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RESEARCH ARTICLE

LISTERIA MONOCYTOGENESIS RAW MILK AND DAIRY PRODUCTS; OCCURRENCE AND TRIALS FOR THEREDUCTION OF THEIR RESISTANCE TO ANTIBIOTICS.

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Abstract

Listeria monocytogenes is a facultative intracellular pathogen responsible for severe infection in animals and humans. This study aimed to determine the occurrence of Listeria monocytogenes in 200 samples of raw milk and dairy products marketed in Egypt, with detection the antimicrobial resistance profiles of the isolates for different antimicrobial agents. The synergistic effects of three plant extracts in combination with the used antimicrobials to control resistant L. monocytogenes isolates were assessed, with evaluation of relationship between the antibacterial activity and composition of plant extracts. A total of 5 (2.5%) L. monocytogenes isolates were identified (3 isolates from raw milk and two isolates from kariesh cheese and ice cream, one each). The isolates were sensitive to ciprofloxacin and sulphadimidine, while for amoxicillin and oxytetracycline, were resistant. The higher antimicrobial effects of basil and cinnamon extracts were detected, in addition, low concentrations of them were enough to show synergistic effect with the amoxicillin and oxytetracycline to control resistant isolates, these might be attributable to their chemical compositions. In conclusion, there is need for effectively pasteurized raw milk consumption or usage in the production of dairy products. In addition, the chemical composition of these plant extracts might help in its use to control resistant isolates with appropriate antibiotics without the need for increasing the used dosage doses.

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Introduction:-

Listeriosis is a serious infectious disease of humans and animals, caused mainly by *Listeria monocytogenes*. The large outbreaks in humans were caused by consumption of raw or post-pasteurization contaminated milk and mainly characterized by low incidence but high case fatality rate (Kasalica et al., 2011). *L. monocytogenes* is susceptible to most antibiotics, except for cephalosporins and fosfomycin. However, major concerns have been raised due to reports of *Listeria* spp. that are resistant to a wide range of antimicrobial agents that are commonly used for the treatment of listeriosis. Thus, as an alternative, combination therapy has been found more effective because synergistic interactions can be faster in therapy, prevent emergence of resistance and increase efficacy of the used combinations (Marr et al., 2004). In addition, it can reduce toxicity, and provide broader spectrum of activity.

The presence of different antimicrobial compounds in plants help scientists to use these plants in treatment of some infectious diseases. However, combination therapy of various plant extracts with antibiotics exhibiting bactericidal synergism is a method leading to new ways for treatment of infectious diseases (Chanda and Rakholiya, 2011).

The current study aimed to determine the prevalence of *L. monocytogenes* in raw milk and dairy products; soft cheese, kariesh cheese and ice cream at Qalyubiyah and Giza governorates, Egypt. The antimicrobial resistance profile of the isolates was determined against four different classes of antimicrobial agents. Moreover, the total phenolic compounds, total flavonoids compounds and antioxidant activity of three plant extracts were evaluated. Also, the synergistic effects of the three extracts in combination with the used antimicrobials to control resistant *L. monocytogenes* isolates were assessed.

Materials and Methods:-**Bacterial isolates:-**

Isolation and identification

Two hundred random samples from fresh milk (n=80) and dairy products (soft cheese, kariesh cheese and ice cream, 40 each) were obtained from different outlets at Qalyubiyah and Giza Governorates, Egypt, during the period of October 2016 to January 2017. The collected samples were aseptically transported in ice box as soon as possible to the laboratory for isolation and identification of *L. monocytogenes* according to ISO (2004).

For isolation of *L. monocytogenes*, 25 grams of collected dairy products were pre-enriched in 225 ml half strength Fraser broth (Oxoid - CM0895) for 24 h at 30°C, then followed by second enrichment of 0.1 ml of full strength Fraser broth for 48 h at 37°C. Similarly, 25 ml of collected fresh milk samples were passed the two step method for enrichment after PH adjusted to neutral. After the enrichment procedure, 0.1 ml of the broth was streaked onto PALCAM agar (Oxoid - CM0877) and incubated for 48 h at 37°C. *Listeria monocytogenes* were preliminary identified by their colonial morphology on PALCAM agar. The suspected colonies were sub-cultured on Tryptone Soya Yeast Extract Agar (TSYEA; Sigma-Aldrich) and subjected to Gram's staining, microscopic examination, catalase test, and oxidase test. The isolates were also sub-cultured on semisolid medium (Nutrient broth supplemented with 0.5% agar, Oxoid) at 25°C for 12-18 h for the detection of the typical tumbling motility (ISO, 2004). For further identification, Microgen™ *Listeria*-ID System (LAB; MID-67) was used according to the manufacturers' guidelines. The phenotypically isolates were confirmed using PCR targeting 16s rRNA (Kumar et al., 2015).

Antimicrobial susceptibility testing:-

Two-fold broth microdilution method was used to determine the minimum inhibitory concentrations (MIC) of the identified strains against the antimicrobial agents amoxicillin, oxytetracycline, ciprofloxacin and sulphadimidine (Sigma-Aldrich), by cultivation in Mueller Hinton broth according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2012). There are no specific international interpretation criteria for susceptibility testing of *L. monocytogenes* for the examined agents. Therefore, the *Staphylococcus* spp. resistance breakpoints were used. The general resistance breakpoints of CLSI (2002) were used in case of amoxicillin, while, CLSI (2016) were used for tetracycline, ciprofloxacin and sulphadimidine. Resazurin dye as a redox indicator was used to detect the MIC values to give a high level of accuracy (Elshikh et al., 2016). Each isolate was examined in triplicate. The reference strain; *L. monocytogenes* (ATCC®35152) was used as a quality control.

Plant extracts:-

Plant extracts preparation and analysis Three plant samples; cinnamon inner bark (*Cinnamomum zylenticum*), coriander leaves (*Coriandrum sativum*) and basil leaves (*Ocimum basilicum*), were obtained from Medicinal and Aromatic Plant Research Department, Horticulture Research Institute, Agricultural Research Center, Egypt. Preparation of the ultrasonic-assisted ethanol 75% extract from plants were applied according to El-Maati et al. (2016).

The plant extracts were analyzed for determination of total phenolic compounds (TPC) by a UV spectrophotometer using Folin-Ciocalteu reagent, and total flavonoid compounds (TFC) using the Folin-Ciocalteu method (Gülçin et al., 2010).

The antioxidant activity of the extracts was determined by measuring 1,1-diphenyl-2-picrylhydrazyl (DPPH[•]) free radical-scavenging activity (Yamaguchi et al., 1998), β -Carotene-linoleic acid bleaching activity (Ordonez et al., 2006), and finally the ferric-reducing antioxidant power (FRAP) activity (Gülçin et al., 2010).

MIC of ethanol plant extracts:-

The influence of plant extracts was evaluated on clinical strains by broth microdilution method using the redox indicator resazurin as a visual marker of the MIC (Elshikh et al., 2016).

Checkerboard assay:-

Interactions between antibiotics and plant extracts were assessed by the broth dilution checkerboard MIC assay in Mueller Hinton broth. Different dilutions of each plant extract (1/2, 1/4 and 1/8) were used separately with different concentrations of each antimicrobial agent (1/2, 1/4 and 1/8 of the MIC value) according to Paparella et al. (2013). Where, the synergistic effect was assessed on the basis of the fractional inhibitory concentration (FIC) index representing the sum of the FICs (Σ FICs) of each drug tested, where the FIC derived from the lowest concentrations of extracts and the antibiotics in combination permitting no visible growth of the test organisms in the Mueller-Hinton broth after incubation for 24 h at 37 °C. In addition, Kamatou et al. (2006) defined synergy to occur when Σ FIC < 1.0, additively when Σ FIC = 1.0 and antagonism when Σ FIC > 1.0.

Σ FIC = FIC (antibiotic) + FIC (extract) = (MIC of extract in combination/MIC of extract alone) + (MIC of antibiotics in combination/MIC of antibiotics alone)

Results:-**Bacterial isolates:-**

Five isolates (2.5%) from raw milk (n=3), kariesh cheese (n=1) and ice cream (n=1) of *L. monocytogenes* were preliminary identified by their colonial morphology. On PALCAM agar, the colonies were small 2-3 mm gray-green surrounded by diffuse black zone (esculin hydrolysis), and on TSYEA agar; pinpoint colonies were observed. The isolates were further confirmed by microscopical and biochemical examination. Molecular confirmation of the five isolates by the amplification of 1200 bp of the 16s rRNA gene; was also carried out.

The antimicrobial resistance profile determined by broth micro dilution method revealed that all *L. monocytogenes* isolates were resistant to amoxicillin and oxytetracycline, while, all the isolates were sensitive to ciprofloxacin and sulphadimidine.

Plant extracts:-

The best yield of extracts was obtained from (24.5%) dry basil leave. The total phenolic compounds, total flavonoid compounds and antioxidant activity of all plant extracts were detected. The results revealed that TPC, TFC, and antioxidant activity was higher in basil leave and cinnamon inner bark extracts than coriander leave extract (Table 1). Regarding antimicrobial activity of plant extracts, the MICs against clinical isolates for dry basil leave, cinnamon inner bark and coriander leave extracts are reported in Table 2.

Checkerboard assay:-

The quantitative effects of different plant extracts with different concentrations in combination with different antibiotics with different concentrations are described as FIC index. Combinations of amoxicillin and oxytetracycline showed synergism with all plant extracts with different concentrations, while, no synergism was detected in combinations with ciprofloxacin or sulphadimidine. The synergism of amoxicillin and oxytetracycline were detected only with (1/2 of the

MIC value) high concentration of coriander, while, low concentrations of basil and cinnamon were needed to show the synergism. The most active concentrations between plant extracts and antibiotics with the fractional inhibitory concentration (FIC) index of each are reported in Table 2.

Table 1:-Extract Yield, TPC, TFC and Bio-Analytical Analysis Of Ethanol Plant Extracts

Plant extract	Extract yield (%)	TPC (mg GAE/g)	TFC (mg QE/g)	β -CLAB (%)	FRAP	DPPH' FRSA (%)		
						0 min	60 min	120 min
Basil leave (Ocimum basilicum)	24.5	186.49	77.2	79.2	1.49	80.4	79.0	79.1
Cinnamon inner bark (Cinnamomum zylenticum)	10.42	116.49	75.2	74.2	1.042	74.7	78.6	76.5
Coriander leaves (Coriandrum sativum)	8.675	20.24	10.0	12.4	0.179	13.7	26.7	31.1

TPC; total phenolic compounds, **TFC**; total flavonoid compounds, **β -CLAB**; β -Carotene/linoleic acid bleaching which measured after 2 h and compared with TBHQ; tertiary butylhydroquinone (as positive control), **FRAP**; Ferric reducing antioxidant power compared with TBHQ (as positive control), **DPPH' FRSA**; 1,1-diphenyl-2-picrylhydrazyl free radical-scavenging activity which measured at 0-time, 60 and 120 minutes

Table 2:-MIC Of Used Antibiotics And Plant Extracts, With The Most Active Combination

Isolate	MIC of antimicrobials (μ g/ml)				MIC of plant extracts (mg/ml)			Amx						Oxy					
	Amx	Oxy	Cip ¹	Sul ¹	Cin	Cor	Bas	Cin		Cor		Bas		Cin		Cor		Bas	
								MIC ²	FICI ³	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI	MIC	FICI
Strain	32	32	1	128	12.5	200	12.5	8 ^{A+}	1.250	8 ^{A+}	1.625	8 ^{A+}	1.375	8 ^{A+}	1.375	8 ^{A+}	1.625	8 ^{A+}	1.250
Strain	16	32	0.5	64	25	200	12.5	8 ^{A+}	1.625	4 ^{A+}	1.750	4 ^{A+}	1.750	8 ^{A+}	1.250	4 ^{A+}	1.750	8 ^{A+}	1.250
Strain	32	16	1	64	12.5	200	12.5	8 ^{A+}	1.250	8 ^{A+}	1.625	8 ^{A+}	1.375	8 ^{A+}	1.625	4 ^{A+}	0.75(S)	4 ^{A+}	1.750
Strain	128	64	1	128	25	200	6.25	8 ^{A+}	1.375	8 ^{A+}	1.625	8 ^{A+}	1.375	8 ^{A+}	1.375	8 ^{A+}	1.625	8 ^{A+}	1.375
Strain	64	32	1	128	25	100	12.5	8 ^{A+}	1.250	4 ^{A+}	1.750	4 ^{A+}	1.500	8 ^{A+}	1.250	4 ^{A+}	1.750	8 ^{A+}	1.375

No synergism was reported between all examined plant extracts with different concentration of ciprofloxacin and sulphadimidine, ² The most active combination (1/8^{A+} + 1/4^P) means 1/8 concentration of MIC of antibacterial agent +1/4 concentration of MIC of plant extract, ³ FICI; the fractional inhibitory concentration index, Amx; amoxicillin, Oxy; oxytetracycline, Cip; ciprofloxacin, Sul; sulphadimidine, Cin; cinnamon, Cor; coriander, Bas; basil, Remarks; (S) means Synergism

Discussion:-

L. monocytogenes is one of the important emerging bacterial zoonotic diseases worldwide. It is associated with consumption of raw milk or dairy products made from unpasteurized milk (Dewé, 2014). The current study aimed to investigate the occurrence of *L. monocytogenes* in raw milk, and dairy products (soft cheese, kariesh cheese and ice cream), obtained from retail outlets at Qalyubiyah and Giza Governorates, Egypt, and to determine the antimicrobial resistance profile. Furthermore, the current study evaluated a trial to reduce the resistance of multidrug resistant isolates by using different plant extracts which have antimicrobial effect with different concentrations.

The isolation rate of *L. monocytogenes* in raw milk samples was 3.75%. In accordance, Meshref et al. (2015) reported that the prevalence rates of *L. monocytogenes* in raw milk samples was 3.92%. However, fresh cow milk samples were free from *L. monocytogenes* in another study (Al Kassaa et al., 2016). Infected animals, poor silage quality and insufficient

hygienic conditions during milking and storage are likely the most common causes of *L. monocytogenes* contamination in raw milk.

The Kariesh cheese is a home-made cheese made in Egypt and Palestine. The current study showed that the prevalence of *L. monocytogenes* was 2.5% in Kariesh cheese samples. Variable isolation rates of *L. monocytogenes* in Kariesh cheese were reported in Egypt; 20% (Hussien et al., 2013) and 6% (Abd El Tawab et al., 2015). On the other hand, *L. monocytogenes* was not detected in soft cheese samples. The same results were recorded in Egypt (Ahmed, 2013), while in Costa Rica, Chaves and Arias (2009) reported the isolation of 27 *L. monocytogenes* isolates from 110 soft cheese samples. Regarding, ice cream samples; the prevalence rate of *L. monocytogenes* was 2.5%. Similar result was reported in Egypt (Tantawy, 2011). On contrary, high prevalence rate (26%) was reported in Greece (Effimia, 2015). The prevalence of *L. monocytogenes* in dairy products might reveal insufficient heat treatment of contaminated milk and improper sanitation of equipment.

The increased rate of *L. monocytogenes* resistance to one or more clinically relevant antibiotics has been reported (Morvan et al., 2010). The current study revealed that all isolates were sensitive to both ciprofloxacin and sulphadimidine. Similar results were previously reported (Karadal and Yildirim, 2014). However, all *L. monocytogenes* in the current study were resistant to amoxicillin and oxytetracycline. In accordance, Peter et al. (2016) reported that 16 *L. monocytogenes* isolates in Nigeria were resistant to amoxicillin and tetracycline. High resistance rates to amoxicillin and oxytetracycline may be partly attributable to overuse of these antibiotics in veterinary field, which increase genes mutation rate, in addition to acquisition of mobile genetic elements.

The continuous increase in microbial resistance to different types of antibiotics increases the need to find new classes of antibiotics. Antimicrobials of plant origin may give a hope to find a solution to this problem. Phenolic compounds are mainly considered as scavengers of free radicals (Gülçin et al., 2005). The bio-analytical analysis of plant extracts used in the current study demonstrated that basil and cinnamon had higher amount of TPC than coriander. In accordance, the high amount of TPC in basil and cinnamon were reported (Snigdha and Monika, 2014). Also, higher amounts of TFC were reported in the current study than coriander. Similar finding was reported (Jangra et al., 2018).

The ability of natural antioxidants to inhibit the growth of bacterial pathogens has been documented to have influence on infectious diseases (Roginsky and Lissi, 2005). In the current study, the highest antioxidant activity was detected in basil followed by cinnamon then coriander. The antimicrobial activities of the ethanol extract of plant extracts against bacterial isolates were determined by evaluating the presence of MIC values, and thus, their potencies were assessed quantitatively (Yildiz, 2016). In the current study, the higher antimicrobial effects of basil and cinnamon were detected. These results might be conducted to the positive relationship between antibacterial activity and composition of plant extracts from TPC and TFC, in addition to antioxidant activity of plant extracts (Yildiz, 2016). The yield of extracts recovered from the examined dried plants in the current study ranged from 8.675 to 24.5%. The variation in the extraction yields from different plants is attributed to their varied chemical composition, efficiency of extraction and the surrounding climatic conditions, in addition to the phytochemical properties and differences among bacterial species (Brant et al., 2008). Also, the variations in the ability of antibiotics and plant extracts combinations to reduce drug resistant in bacteria might be conducted to their effect on efflux pumps, which need to be evaluated (Wagner and Ulrich-Merzenich, 2009).

The interaction between antibiotics and bioactive plant extracts is a promising concept (Adwan and Mhanna, 2008). The development of improved management of microbial infections depends mainly on the clear differentiation of synergistic from antagonistic interactions. It was assessed on the basis of the fractional inhibitory concentration (FIC) index. The antimicrobial activities of basil, cinnamon and coriander extracts alone were evaluated (Shaaban et al., 2013). Betoni et al. (2006) reported that although the antimicrobial activity of a plant extract has not been relatively high, the synergism rates with other antibiotics were relatively high. In the current study, low concentrations of amoxicillin and oxytetracycline showed synergism with all plant extracts used against resistant isolates, especially, with basil or cinnamon extracts showed relatively high synergism than coriander extract. These findings confirmed that the activity of antibacterial combinations were more effective than individual agents. The same findings were reported in other studies. These results are consistent with a previous study mentioned that the double attack of two agents on different target sites of the bacteria could theoretically lead to synergistic effect (Olajuyigbe and Afolayan, 2012). For instance, agents that inhibit protein synthesis (tetracycline) or interfere with cell wall synthesis (amoxicillin) had strongest synergistic effect with plant extracts (Betoni et al., 2006; Marzoug, 2013). Also, the antagonistic effect in vitro between coriander in combination with amoxicillin

/clavulanic acid against *L. monocytogenes* was reported (Shaaban et al., 2013). Ciprofloxacin and sulphadimidine showed neither synergistic nor additive effect against *L. monocytogenes* in the current study with the investigated plants. These results are consistent with those obtained by Betoni et al. (2006) who reported that none or weak synergism activity between nucleic acid synthesis inhibitors and plant extracts. In contrary, Shaaban et al. (2013) reported antagonistic effect between norfloxacin (Quinolones) and coriander. The absence of effect between plant extracts and ciprofloxacin or sulphadimidine in the current study might be because the isolates were sensitive to them. In conclusion, this study indicated that raw milk and dairy products could be considered as sources of *L. monocytogenes*. Therefore, raw milk must be effectively pasteurized before consumption or usage in the production of dairy products. The chemical characters of ethanol extracts of basil, cinnamon and coriander, including; TPC, TFC and anti-oxidant properties, was in parallel to their synergistic effect with antibiotics. Furthermore, their concomitant administration together could be useful in treating *L. monocytogenes* infections with minimal concentrations of antibiotics, and subsequently minimal side effects from antibiotics.

Disclosure Statement:-

No competing financial interests exist.

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