.00f	1.95±0.00b
Staphylococcus aureus	3.90±0.00a	3.38±0.26b	1.43±0.06c	1.43±0.13c	2.34±0.00d	3.12±0.00b	1.30±0.13c	0.78±0.00e

Means with different small letters within the same row indicate significant difference (P<0.05).

Table 5. Mean ± SEM minimum bactericidal concentration (mg/ml) of olive leaf extract against *Escherichia coli, Salmonella typhimurium and Staphylococcus aureus*.

Pathogens	Olive leaf varieties							
	BARI-1	**BARI-2**	**Sevillano**	**Manzanilla**	**Azerbaijan**	**Earlik**	**Hamdi**	**Gemlik**
Escherichia coli	15.620.00a	9.37±0.00b	6.25±0.00c	4.68±0.00d	12.50±0.00e	4.68±0.00d	7.81±0.00f	9.37±0.00b
Salmonella typhimurium	7.81±0.00a	6.25±0.00b	9.37±0.00c	3.12±0.00d	3.90±0.00e	7.81±0.00a	9.37±0.00c	1.56±0.00f
Staphylococcus aureus	6.26±0.00a	7.81±0.00b	1.95±0.00c	9.37±0.00d	4.68±0.00e	3.12±0.00f	6.25±0.00a	2.34±0.00g

Means with different small letters within the same row indicate significant difference (P<0.05).

Table 6. Effect of OLE coating on mean ± SEM thickness, oxygen transmission rate (cc/m^2/day) and water vapor transmission rate (g/m2/day at 37.8°C & 100% RH) of plastic films designed for active packaging.

Sheet Type	Film Thickness		OTR		WVTR	
	Control (mm)	**Coated film (mm)**	**Control**	**Coated films**	**Control**	**Coated films**
A	0.055±0.002	0.064±0.005	1.24±0.01	0.80±0.01	2.46±0.11	1.53±0.01
B	0.085±0.001	0.095±0.006	3.00±0.01	2.53±0.25	4.38±0.04	3.47±0.04
C	0.081±0.004	0.092±0.006	61.66±0.57	60.07±0.57	3.82±0.13	3.99±0.13

Means with different small letters within the same row indicate significant difference (P<0.05).

lowest (1.30±0.13) than all other varieties. The mean MIC of OLE increased in the following order: Manzanilla, Hamdi, Gemlik, Sevillano, BARI-1, both Earlik and BARI-2, and Azerbaijan. MIC differed significantly among all the varieties but between BARI-2 and Earlik. Azerbaijan was the least antibacterial with significantly highest MIC of 6.25±0.00. The mean MIC of OLE against *S. typhimurium* (Table 4) was the lowest for both Gemlik and Manzanilla (0.78±0.00) and was significantly different from all the other varieties. The least effective variety was BARI-1 with significantly highest MIC of 4.68±0.00. MIC different among all the varieties except between Gemlik and Manzanilla, and between Azerbaijan and Hamdi. MIC against *S. typhimurium* increased in the following order: Gemlik and Manzanilla, Azerbaijan, Hamdi, BARI-2, Earlik, Sevillano and Bari-1. Against *S. aureus*, mean MIC of OLE (Table 4) from Hamdi was significantly lowest (0.78±0.00) than all other varieties. The mean was significantly highest (3.90±0.00) for BARI-1. The mean MIC differed among all varieties except between Azerbaijan and Gemlik, and among BARI-1, Sevillano and Manzanilla.

***Minimum Bactericidal Concentration*:** Against *E. coli*, mean MBC of OLE (Table 5) from Manzanilla and Earlik was significantly lowest (4.68±0.00) than all other varieties. The mean MBC of OLE increased in the following order: Manzanilla and Earlik, Sevillano, Hamdi, Gemlik and BARI-2, and BARI-1. MIC differed significantly among all the varieties except between Manzanilla and Earlik and between Gemlik and BARI-2. BARI-1was the least bactericidal with significantly highest MIC of 15.62±0.00. Mean MBC of OLE against *S. typhimurium* (Table 5) was the lowest for Gemlik (1.56±0.00) and was significantly lowest from all the other varieties. The least effective were Sevillano and Hamdi with significantly highest MBC of 9.37±0.00 for both. MBC differed significantly among all the varieties except between BARI-1 and Earlik, and between Sevillano and Hamdi. MBC against *S. typhimurium* increased in the following order: Gemlik, Manzanilla, Azerbaijan, both Earlik and Bari-1, and both Hamdi and Sevillano. Against *S. aureus*, mean MBC of OLE (Table 5) from Sevillano was significantly lowest (1.95±0.00) than all other varieties. The mean was significantly highest (9.37±0.00) for Manzanilla. The mean MBC differed significantly among all the varieties except between BARI-1 and Hamdi. The mean increased in the following order: Sevillano, Gemlik, Earlik, Azerbaijan, BARI-1 and Hamdi, BARI-2 and Manzanilla.

***Olive leaf extract as packaging material*:** OLE coated Sheet A (PET/ Barrier PET/ Polypropylene), sheet B (PET/ EVOH-Polyethylene) and sheet C (mLLDPE/ LLDPE/ LLDPE/ Nylon/ LLDPE/ mLLDPE/ mLLDPE) had increased thickness when compared to their respective control sheets (Fig. 1). A significant decrease in OTR values was observed for all OLE coated packaging sheets compared to the respective non-coated controls (Fig. 1). Hence, oxygen barrier

properties were increased after application of coating material. All three types of OLE coated packaging sheets had significantly lesser mean WVTR compared to the respective mean WVTR before coating (Fig. 1). Antibacterial activity of OLE coated films against *Salmonella typhimurium, Staphylococcus aureus* and *E. coli* for the three sheet types (packaging material) was evident by the presence of inhibition zone (Fig. 1). However, the respective control sheets without OLE used as negative control showed no zone of inhibition. Thus, OLE coated films had antibacterial activity against the studies pathogens when compared to negative control. Overall, the highest inhibition zone was found against *staphylococcus aureus* followed by *salmonella typhimurium* and *Escherichia coli*, for all three sheet types.

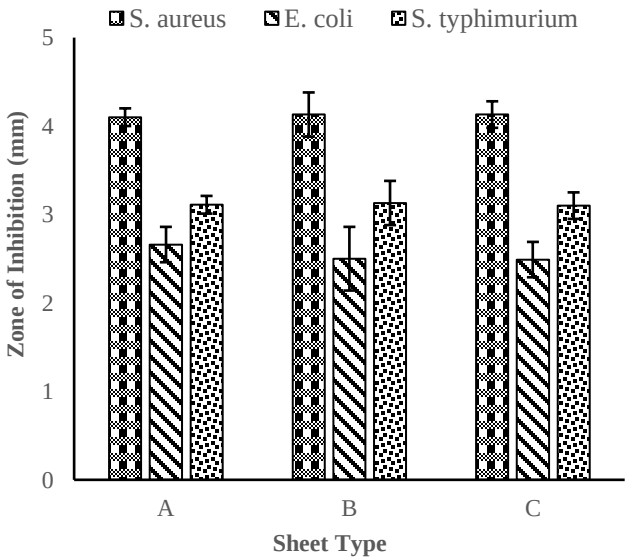

Figure 1. Effect of OLE coating on antibacterial activity of plastic films designed for active packaging in terms of mean ± SEM zone of inhibition (mm).

DISCUSSION

Studies on natural antioxidants and antimicrobials from GRAS plant extracts have gained popularity in the last decade. The present study confirms the antioxidant and antibacterial potential of locally grown OLE recommending its use as in functional packing in the processed food industry. Our results on antioxidant potential of OLE are supported by the findings of Cano *et al*. (2002), Lee and Lee (2010), Salah *et al*. (2012), Cao *et al*. (2013), Mitsopoulos *et al*. (2016) and Yancheva *et al*. (2016). The slight variation, in the mean values of antioxidant agents of OLE, between the previous studies and ours can be due to differences in cultivars, harvesting time, growing conditions, geography, geology, location, climate (Yateem *et al.,* 2014), extraction method and extraction solvent. Importantly, genetic makeup cannot be ignored as a factor as native or adapted Pakistani olive varieties were used in the present study. Cano *et al*. (2002) showed that the pH of extraction solvents affects the values of antioxidants compounds from OLE, however we had constant pH of extraction solvent across all varieties. Yateem *et al*. (2014) revealed that the geographical region has a significant effect on antioxidant values of olive leaves. They concluded that Palestinian olive varieties had higher antioxidant potential as compared to the varieties from Italy, Iran and Greece. Anyhow, determining the factors affecting the antioxidant potential of olive was beyond the scope of this study. We found differences among varieties in terms of mean concentration of bioactive compounds. Salah *et al*. (2012) studied the antioxidant activity of olive leaf extract from various varieties and found significant variation among varieties. They suggested that the variation in results might be due to environmental (climate, geographical and geological) and cultural (harvesting, pruning and watering) conditions. However, in our study varieties were grown under the same climatic and cultural conditions so the variation among varieties could be due to genomic differences among the varieties.

Pereira *et al*. (2007) determined the antibacterial potential of OLE against several bacteria and observed that the extract was more effective against *Bacillus cereus* followed by *E. coli, Staphylococcus aureus, C. neoformans and Bacillus subtilis.* Gokmen *et al*. (2014) determined the antibacterial potential of OLE by disc diffusion method and observed inhibition zone of 13.33±2.08 mm against *S. typhimurium.* Aliabadi *et al*. (2012) investigated the antibacterial activity of OLE against *Salmonella typhimurium* PTCC 1639 showing an inhibition zone of 11.53±0.98 mm. The results of all the above researchers are in general agreement with the present research work where OLE is proven to have antibacterial effect. The slight difference between our findings and the previous literature may also be due to using different serotypes of the bacteria.

Our findings of OLE as a potential functional coating for packaging are supported by Bedane *et al*. (2012 and Buntinx *et al*. (2014). They showed that the functional coating of modified cellulose material on mono and multilayer sheets with variable depth improved the barrier properties of sheets. The decrease in OTR and WVTR values observed in the present study might be due to an increased sheet thickness because OTR and WVTR of a sheet are affected by the sheet thickness (Erdogan and Eksi, 2014). Based on our findings, OLE coating can be used industrially to improve quality of packing sheets as the packing industry requires the polymer constituents with lower permeability to oxygen and water vapors. Better gas and vapor barrier properties by functional coating provides a surface layer with reduced penetration of the packed substances through the sheet (Andersson, 2008); and better-quality barrier of the packaging films can help to enhance the shelf life of food items (Del *et al.,* 2006; Koide

and Shi, 2007). Low oxygen barrier of the packaging film can cause the initiation of oxidative reactions that damage lipid and proteins leading to food spoilage. Our finding of antibacterial properties of OLE coated films is supported by An *et al*. (2000) and Neetoo *et al*. (2007). An *et al*. (2000) found effective antibacterial potential of bacteriocins coated polyethylene film against *E. coli, B. subtilis, B. cereus, M. flavus* and *L. monocytogenes*. Neetoo *et al*. (2007) showed that nisin coated plastic films had antibacterial activity against *Listeria monocytogenes*. Neetoo *et al*. (2007) determined that the type of film had no effect on retention and release of active agents; similarly, we also observed non-significant effect of sheet type on the antibacterial potential of the sheets.

Oleuropein and rutin are the most antioxidant compounds in olive (Benavente *et al.*, 2000). Logically, Oleuropein and Rutin contents of Pakistani OLE can be the basis for antioxidant and antibacterial activities observed in the present study.

***Conclusion*:** Based on our findings, we recommend that cultivation of Gemlik be favored over other olive varieties in the country especially in Potohar "olive" valley. In the present study, all sampling was from Potohar valley, further studies are needed to study olive varieties grown in other areas of Pakistan with different growing conditions. Furthermore, we found significant antioxidant and antibacterial potential of the varieties grown in Pakistan, studies are needed to determine health promoting the effect of locally grown olive fruits and leaves in the laboratory animal model.

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[Received 19 Nov 2018: Accepted 15 April 2019: Published (online) 08 June 2020]